Elucidating the Role of the Humoral Response in *Mycobacterium tuberculosis* infected Cynomolgus Macaques

by

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ABSTRACT

Mycobacterium tuberculosis remains as a global burden today with an estimated one-third of the global population being infected and at risk of developing active infection. Most studies in the immunology and pathogenesis of tuberculosis have revealed the importance of cellular immunity in controlling the infection. However, the role of the humoral immune response is poorly understood, particularly in the primate models of *M. tuberculosis* infection. The primary goal of this thesis was to understand how B cells and antibody contribute towards containment of *M. tuberculosis* within the cynomolgus macaque model of infection. The thesis starts off by characterizing B cells and antibody profiles within the granulomas of *M. tuberculosis* infected cynomolgus macaques. B cells were noted organize themselves into clusters that resemble germinal centers found in lymphoid organs or in chronic autoimmune conditions, within the granuloma. The effect of B cell depletion on the outcome of *M. tuberculosis* infection in cynomolgus macaques was also performed. The study findings suggest that B cells and antibody contribute very little in terms of disease control in the early stages of natural *M. tuberculosis* infection. However, subtle differences such as a slight increase in bacterial burden within individual granulomas and altered cytokine correlations within individual granulomas were noted. Macrophage behavior in the absence of B cells and antibody was also studied using

granuloma tissue derived from the B cell depletion study. No differences were found within the macrophages in the absence of B cells at least at the acute stage of infection. The experiments detailed in this thesis suggest that the humoral response is not crucial towards *M. tuberculosis* control in the early stages of natural infection. However, the findings in this thesis suggest that B cells and antibody may play a role in long term chronic control of tuberculosis infection.

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1.0 ACKNOWLEDGEMENTS

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2.0 AN INTRODUCTION TO TUBERCULOSIS

2.1 A BRIEF HISTORY OF A SUCCESSFUL PATHOGEN

Tuberculosis (TB) occupies a unique position in human history as one of the most successful diseases that has plagued humanity until today. *Mycobacterium tuberculosis*, the primary causative agent of TB in humans, is thought to have been infecting early humans migrating out of East Africa approximately 3 million years ago and has continued to persist throughout history[1]. Characteristic bone deformations found in mummified remains from Egypt around 3000 BC showed very clear signs of Potts disease (spinal form of TB), suggesting that TB itself has been present since early human history[2]. The disease itself has earned many names throughout history, phthisis by the ancient Greeks[3], King's Evil[4] during the medieval era and consumption closer to the industrialization period of Europe. All these terms share a common theme of sufferers being chronically ill, bedridden by bloody coughs and wasting away as if being consumed by the disease. Further findings in paleoarchaeology would undoubtedly reveal further evidence that *M. tuberculosis* has not only persisted but co-evolved alongside humans throughout history.

M. tuberculosis was eventually isolated as the causative agent in 1882 by a German physician turned scientist, Robert Koch and proved to be a landmark discovery in microbiology[5]. Robert Koch was hailed as the forefather of microbiology due to his scientific principles in

identifying infectious agents known as the Koch's postulates, that were instrumental in identifying many other pathogens such as *Vibrio cholera*[6]. With regards to TB, Robert Koch's work also provided the means to diagnose patients with the disease such as the tuberculin skin test which is still routinely used today[7].

Despite the isolation of *M. tuberculosis*, adequate treatment and vaccination modalities were not discovered until the early 20th century. Until then, various treatments for TB were present from the ingestion of laudanum[8] to admitting patients in sanatoriums in the belief that fresh air, graduated labor, exercise and diet would ameliorate the disease[9]. It wasn't until 1908 that Albert Calmette and Camille Guérin developed an attenuated strain of *Mycobacterium bovis*, a cousin of *M. tuberculosis* that infects primarily cattle and badgers, to serve as a vaccine. This was accomplished by passaging the virulent *M. bovis* on an alkaline glycerin-ox bile-potato media over 200 times until the strain becomes sufficiently attenuated for human use[10, 11]. This vaccine, termed the bacille Calmette-Guérin (BCG), is still in use by many countries worldwide and has lowered the incidence of more sever extra-pulmonary dissemination of disease such as tuberculous meningitis especially in children[12, 13].

With the advent of antibiotics, particularly streptomycin in 1943[14, 15], and then the introduction of the anti-tuberculosis drug cocktail starting from the 1960s[16], global attention towards TB begun to wane as the disease was seemingly brought under control. However, TB has made a dramatic return in recent decades in part due to the human immunodeficiency virus (HIV) pandemic ravaging across the world[17]. To compound matters further, antibiotic resistant strains of *M. tuberculosis* are also on the rise due to a combination of inadequate drug treatment regimens, socio-economic collapse and poor monitoring programs in various involved

countries[18, 19]. Given how TB has persisted alongside humans throughout history, it is not surprising that eradication of such a tenacious pathogen remains difficult.

2.2 MICROBIOLOGY AND EPIDEMIOLOGY OF *M. TUBERCULOSIS*

The causative agent of TB in humans, *M. tuberculosis*, is part of a group of phyllogenetically related group of bacteria termed the *M. tuberculosis* complex (MTBC)[20]. Aside from *M. tuberculosis*, *Mycobacterium africanum* is another member of the MTBC which has adapted as a human pathogen[21]. There are also members of the MTBC that has adapted to using both wild and domesticated animals as hosts such as *M. bovis* (cattle and badgers), *Mycobacterium caprae* (sheep and goat), *Mycobacterium microtii* (voles) and *Mycobacterium pinnipedii* (seals and sea lions)[22]. The animal-adapted MTBC members are capable of causing disease in humans, as demonstrated by *M. bovis* infections in children from consuming tainted raw milk[23], but has limited human to human transmission capabilities[24].

The human adapted MTBC members, *M. tuberculosis* and *M. africanum* can be further differentiated into distinct genetic lineages[25]. The geographical distribution of these lineages of human adapted MTBC correlate very closely with certain human populations, suggesting that human adapted MTBC lineages have adapted to utilize certain human genotypes as hosts[25]. Africa is unique as it harbors the highest diversity in human adapted MTBC lineages. This observation suggests that human adapted members of MTBC originated in Africa and have coevolved along with their respective human population after the migration out of the continent approximately 70000 years ago[26] (Fig. 1.). The resultant spread of the different lineages of

human adapted MTBC members in modern times can be attributed to trade, exploration and conquest throughout recorded history[26].

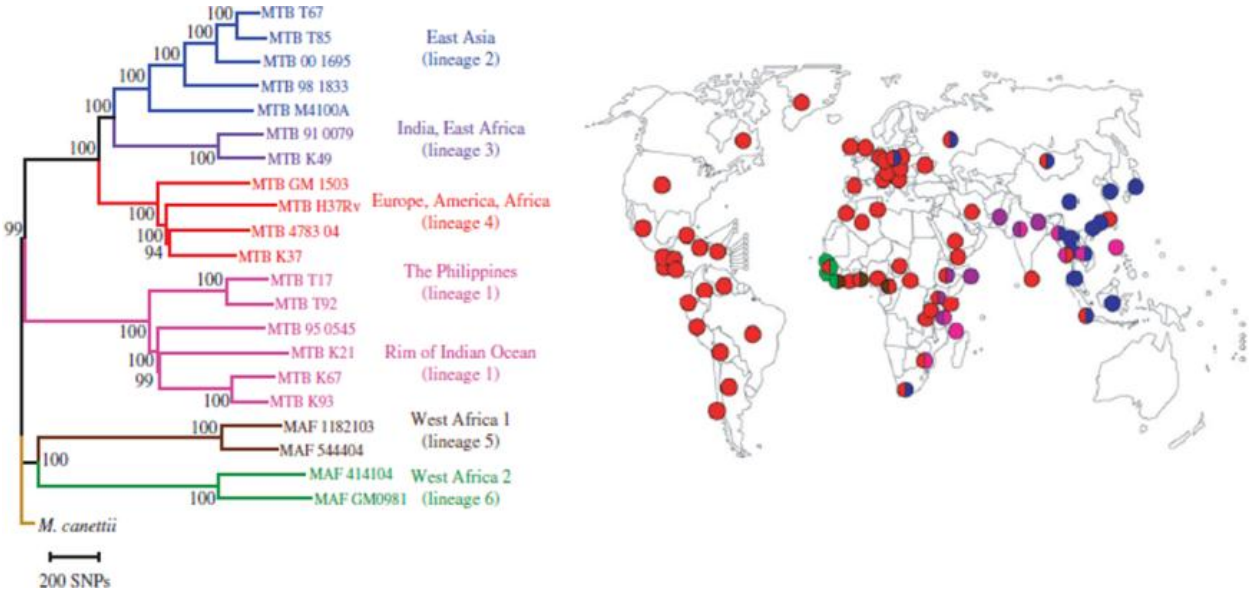

Figure 1. *Phylogenetic tree and geographic distribution of the various global lineages of Mycobacterium tuberculosis complex.* Image adapted, with permission, from Gagneux *et al.*[25] Copyright (2006) National Academy of Sciences, USA.

TB is very much a disease of the poor and correlates very closely with poverty levels within any given population[27]. During the period of the Industrial Revolution of Europe from the late 18th century to the early 20th century, mortality rates due to TB skyrocketed from the rampant poverty, malnutrition and overcrowding[28]. Today, the same socioeconomic factors influence the distribution of TB globally. Current estimates suggest that roughly one third of the human population is infected with *M. tuberculosis*, with a smaller fraction (around 10 percent) presenting with clinical signs of active disease[29]. Global active TB incident cases currently stands at 8.7 million with 1.5 million deaths per year. Countries with the highest incidence and mortality rates in 2011 are in sub-Saharan Africa, South East Asia and North Korea. Aside from poverty, some of these areas also contain the highest HIV prevalence rate which exacerbates the already heavy TB burden present[29] (Fig. 2).

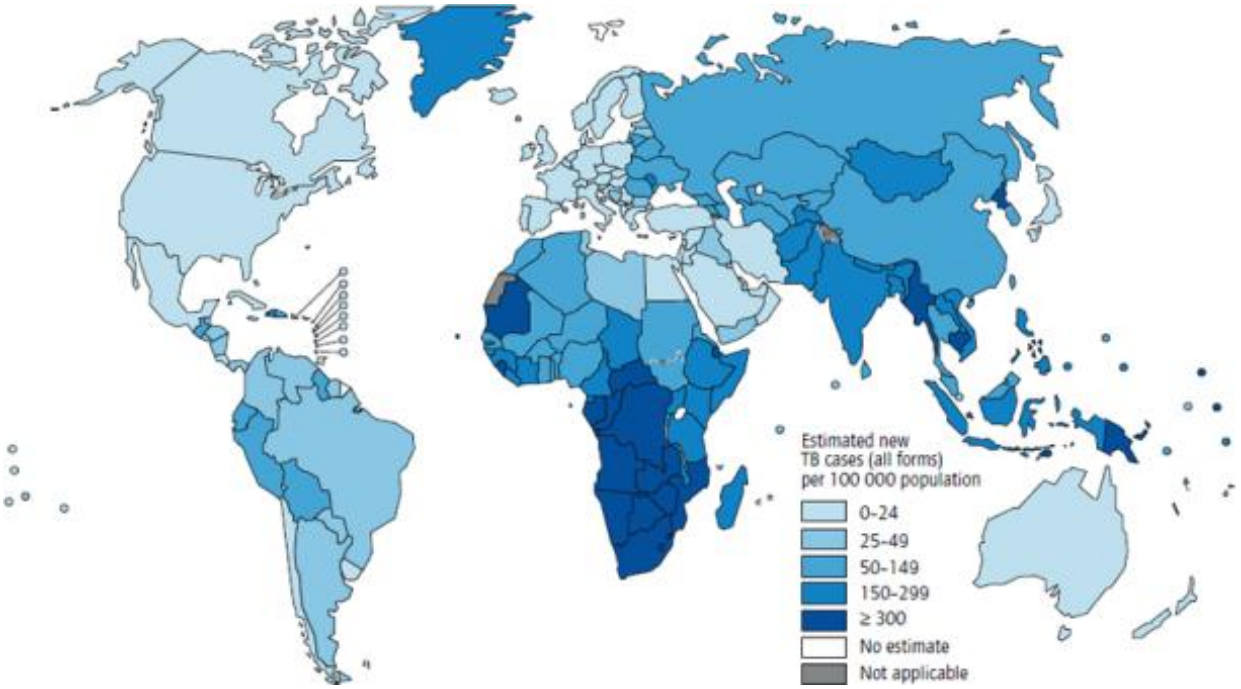

Figure 2. *World map depicting the incidence rates of tuberculosis.* Figure was reused, with permission, from the World Health Organization (WHO) Global Tuberculosis Report 2012.[29]

2.3 HOST IMMUNOLOGIC RESPONSES TO *M. TUBERCULOSIS*

M. tuberculosis is primarily spread via aerosol droplets generated from an actively infected individual. The primary site of infection is typically the respiratory tract, more specifically the alveolar macrophages or alveolar dendritic cells upon phagocytosis of bacilli containing droplets. Deposition of bacteria within the lung alveolar spaces are less likely to be cleared compared to deposition within the airway due to the tendency of resident APCs to induce tolerance[30, 31]. Hence, *M. tuberculosis* has a better chance at establishing a productive infection if the bacilli-laden aerosol is deposited within the lung parenchyma than the airway as immune cells of the airway mucosa tends to be more microbicidal[30, 31]. Upon detection of the bacilli, pathogen recognition receptors are activated resulting in the release of pro-inflammatory cytokines and

chemokines that recruit other monocytes and dendritic cells to the site of infection[32]. This wave of cellular recruitment is thought to be an attempt to contain the infection[33, 34] while allowing for the infected dendritic cells to migrate to the draining lymph nodes to begin the priming of the adaptive immune system to recognize *M. tuberculosis* antigens[35, 36]. Primed lymphocytes, both T and B cells then migrate to the lungs between 2 to 6 weeks post infection leading to the formation of a granuloma[37, 38]. Successful containment of the bacilli within granuloma can lead to either bacterial clearance or latent infection whereby the infection remains clinically dormant, sometimes for the lifetime of the host. However, failure to contain the bacilli results bacterial dissemination, leading to granulomas forming throughout the lungs and other organs. More severe forms of the disease result in lung cavitation as the granulomas begin to erode away normal tissue or even miliary disease, where systemic bacterial dissemination causes granulomas to form all over the body[39].

A functional granuloma is thus essential in containing *M. tuberculosis* and requires the cooperation of a myriad of cells ranging from macrophages, neutrophils, fibrocytes and lymphocytes[40, 41]. In primate TB, there is a huge variation in the pathology of the granuloma ranging from a spectrum of solid cellular, caseus necrotic, fibrotic or mineralized[42, 43]. Furthermore, the outcome of each individual granuloma is unique and independent of each other even within granulomas from the same individual[44]. This suggests that the local interplay between host and pathogen is crucial in dictating the outcome of each granuloma.

2.3.1 Roles of the macrophages.

The primary function of alveolar macrophages is to keep the airway clear by phagocytosis. They are thus the first to encounter *M. tuberculosis* upon ingestion of the bacilli laden aerosols[45]. Entry

into the macrophage is typically mediated by the binding of mannose receptors, notably DC-SIGN[46, 47], complement receptors such (CR) as CR1[48], 3[49] and 4[48] or via Fc receptor engagement with antibody, specifically of the FcγR family[50]. DC-SIGN engagement typically promotes an anti-inflammatory phenotype in macrophages which is beneficial to the invading bacilli[51]. Engagement of activating FcγR promotes bacterial killing and the secretion of pro-inflammatory cytokines by the macrophage[52]. The mode of entry into the macrophages is thus crucial as it can modulate macrophage activation and affect the bacterial clearance efficacy downstream.

Within the cell, *M. tuberculosis* is detected by several groups of pathogen recognition receptors such as members of the Toll-like receptor (TLR) family and NOD-like receptors[53-56]. TLR2 has been shown to interact with a 19kDa lipoprotein[57] while TLR4 recognizes cell associated factors specific for *M. tuberculosis*[58, 59]. TLR activation typically leads to the secretion of pro-inflammatory cytokines such as IL1β[60, 61] and tumor necrosis factor (TNF) and chemokines leading to the recruitment of more monocytes and macrophages to the site of infection to begin granuloma formation[35, 36, 62]. TLR activation can also be subverted by *M. tuberculosis* as a means to mediate immune evasion by down-regulating IFNγ induced killing[57] or reduce apoptosis within infected macrophages[63].

M. tuberculosis may secrete virulence factors that actively result in macrophage recruitment initially in order to increase the number of infected cells[33-35]. This is done by inducing the infected cells to undergo necrosis by directly damaging the cell membrane and blocking the membrane repair mechanism[34]. As necrotic cells induce active inflammation, more phagocytotic cells are recruited leading to more potential targets for *M. tuberculosis* infection[64].

Within the granuloma in humans and non-human primates, infected macrophages are typically confined to the center while being surrounded by a layer of epitheliod macrophages, in

an attempt to wall off the bacteria and prevent dissemination[41-43]. The center of the granuloma thus becomes necrotic and highly hypoxic in an attempt to impair mycobacterial growth[65].

M. tuberculosis has a variety of ways to evade the immune response and survive within the macrophage. Secreted virulence factors such as the 19kDa lipoprotein are capable of down regulating antigen presentation via MHC class II within macrophages and dendritic cells[66] and downregulate IFNγ dependent killing mechanisms[57, 67]. Mycobacterial cell wall components are also capable of arresting phagosome maturation and fusion with the lysosomal compartment into the phagolysosome[68], allowing for internalized bacilli to survive within the phagosome of macrophages[69].

Macrophages thus need to be primed for effective bacterial killing. Interferon γ (IFNγ) and TNF secreted by activated lymphocytes, typically $CD4^+$ T_h1 T-cells are capable of activating macrophages into a classically activated phenotype (M1)[70] to kill any ingested bacilli[71]. These M1 activated macrophages tend to produce more reactive oxygen and nitrogen species[72] and are capable of further acidifying the phagolysomal compartment where internalized *M. tuberculosis* are present[73]. However, macrophage activation needs to be confined and balanced within the granuloma as extensive tissue damage can occur otherwise, leading to extensive inflammation, impairment of bacterial containment and bacterial dissemination[41, 74].

Macrophages can also take on an alternatively activated (M2) phenotype in response to IL4, IL5, IL10 and IL13[70, 75]. M2 macrophages are more closely associated with down regulating immune responses by secreting anti-inflammatory cytokines such as IL10 and transforming growth factor β (TGFβ)[76]. M2 macrophages are also capable of promoting fibrosis to begin the resolution phase of the inflammatory process[70, 77-79]. Within the granuloma, M2 macrophages are crucial as

they may limit the amount of tissue pathology that results from excessive pro-inflammatory microbicidal responses.

2.3.2 T-cells.

T-cells are an essential component in any immune response against pathogens. In the case of tuberculosis, T-cells have been found to be an indispensable component in controlling the infection. This is most evident in αβ T-cell receptor mutant mice where these mice succumb very quickly to *M. tuberculosis* infection much earlier compared to wild type counterparts and present with greater pathology and higher bacterial burden[80, 81]. Further knockout mice studies have since revealed the relative importance of various subsets of T-cells in shaping the immune response to *M. tuberculosis* over the course of infection. From the mouse studies, CD4 T-cells were noted as key players in organizing the immune response within the granuloma[82, 83] via cytokine secretion, in particular IFNγ[84] and TNF[85]. CD8 T-cells were primarily noted for cytotoxic functions such as mediating apoptosis of heavily infected macrophages or dendritic cells via granzyme B, perforin[86-89] and in the case of humans, granulysin production[90].

In humans, the importance of T-cells in the control of *M. tuberculosis* is evident from several clinical observations. HIV-1 infected patients have an increased risk of *M. tuberculosis* infection, disease, and increased risk of reactivation from latent infection, highlighting the importance of CD4 T-cells in controlling *M. tuberculosis* as HIV primarily targets CD4 T-cells and macrophages[91-93]. Another observation is that patients with recurring mycobacterial infection tend to have genetic lesions within certain T-cell cytokine signaling pathways particularly within the IL12, IL23 and IFNγ axis[94, 95]. These genetic studies highlight the importance of cytokine signaling in orchestrating an effective response towards controlling intracellular infection by T-

cell dependent pathways. Furthermore, patients taking pharmacological inhibitors of TNF are at high risk of reactivation of latent tuberculosis, which again reinforces the importance of adequate cytokine signaling in controlling *M. tuberculosis* infection[96, 97].

2.3.3 Cytokines orchestrate cellular interactions within the granuloma.

Successful containment of *M. tuberculosis* depends upon adequate cellular signaling within the granuloma for proper bacterial clearance and to confine damaging pro-inflammatory reactions to reduce tissue damage[41]. The complex interplay of cytokine signaling continues to be unraveled with various cytokines taking up roles in mediating pro- or anti-inflammatory responses or specific cellular activation in the case of T-cells and macrophages[40]. Thus, granulomas are incredibly complex and dynamic immunologic structures with very unique microenvironments for cellular behavior due to the diversity of cytokine interactions present.

Cytokine signaling is thought to set the context of subsequent immunologic responses upon detection of specific pathogens by tissue resident macrophages or dendritic cells. In the case of tuberculosis, detection of the pathogen via the TLR2, TLR4 and TLR9 [54]is thought to promote IFNγ and IL12 production to prime CD4 T-cells towards the T_H1 lineage for further release of IFNγ and TNF[48]. These cytokines are essential as they synergize to further activate macrophages for anti-microbial functions through the release of nitric oxide synthase (iNOS) and reactive radical species[98, 99]. IFNγ also serves to enhance the antigen presentation capabilities of macrophages towards intracellular antigens, thereby promoting further immune activation against *M. tuberculosis*[73]. Furthermore, CD4 T-cells are helper cells and would thus signal to other cell types such as CD8 T-cells to mount the appropriate response to the specific pathogen,

in the case of tuberculosis, more IFNγ and TNF production for enhanced macrophage activated killing[71, 75] or direct cytotoxic capacity[100].

TNF and other members of the TNF superfamily are also indispensable in the establishment of the granuloma as TNF deficient mice are highly susceptible to *M. tuberculosis* infection[85, 101]. In humans, reactivation of tuberculosis is a major risk for patients under anti-TNF therapies, highlighting the crucial role TNF occupies within the immune response to *M. tuberculosis*[96, 102]. Aside from its role in activating macrophages along with IFNγ, TNF is crucial as a signaling molecule to initiate further immune activation via cytokine and chemokine signaling responsible for cellular recruitment. In TNF deficient mice, granuloma formation was delayed due to less monocyte recruitment to the site of infection and reduced chemokine secretion by infected macrophages[103, 104]. Furthermore, TNF signaling is crucial for the maintenance of granuloma function, particularly the chemokine gradients necessary to orchestrate macrophage and lymphocyte localization and function within the granuloma[101]. However, the originally assumed role of TNF in granuloma formation and maintenance identified in the mouse was not observed in non-human primates treated with anti-TNF antibody[105]. Although anti-TNF antibody resulted in exacerbated acute infection and reactivation of latent infection in macaques, granuloma structures formed and remained intact. However, these granulomas were not apparently capable of controlling infection, and dissemination was rampant. Normal granuloma structure was also observed in human patients treated with anti-TNF for inflammatory diseases that subsequently developed reactivation tuberculosis.

Other inflammatory cytokines involved in *M. tuberculosis* infection include IL1 family members, IL6 and IL17. Macrophages are known to produce IL1 and IL6 upon interaction with *M. tuberculosis*[106, 107]. IL1 members particularly IL1β and IL18 are essential in activating

inflammatory responses via inflammasome assembly and promote IFNγ production by T-cells[108-110]. IL6 in particular has prominent roles in activating T-cell, particularly in the development of the T_H17 T-cell subset and B cell responses. T_H17 T-cells secrete IL17, a potent cytokine that helps to recruit neutrophils to the site of immune activation[111].

Macrophages also need to be activated in a controlled manner to limit tissue pathology as uncontrolled inflammatory processes can result in bystander tissue damage. IL10 is an example of an immune suppressive cytokine that down-regulates immune cell activation[112]. CD4 T-regulatory cells, formed during T-cell activation via antigen recognition, and macrophages are capable of producing IL10[113, 114]. However, adequate control of IL10 production is crucial as the suppressive activities of this cytokine can also hinder proper pathogen control[112, 115].

2.4 TREATMENT MODALITIES FOR TUBERCULOSIS

Antibiotics are available for the treating tuberculosis but suffer from several issues. The most pressing is that adequate treatment requires the administration of several antibiotics over a long period of time. The standard short course chemotherapy consists of 4 first-line drugs, isoniazid (INH), rifampicin (RIF), ethambutol (ETH) and pyrazinamide (PZA) taken in combination daily over a period of 6 months[116]. The long treatment period was deemed necessary due to the inherent slow growing nature of *M. tuberculosis*. However, patient compliance in adhering to the drug regiment over such a long period proved to be difficult, which frequently results in the emergence of drug resistant tuberculosis[117, 118].

Second-line antibiotics are available but are typically reserved for cases of multi-drug resistant *M. tuberculosis* strains (MDR-TB). MDR-TB strains are defined as *M. tuberculosis*

strains resistant to two or more first line drugs, usually INH and RIF[118]. These strains require even longer treatment periods that can exceed 2 years with varying efficacy[119, 120]. The second-line drugs include aminoglycosides such as kanamycin, fluoroquinolones including levofloxacin, thioamides and various polypeptide based drugs such as capreomycin[121]. These drugs are more toxic, can be more difficult to administer and are not widely available in low income countries, making them prohibitively expensive[122, 123].

Multidrug resistant strains of *M. tuberculosis* are of great concern especially with the emergence of extremely drug resistant varieties (XDR-TB) which can be impervious to even the second-line drugs. In light of such highly resistant strains, finding new drug targets and developing new antibiotics is crucial to keep treatment options open[124].

2.5 ANIMAL MODELS FOR TUBERCULOSIS

Animal models of human diseases are essential in providing invaluable insight into host-pathogen interactions essential for establishing new treatment modalities, vaccines and drug research. In the case of tuberculosis, many animal models are available for research purposes, each with its own set of advantages and shortcomings. However, data from all the animal models have provided invaluable data into defining host-pathogen interactions of tuberculosis with humans.

2.5.1 Murine model of tuberculosis.

The mouse model (*Mus musculus*) is currently the most widely used model of study in tuberculosis research. This animal model has many advantages. The mouse is a versatile model due to the ease of genetic manipulation which allows for the identification of host genetic factors via gene knockouts or overexpression systems. Through use of knock-out mice models, many genetic determinants such as IFNγ, TNF and various cell populations crucial to the control of tuberculosis were identified[84, 85, 125, 126]. Furthermore, research reagents are very widely available due to the widespread use of the mouse model in other fields of study.

The mouse is also cheap as they can be housed easily, thus reducing the overall research costs. This model is easily infected with a variety of *M. tuberculosis* strains via intravenous or aerosol routes of infection which allows for vaccine and drug studies to be conducted with minimal complications[127]. Thus, the mouse model is the most widely used disease model in *M. tuberculosis* research.

Despite these advantages, the murine model does present with certain shortcomings in terms of disease progression and pathology. *M. tuberculosis* infection is progressive in all mouse strains and mice do not develop latent infection as in the case of human tuberculosis[128, 129]. Although several manipulations using drugs to mimic latency are available, this still limits the understanding of factors involved in controlling the disease at the latent stage in humans[125].

In addition, granuloma formation in mice does occur but lacks the highly organized cellular structures seen in human granulomas. Caseus necrosis is absent unless several genetic factors crucial to immune function are ablated leading to abnormally high bacterial burden in the lung. Even then, the disease pathology does not resemble actual human caseous granuloma pathology. Furthermore, murine *M. tuberculosis* infection typically results in poorly formed

cellular ‘granulomas’ and eventually progresses to fatal cellular infiltration of the lungs by macrophages, neutrophils and lymphocytes[130].

It should thus be noted that the mouse model can be useful if the research question is properly phrased. The eventual goal is to translate the findings in the murine model for use in human disease treatment.

2.5.2 Non-human primate model of tuberculosis.

Research of tuberculosis in non-human primates are generally performed on macaques, particularly using rhesus (*Macaca mulatta*)[131] and cynomolgus (*Macaca fasicularis*) macaques[42]. Characterization of infection within these animals revealed that disease aetiology and pathology very closely mimic the spectrum of infection in human tuberculosis, including both active and latent disease. Although tuberculosis has been characterized more in cynomolgus macaques, rhesus macaques are still used due to the extensive work done in related infectious disease fields of simian immunodeficiency virus (SIV) as a surrogate model of HIV.

Infections in monkey models are done via bronchoscope instillation of bacteria (generally 20 to 200 CFU) or aerosol route. Infected animals can be followed up using a range of clinical indicators for inflammation and infection such as erythrocyte sedimentation rates (ESR), C-reactive protein, chest X-ray to detect for pulmonary infiltration, or culture positivity of gastric aspirate or bronchoalveolar lavage fluid (BAL). All infected animals also can be confirmed for infection using the tuberculin skin test 4 to 8 weeks posts infection[42, 43].

Pathology in the non-human primate model recapitulates the entire spectrum of granulomas observed in human tuberculosis. Granulomas from non-human primates are similar, even indistinguishable from human granulomas in terms of gross appearance and microscopic

presentation, including the broad range of granuloma types from caseus necrotic, fibrotic, mineralized or solid cellular granulomas. In terms of disease presentation, tuberculosis in non-human primates also progresses in a similar manner to humans with the classical Ghon complex presentation (pulmonary granuloma with associated lymph node involvement), lung cavitation, and even atypical presentations such as Pott's disease and tuberculous meningitis to reflect disseminated disease[42, 43]. These marked similarities thus allow for findings in research to be easily applied for human use. The availability of real-time imaging technology such as PET/CT in tracking disease progression also facilitates the use of the monkey model in the development of vaccines and drugs against tuberculosis[132-134].

The most important hallmark of the non-human primate model is latent infection. An animal model for latent tuberculosis infection is invaluable in dissecting the mechanisms that induce latent infection, and the associated risk factors that govern disease reactivation[135-137]. This is crucial as the majority of humans infected with *M. tuberculosis* are latently infected. Furthermore, the non-human primate model may be helpful in dissecting the problem of HIV-TB co-infection where tuberculosis remains a major killer among people with HIV[138, 139].

However, non-human primates are far more costly to maintain for research compared to small animals such as mice. The economic factor alone to maintain monkeys in BSL3 conditions is staggering, thus prohibiting widespread use. Monkeys are also highly outbred unlike the laboratory mouse which necessitates the use of more animals in research studies, adding to the cost. However, the outbred nature of the animals themselves allow for the research data to be more easily applied to humans as humans are an outbred population and could explain the variable outcome of disease presentation in both monkey and human groups.

Another limitation is the availability of research reagents tailored for monkey use. However, many human specific reagents can cross react with non-human primate samples and the increasing use of macaques in other infectious disease fields may help spur further reagent development specifically for non-human primate research use.

2.5.3 Other mammalian models of tuberculosis.

The New Zealand White rabbit is currently the most commonly used rabbit species in tuberculosis research. Like mice and non-human primates, rabbits are also susceptible to *M. tuberculosis* infection via various routes depending on the model being studied[65, 140-144], although they are much more susceptible to *M. bovis*[144, 145]. Rabbits are also used in tuberculosis research to as models for tuberculosis meningitis when the bacilli gains access to the central nervous system (CNS)[146, 147]. CNS complications from tuberculosis in humans are the most complicated to treat, with a mortality rate as high as 50 percent[148]. The tuberculous meningitis rabbit model has provided insight into treatment modalities to reduce brain damage by including immunomodulatory drugs in addition to standard anti-tuberculous drugs[146, 148].

The major drawback for the rabbit model is the availability of reagents for research which limits the scope and depth of questions being addressed. Rabbits are also more costly to handle compared to mice which limits the number of people willing to invest in the model.

Other common tuberculosis models include the guinea pig[149], cattle[150, 151], and zebrafish[152]. Each has strengths and weaknesses, but all have been used to further our understanding of the host-pathogen relationships in mycobacterial infections.

3.0 AN INTRODUCTION TO B CELLS:

3.1 THE HUMORAL IMMUNE SYSTEM

The humoral immune system is a component of the adaptive immunity that is responsible for protecting the extracellular spaces of the host from pathogens. B cells are the main players of the humoral immune system as they differentiate into plasma cells upon activation with appropriate cognate antigen and associated signals to produce antibodies[153]. Antibodies are secreted immunoglobulins and are the primary proteins that mediate the interactions of the humoral response with the rest of the immune system. Antibodies function by binding to their antigenic targets upon recognition and mediate clearance via neutralization[154, 155], enhancing opsonization[156] or complement activation[157]. Antibodies, when bound to cognate antigen, form immune complexes which are capable of mediating cellular signaling to cells bearing the appropriate Fc receptors[158]. Thus, Fc receptors provide a mechanism by which components of the humoral immune system such as antibody-antigen immune complexes can modulate the immune response[159].

B cell activation is a key process in the initiation of the humoral response and is tightly regulated. As mentioned above, B cell activation requires both cognate antigen recognition by the B cell receptor complex (BCR) and outside help in the form of helper $CD4^+$ T-cell signaling[160] or TLR activation via pathogenic components such as CpG or LPS[161]. The BCR

itself is a membrane bound immunoglobulin, similar to antibodies, and is highly variable in sequence, allowing for diverse antigen recognition capabilities[162]. Upon activation, B cells undergo a complex set of differentiation and specialization processes to form plasma cells whose sole function is to secrete huge quantities of antibodies[153]. A subset of activated B cells also differentiates into memory B cells to confer long term protection to the host against previously encountered antigen[163].

B cells are also recognized to provide other functions aside from antibody generation. B cells are capable of secreting cytokines for cell signaling during infection and have been observed to release a diverse repertoire of signaling mediators that can both enhance or blunt the inflammatory response such as IL17 and IL10 respectively[164]. Cytokine production by B cells within the context of infection and autoimmunity is an ongoing field of interest[164, 165]. In addition to providing cytokine help, B cells have also been shown to be capable of presenting antigen to T-cells, thereby boosting local immune reactive processes in the case of infections or autoimmunity. Activated B cells have been shown to increase the expression of antigen presenting molecules such as MHC class II which allows for peptides to be presented to $CD4^+$ T-cells[157, 158]. However, antigen presentation by B cells is relatively inefficient compared to professional antigen presenting cells such as dendritic cells or macrophages. Furthermore, B cells can only boost the response towards cognate antigens but are unable to prime a response towards a novel antigen[166].

3.2 GENERATION OF THE B CELL

B cells are lymphocytes that develop within the bone marrow following interactions of the BCR with stromal cells present within the compartment[167]. The BCR is the primary signaling molecule that allows for B cells to detect and respond to antigen. During B cell development, the BCR is generated via gene rearrangement of germline DNA to generate receptors capable of recognizing a colossal range of antigenic targets, giving rise to multiple different clonal B cell populations[168-170]. After eliminating self-reactive B cells, these immature B cells then migrate into circulation to begin patrolling for cognate antigen[171]. Upon binding with cognate antigen and receiving secondary activation signals via T-cell help or TLR signaling, B cells start proliferating and begin the process of somatic hypermutation[172] and class switching to enhance BCR recognition and binding to cognate antigen. B cells also start terminally differentiating into plasma cells for the sole purpose of generating highly antigen specific antibodies[173, 174].

As mentioned, the BCR is responsible for antigen recognition by B cells against a diverse array of possible antigenic permutations[162]. This ability to recognize a diverse set of antigens is due to the process of gene arrangement of several segments of germline DNA coding for the BCR during B cell development within the bone marrow[168]. The colossal variability that arises from the possible permutation of the BCR gene segments allows for B cells to recognize any possible antigenic configuration that can be encountered by the host[175].

B cells develop within the bone marrow from progenitor B cells (pro B cell) which requires direct contact with IL7 secreting stromal cells for continued survival and proliferation. Interactions of the pro B cell with the stromal cell also induces the expression of DNA recombinases RAG1 and RAG2 which are required for gene arrangement[167, 168].

Further development requires pro B cells to begin rearrangement of the immunoglobulin gene locus to generate the heavy chain portion of the BCR. Each pro B cell has two consecutive chances to rearrange the heavy chain (one on each chromosome) to generate a productive heavy chain. A functional heavy chain allows for continued survival of the pro B cells and generates signals that terminate further gene rearrangement on the heavy chain locus[170].

Upon successful rearrangement of the heavy chain, pro B cells are now classified as pre B cells. At this stage, the pre B cell undergoes multiple cell divisions to generate a clonal population of the same functional heavy chain. Each progeny cell would then begin rearrangement of the light chain locus, resulting in increased diversity of BCR arrangement from the same heavy chain. A productive light chain rearrangement results in the generation of an immature B cell bearing a functional BCR in the form of membrane bound IgM[176]. Immature B cells undergo negative selection within the bone marrow where self-reactive B cells are either eliminated via mIgM crosslinking after binding with cognate self-antigen[173] or undergo further gene editing of the light chain locus[177]. The immature B cells that remain then exit the bone marrow compartment into circulation as naïve B cells, ready for activation upon recognizing cognate antigen. Typically, only around ten percent of B cells generated enter the pool of B lymphocytes circulating in the bloodstream[178].

3.3 B CELL ACTIVATION AND THE HUMORAL RESPONSE

Most naïve B cells in the periphery never encounter their cognate antigen and therefore die by apoptosis within a few weeks[178]. B cell activation typically requires the two signals, antigen crosslinking of the BCR as the primary signal[160] and T-cell help via CD40 and CD40L

interactions[179]. This mode of activation is known as thymus-dependent (TD) antigen activation. However, thymus-independent (TI) antigens are also present which do not require T-cell help but rely on multimeric BCR crosslinking by antigen. The mode and action of TI antigens are less complex and different than TD antigens, with TI antigenic responses being closer to those of the innate immune system[180, 181].

3.3.1 Induction of the humoral response.

B cell activation typically occurs within lymph nodes and involves TD antigens. The lymphatic system drains excess tissue fluid from various tissues and is thus an ideal site for lymphocytes such as B cells to encounter antigen[174].

The binding of cognate antigen with the BCR results in the assembly of large signaling complexes on the surface of the B cell, involving various molecules such as CD19, CD21 and CD81, that results in the increased expression of MHC class II and costimulatory molecule B7 on the B cell surface[162]. After BCR recognition and binding, the antigen is internalized via receptor mediated endocytosis, processed within the endocytic pathway and presented on the MHC class II molecules to recruit T-cell help. Once a corresponding T-cell recognizes the presented peptide, a T-B cell signaling conjugate is formed to facilitate further cellular interaction via CD40/CD40L and B7/CD28 interactions or cytokine release. This process typically occurs at the interface of the cortex and the paracortex of a lymph node which is rich in B cells and T-cells respectively[182-184].

Once antigen mediated B cell activation occurs, the activated B cell undergoes intense proliferation, forming a clonal population of B cells[185]. These B cells also begin to move further into the cortex, forming small foci of activated cells. Some of the activated B cells differentiate

into plasma cells within the foci to begin producing IgG and IgM antibodies which constitutes the primary antibody response[172]. These foci of cells are thought to migrate into the follicles present within the cortex of the lymph node which contains follicular dendritic cells (FDC). The follicular dendritic cells are uniquely suited for presenting antigen to B cells as they possess long cellular extensions with extensive Fc receptors and complement receptors, allowing for retention and presentation of antigen-antibody complexes over long periods on the cell surface[186]. The interaction of activated B cells with these FDCs is essential for further B cell activation, antibody class switching and differentiation into plasma cells[185].

3.3.2 Antigen induced B cell Differentiation.

The migration of activated B cells into the follicles of the cortex eventually results in the formation of a germinal center, which are regions of intense B cell proliferation. Germinal centers are crucial structures in the course of B cell differentiation as these are sites where B cells undergo affinity maturation via somatic hypermutation of the BCR and antibody class switching. Formation of B memory cells and terminal differentiation into plasma cells also occur concurrently as B cells alter the BCR specificity[174, 185]. These structures are typically formed within the lymph node upon B cell encounter with antigen. However, within instances of chronic inflammation due to persistence of antigen, germinal centers can form ectopically within involved tissues[187].

Antibody affinity maturation describes the observation whereby the antigenic specificity of an antibody towards its cognate antigen *in vivo* increases significantly over time from the moment antigen is encountered. This observation was eventually attributed to somatic hypermutation, a process whereby activated B cells utilize the enzyme activation induced

deaminase (AID) to deliberately introduce random mutations specifically within the rearranged V(D)J segments of the immunoglobulin gene[188, 189]. The randomness of such mutations within the variable region of the immunoglobulin gene coupled with the high rates of cell proliferation allows for the B cell to modify the affinity of the BCR receptor to its cognate antigen[174, 185].

A selection mechanism is in place to direct somatic hypermutation, ensuring that the resulting BCR binds cognate antigen with greater affinity. Within germinal centers, FDCs take on the role of presenting antigen to B cells undergoing somatic hypermutation. Membrane BCR crosslinking of B cells by the antigen on the FDC permits continued B cell survival. Due to the miniscule amount of antigen present on the FDCs, only B cells that have higher affinity BCR for cognate antigen are successful in binding antigen and survive[186]. Upon binding antigen on the FDC, activated B cells need to be able to internalize the antigen and present them for T-cell help. The interaction with T helper cells by B cells is also an essential component for continued B cell survival[182]. After repeated process of somatic hypermutation and selection, subsequent B cells would have BCR far more efficient at antigen recognition and binding than the initial B cell that first encountered the antigen[185].

Activated B cells may further modify the BCR by altering the isotype of the heavy chain constant domain, a process termed antibody class switching. Class switching allows the antibody to retain its antigen recognition specificity but enables different biological effector activity depending on the heavy chain isotype. Class switching is thought to occur when the heavy chain DNA undergoes further rearrangement by a class of switch recombinase enzymes upon cytokine signaling[190-192]. CD40/CD40L interactions are also required for class switching to occur as patients with defects with CD40L on T-cells suffer from X-linked hyper-IgM syndrome. These

patients lack memory B cells, lack germinal centers and are only capable of generating IgM but not the other isotypes[193].

The humoral response to thymus dependent antigens relies very heavily on class switching from IgM to other antibody isotypes. To date, there are five known classes of antibody isotypes, IgM, IgD, IgG, IgA and IgE, each with well-defined biological roles regarding pathogen mediated responses[190] (Fig. 3).

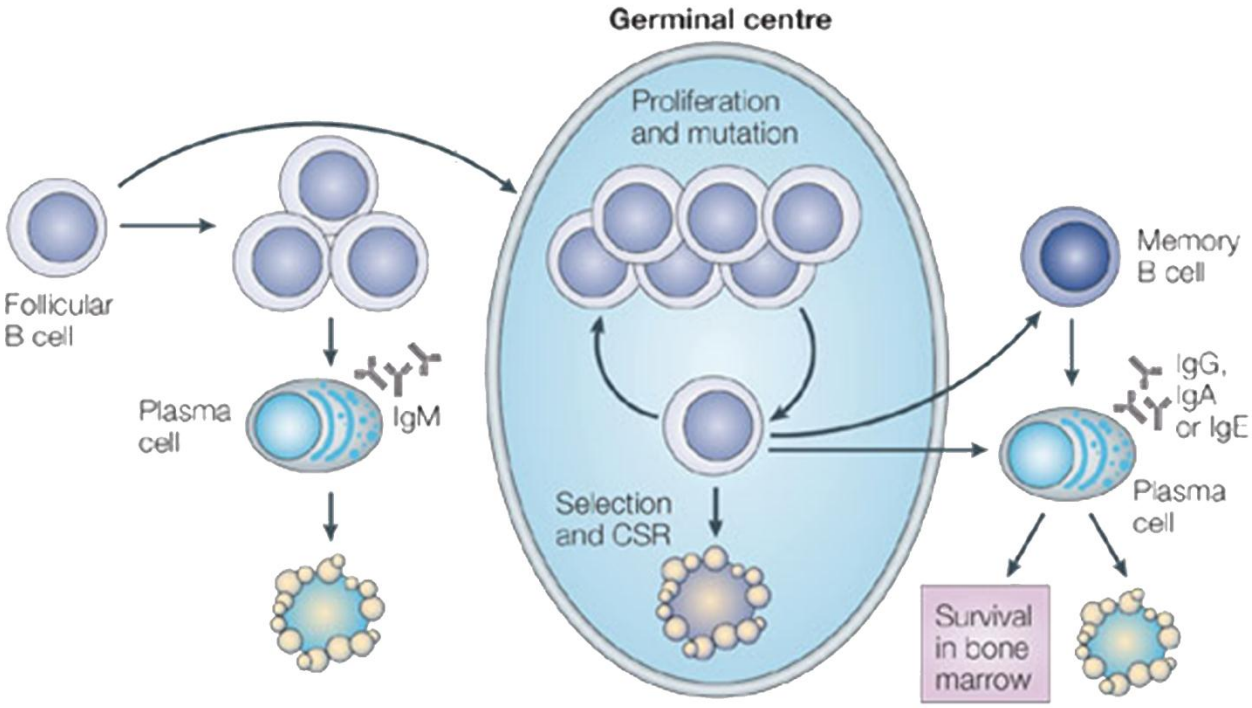

Figure 3. *B cell activation and differentiation into plasma cells upon antigen encounter.* Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Immunology[153], copyright 2005.

3.3.3 Antibody-mediated interactions.

Antibodies mediate a wide range of immune activities against encountered antigen and by extension pathogens. As mentioned, the antibody isotype can specify for distinct effector functions but antibodies also elicit shared biological activities.

Antigen recognition by antibodies is a means for the immune system to tag and bind foreign matter for elimination via the formation of antibody antigen immune complexes[155, 156, 158]. Binding of the Fc portion on antibodies of such complexes with Fc receptors on immune cells allow for targeted cytotoxic activities on antibody coated pathogens or cells via antibody-dependent cell-mediated cytotoxicity (ADCC). This process involves the targeted delivery of lytic enzymes that kill antibody bound targets or can involve the delivery of apoptotic signals to antibody coated cells. ADCC is typically mediated by natural killer (NK) cells, macrophages and eosinophils. In the case of ADCC, antibodies function as receptors that direct clearance actions on bound targets[155].

IgE antibodies are primarily associated with the clearance of multicellular parasites and helminthes and allergic responses. IgE functions by priming nonphagocytic cells such as mast cells, basophils and eosinophils via the binding of Fcε receptors[194, 195]. Upon recognition of antigen, the prebound IgE on the Fcε undergoes crosslinking and receptor ligation, resulting in cell degranulation of vesicles loaded with inflammatory mediators such as TNF and leukotriene C4 along with proteins affecting vascular permeability such as histamine[196].

Antibodies are also capable of activating complement, a collection of serum glycoproteins with several different functions. Complement proteins, when properly activated, can perforate cell membranes leading to cell death. Byproducts of the complement activation cascade such as C3b can mediate antigen clearance by binding to the complement receptor on macrophages. Complement activation is primarily mediated by IgM and several subclasses of IgG in humans[157].

IgA antibodies are the main components of humoral immunity which protect mucosal surfaces such as the gastrointestinal and reproductive tract from pathogenic invasion. Circulating

IgA antibodies and those synthesized by plasma cells of the lamina propria in the gut are transported across the epithelial layer by polymeric immunoglobulin receptors (pIgR) expressed on the basolateral surface into the lumen[197]. Within the luminal space, IgA acts by binding and neutralizing pathogens and any pathogen derived toxins from causing direct damage to the epithelium[154].

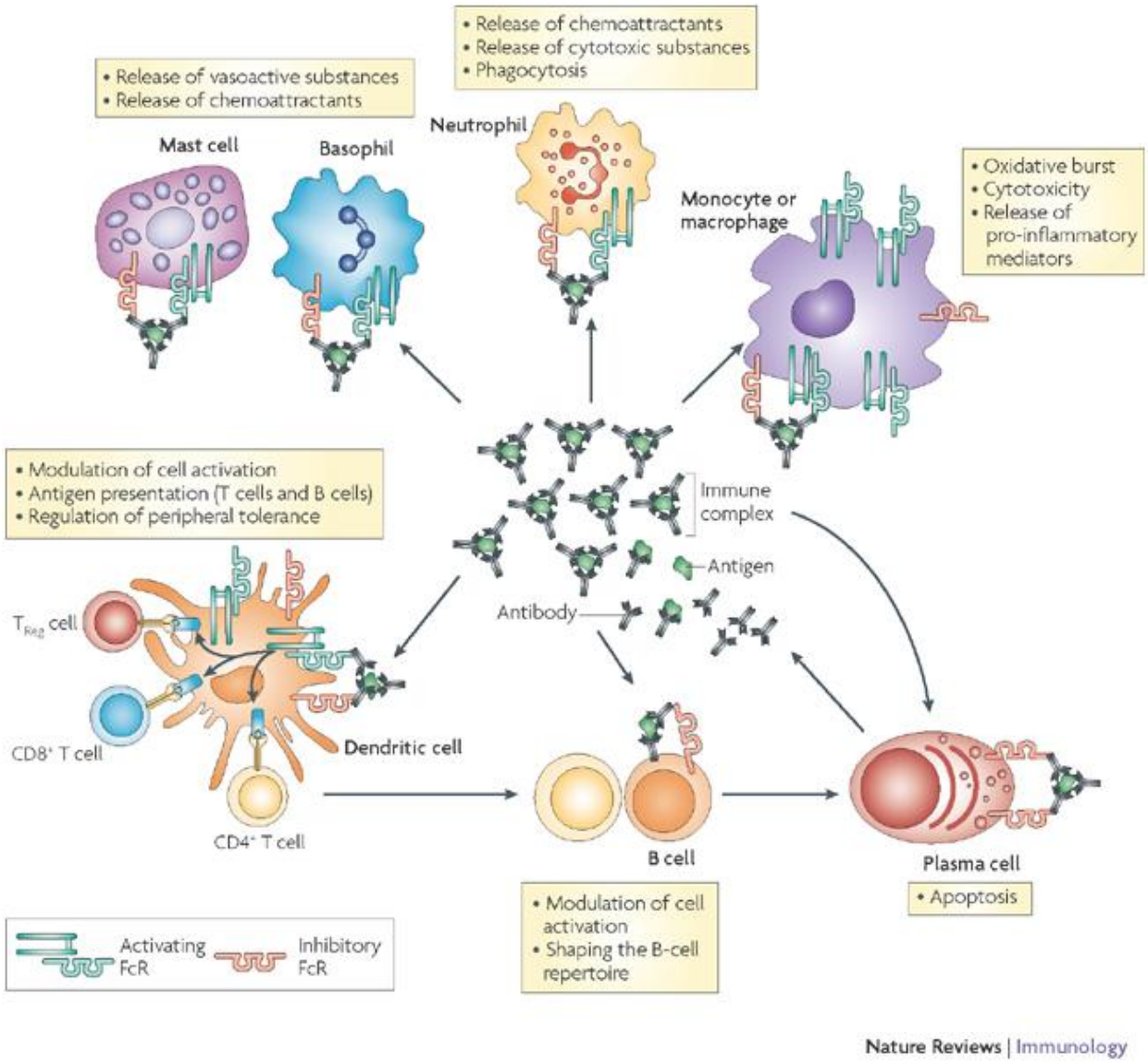

Figure 4. *Possible mechanism of action for antigen antibody complexes and FcγR receptors on various immune cells.* Reused with permission from Macmillan Publishers Ltd: Nature Reviews Immunology[198], copyright 2008.

Antibodies bound to antigen can also directly enhance phagocytosis by macrophages and neutrophils via opsonization. Due to the multimeric nature of antibodies, antigen antibody binding leads to the formation of immune complexes which agglutinates antigenic particles, allowing for more efficient clearance by phagocytic cells. Uptake of antibody bound complexes requires the crosslinking of Fc receptors present on macrophages and neutrophils to signal for phagocytosis[156, 158] (Fig.4).

3.3.4 Fc receptor biology.

Each antibody isotype has its respective Fc receptor that binds to the Fc portion of the antibody[199, 200]. As mentioned above, these receptors allow the various antibody isotypes to elicit specific immunologic responses designed to enhance pathogen clearance. Fc receptors also allow for immune complexes to be internalized by antigen presenting cells to mediate further immune activation particularly for T-cell activation. Lastly, Fc receptors help to clear the extracellular environment of antigen to mediate anti-inflammatory responses after immune activation to prevent excessive damage to host tissue[52, 200, 201].

Fc receptors are typically members of the immunoglobulin superfamily and recognize the Fc portion of the heavy chain of each antibody isotype. Each Fc receptor has a distinct cell expression profile that corresponds to the specific biological activity of the antibody isotype. All Fc receptors require crosslinking and ligation of multiple receptors to initiate cell signaling. Receptor crosslinking is typically achieved by the association of Fc receptors with immune complexes as it contains multiple Fc ends that bind to multiple Fc receptors on the cell surface, initiating receptor ligation and cell signaling[52, 159, 201].

Almost all Fc receptors, upon ligation, signal for immune activation except for FcγRIIB which negatively regulate cell activation upon recruitment and ligation. Fc receptors are fairly conserved between mouse and humans although humans have an expanded repertoire of 6 activating FcγR members compared to the 4 found in mice. FcγRIIB is primarily found on B cells, plasma cells, macrophages, mast cells, and neutrophils and is thought to terminate cell activation upon recruitment into the Fc receptor signaling complex by immune complexes[201, 202] (Fig. 5.).

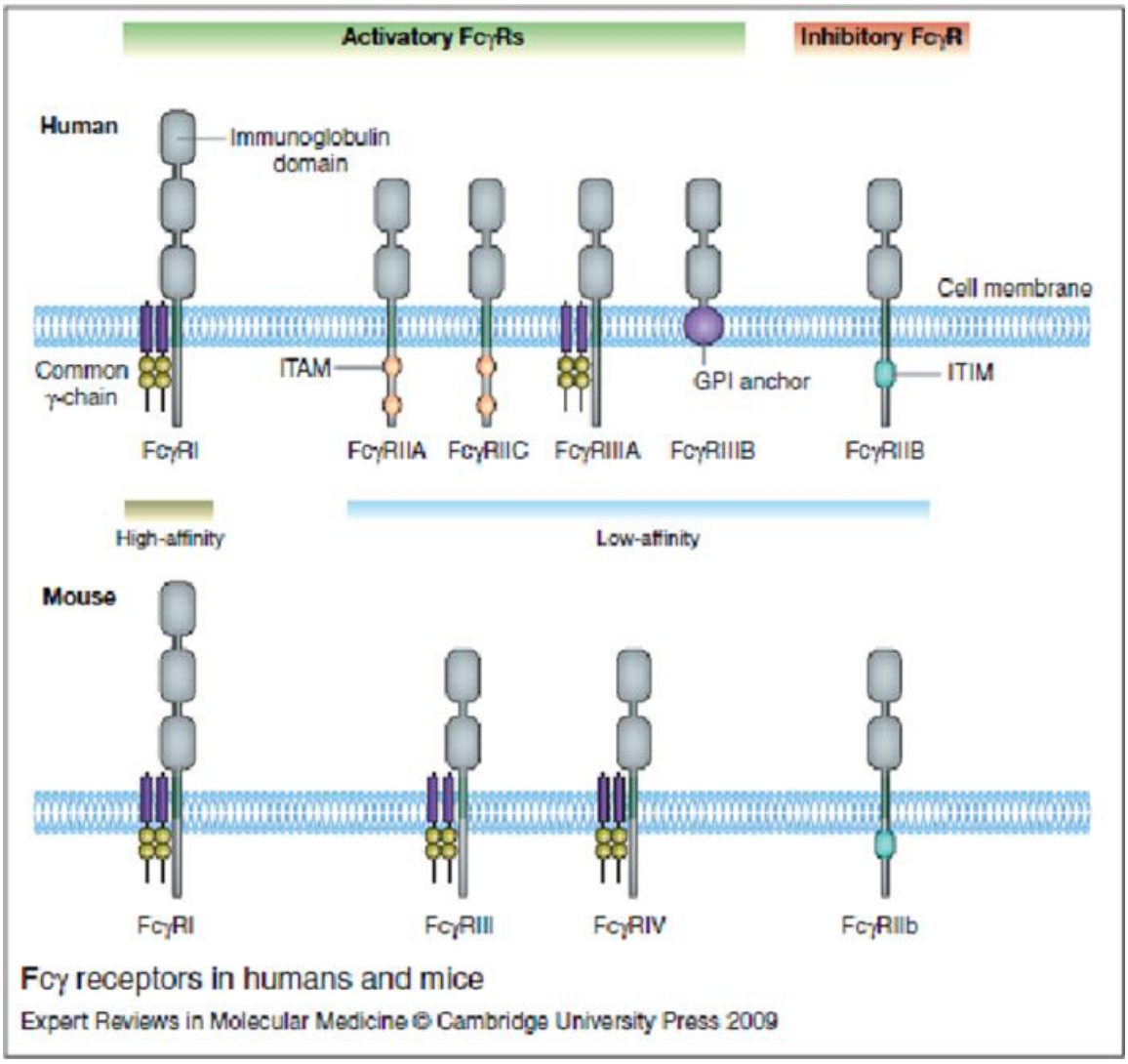

Figure 5. *Members of the FcγR receptor family of humans and mice.* Reused with permission from Cambridge University Press: Expert Reviews in Molecular Medicine[52], copyright 2009.

Cell activation by Fc receptor complexes depends on several factors which dictate the signaling context for the cell. These factors are the type and ratio of Fc receptor expressed on the immune cell, the extracellular microenvironment which the cell resides in and also the size of immune complexes present[198, 201-203].

As mentioned above, the presence of inhibitory FcγRIIB in the signaling complex impairs cell activation. Thus, a signaling complex with FcγRIIB would require a larger amount of other activating Fc receptors present within the complex to elicit cellular activation. The ratio of activating and inhibitory receptors present on the cell surface helps to illustrate the ease of activation for the particular immune cell upon ligation with antibody antigen immune complexes[52, 159, 198].

The extracellular microenvironment is a major determinant for Fc receptor activation thresholds as cytokines are able to influence the expression pattern of Fc receptors on immune cells such as macrophages and B cells. IFNγ has been shown to upregulate activating FcγRIIA and FcγRIII on macrophages while IL4 tends to increase FcγRIIB expression on the cell surface of macrophages but decrease FcγRIIB on B cells. Such responses towards cytokine signaling thus alters the activation inhibition ratio and hence, the activation threshold of specific immune cell lineages[52, 204].

Cellular activation can also be influenced by the size of the antibody antigen immune complex[205]. Large immune complexes are formed when there is an excess of antibody compared to cognate antigen while smaller immune complexes results from antigen excess. These large complexes are thought to possess more free Fc ends that can recruit more Fc receptors into a signaling complex on the cell surface. Thus, the probability of crosslinking FcγRIIB in a cell signaling complex is higher compared to interactions of smaller immune complexes with less Fc

ends, which favors cellular activation. Macrophages have been shown to respond differently when fed with immune complexes of varying sizes *in vitro*. Larger immune complexes tend to favor IL10 production by macrophages while smaller complexes tend to result in IL12 production[206]. The different modes of cell behavior elicited by immune complexes of different sizes may be a means by which the immune system detects antigen concentration via antibodies and relay the appropriate response via Fc receptor recruitment depending on immune complex sizes.

Fc receptors are also known to be cross-linked by pentraxins, a family of acute phase immune proteins such as serum amyloid P (SAP) and C reactive protein (CRP). Both examples are involved in innate immune recognition of bacterial and fungal pathogens and are capable of inducing complement activation[196, 197]. Hence, Fc receptors are a robust sensing system that allows immune cells to respond to cues from both the innate and adaptive arms of the immune system.

3.4 THE ROLE OF HUMORAL IMMUNITY IN TUBERCULOSIS

The humoral response has not been a focus of anti-tuberculous immunity. The intracellular nature of the pathogen, hiding within the phagocytotic cells, would logically render the immunogenic epitopes of the bacteria invisible to circulating antibodies. In any case, antibody binding is a mechanism to promote and enhance phagocytosis. Even with antibody recognition and binding to *M. tuberculosis*, such processes would only facilitate the entry of the pathogen into phagocytic cells to propagate infection[50].

Historical attempts to develop passive immunization therapies against *M. tuberculosis* infection by introducing antisera from various animals into human patients provided inconsistent results ranging from no improvement to disease exacerbation, likely attributed to the different preparations of antisera for treatment[207]. As antibiotics were introduced and were found to be more effective as a treatment agent, interest in defining humoral immune responses against tuberculosis as a possible treatment modality began to wane[208].

With the discovery of the T-cell arm of cellular immunity and the central role they play in neutralizing intracellular pathogens, scientific interests were more focused on defining the protective responses of T-cells rather than humoral components of tuberculosis immunity. As T-cell responses such as IFNγ and TNF signaling pathways were characterized, humoral immunity against TB was not as actively pursued beyond defining antibodies as biomarkers to distinguish between active and latent infections[69, 84, 85, 88, 89, 135].

However, with recent findings of humoral immunity being capable of influencing antigen presentation, T-cell activation and macrophage activation phenotypes, there is renewed interest in defining the role of B cells and antibody in tuberculosis immunity. By better defining the role of humoral immunity towards *M. tuberculosis*, vaccine development can be enhanced by incorporating components that elicit specific modes of cellular activation better suited for dealing with intracellular pathogens such as *M. tuberculosis* or providing better readouts for correlates of protection. Furthermore, genetic variation pertaining to humoral immunity might very well influence individual risks of infection and disease outcome in terms of developing active and latent disease. Such findings are crucial as it can help identify populations at risk of infection or developing active disease which ultimately aids in providing better diagnosis and treatment[208-210].

3.4.1 B cell contributions to tuberculosis control.

The role of B cells and antibody in tuberculosis remains largely undefined compared to the contribution of T-cells towards disease control. Tissue sections from lung granulomas of infected animals or lung tissue resections of *M. tuberculosis* infected patients had B cells present within the site of the lesion[211, 212]. These observations suggest that B cells are activated and are trafficking into the granuloma. Further characterization of these B cells suggests that they are highly proliferative, via Ki67 staining, and express CD21, a marker of germinal center B cells, within the granuloma[213, 214]. These data further suggest that B cells appear to be forming ectopic germinal centers at the site of the infection. However, the precise mechanism of how B cells contribute towards *M. tuberculosis* containment is still undetermined.

Cytokine production by B cells has not been characterized as extensively as T-cells although B cells are known to produce a variety of cytokines such as IL6 and IL10[164]. Indeed, the contribution of B cell cytokine production towards a variety of autoimmunity conditions such as multiple sclerosis is increasingly being recognized[165]. However, B cell cytokine production in infectious disease immunity is relatively unknown although B cells are known to influence cytokine production by other immune cells[164, 165]. In the context of tuberculosis, μMT mice lacking B cells were observed to have elevated IL10 production within infected lungs compared to infected wild type control mice. In addition, infected B cell depleted mice had substantial infiltration of neutrophils into the lungs, indicating that rampant inflammation is occurring. The massive inflammatory response was absent within μMT mice reconstituted with B cells from tuberculosis infected wild-type mice. This suggests that B cells are providing some amount of immunologic control towards tuberculosis infection[211, 215]. In humans, B cells were found to be able to alter IL17 and IL22 cytokine production of lymphocytes from pleural fluid of *M.*

tuberculosis infected patients *in vitro*[213]. Thus, B cells have an enormous capacity to alter the cytokine milieu at the site of infection either by providing signaling cues to other cells or by producing the cytokines themselves.

3.4.2 Antibody contributions towards Tuberculosis control.

Although antibody is a crucial component in immunity against pathogens, its contribution towards tuberculosis control is unknown. Many studies involving antibody attempted to identify possible antibody signatures within serum from human patients as potential biomarkers for diagnosing active and latent tuberculosis infection. Although certain *M. tuberculosis* antigens were identified as being very immunogenic in terms of inducing antibody titers, very few studies were done to elucidate the mechanism of action by antibody against the bacilli[207, 210].

What is known is that antibodies can alter macrophage microbial killing capacity as antibody coated *M. tuberculosis* is more readily killed after phagocytosis *in vitro* compared to uncoated bacilli. This suggests that the presence of antibody induces the bacilli to be taken up via a separate pathway, possibly via FcγR, leading to more bacterial killing[50]. *In vivo* experiments involving transfer of infected wild-type mice serum into infected μMT mice revealed that infected μMT did better with lower inflammation within the lungs when treated with infected mouse serum. These findings clearly delineate a role of antibody in regulating macrophage activation and tissue inflammatory reactions[215].

As mentioned previously, antibody interacts with the rest of the immune system via the Fc receptor family, particularly FcγR for IgG. FcγR has been implicated in a variety of autoimmune conditions and infections where variations within specific FcγR molecules are associated with susceptibility to systemic lupus erythematosus[203] and malaria[52]. Findings of

tuberculosis infection in mice deficient in particular FcγR revealed similar susceptibilities towards tuberculosis infection; mice lacking in the inhibitory receptor FcγRIIb had less pathology in the lungs compared to both wild-type mice and mice lacking all activating FcγR[216].

Taken together, these findings suggest that antibodies are providing a certain degree of control over inflammatory processes that influences the outcome of tuberculosis infection, at least in mice. Humans and non-human primates have an expanded repertoire of FcγR compared to mice[52]. By understanding how anti-tuberculosis antibodies can manipulate immune cell activation, future vaccine candidates can be improved to maximize anti-microbial capacity of the immune system while ameliorating excessive inflammation to reduce tissue damage from bystander responses.

4.0 STATEMENT OF THE PROBLEM

M. tuberculosis is an intracellular pathogen that is the causative agent of tuberculosis, affecting a third of the world's population. 10 percent of infected individuals develop clinical symptoms of active tuberculosis while the remaining 90 percent are latently infected as they successfully contain the disease and remain clinically asymptomatic. In order to contain the bacilli, the host immune system forms an organized conglomeration of cells known as a granuloma. The granuloma is thought to function as a physical and immunological barrier, keeping *M. tuberculosis* contained by walling off infected macrophages at the center of the structure. However, the granuloma is a dynamic structure that depends on host-pathogen interactions to maintain itself. Failure to control the bacilli within the granuloma allows for bacterial proliferation and dissemination throughout the host resulting in reactivation within latent individuals. To date, the contribution of humoral immunity towards containing *M. tuberculosis*, particularly the role B cells and antibody, remains largely unknown.

The cynomolgus macaque model of *M. tuberculosis* infection has been shown to recapitulate human tuberculosis very closely, allowing for the dissection of immunologic and microbiologic events during infection not possible in human infection. This model would thus permit us to examine the role of the humoral immune system in the contribution towards *M. tuberculosis* containment. The first aim of the study focuses on defining B cells present within the granuloma and examining the antibody content within infected tissue from the lung at

necropsy. We found that B cells are organizing into ectopic germinal centers within the lymphocytic cuff of the granuloma. Antibodies specific to *M. tuberculosis* antigens are also present at higher quantities within granulomatous tissue compared to normal lung. By understanding how the humoral immune system is organized within sites of infection, we can thus determine how B cells and antibody interact with other immune cells such as macrophages and T-cells to contain the infection.

The second portion of the study focuses on examining how *in vivo* B cell depletion alters the course of infection within the non-human primate model of tuberculosis. B cell depletion was achieved using Rituximab, a chimeric monoclonal anti-CD20 antibody that is widely used in clinical cases of autoimmunity and lymphoma. B cells numbers were tracked throughout the course of the study to ensure adequate depletion within the peripheral and the lymphoid compartment. *M. tuberculosis* infected animals treated with saline were used as controls. We used tissue bacterial burden, disease presentation at necropsy, T-cell responses and tissue cytokine content as a basis of comparison between both B cell depleted and control groups. The findings from this study indicate no difference in the control of *M. tuberculosis* within the first 10 weeks after infection with or without B cells. These findings address the second aim of the study in determining the effects of *M. tuberculosis* infection with B cell depletion.

The third portion of the study looks at how macrophages behave in the absence of B cells and antibody. This part of the study aims to examine the content of two enzymes that specify for different modes of activation for macrophages, inducible nitric oxide synthase (iNos) for pro-inflammatory macrophages and Arginase-1 (Arg1) for anti-inflammatory macrophages and how these enzymes are affected by levels of antibody within the granuloma.

4.1 SPECIFIC AIM 1: DEFINE THE ROLES OF B CELLS WITHIN THE GRANULOMAS OF CYNOMOLGUS MACAQUES INFECTED WITH *M. TUBERCULOSIS.*

Hypothesis: Granulomas of infected cynomolgus macaques have activated B cells and more antibodies specific to M. tuberculosis antigens relative to normal lung tissue

Understanding how the humoral response organizes itself within the granuloma will allow for further investigation into the interaction of B cells and antibody along with other cells within the granuloma.

4.2 SPECIFIC AIM 2: EXAMINING THE EFFECTS OF *IN VIVO* B CELL DEPLETION VIA RITUXIMAB IN *M. TUBERCULOSIS* INFECTED CYNOMOLGUS MACAQUES.

Hypothesis: B cell depleted animals would have a worse disease outcome compared to infected control animals.

B cells are hypothesized to be essential within the granuloma in terms of generating antibodies specific to *M. tuberculosis* and cytokines specifying for adequate cellular activation. By depleting B cells, granulomas are less able to organize an effective immune response in containing the bacilli. B cell depleted animals are hypothesized to present with more disease burden at necropsy compared to control animals.

4.3 SPECIFIC AIM 3: EXAMINING THE EFFECT ON MACROPHAGE ACTIVATION WITHIN THE GRANULOMAS OF B CELL DEPLETED ANIMALS.

Hypothesis: Macrophages within granulomas of B cell depleted animals will have higher levels of Arg1.

Antibodies are known to set the threshold of activation of macrophages via signaling through Fc receptor crosslinking. In the absence of antibodies, as in the case of B cell depleted animals, macrophages are hypothesized to be less capable of activation for bacterial killing via the production of iNos.

5.0 ACTIVATED B CELLS IN THE GRANULOMAS OF NONHUMAN PRIMATES INFECTED WITH *MYCOBACTERIUM TUBERCULOSIS*

This chapter is adapted from the original publication

Phuah JY, Mattila JT, Lin PL, Flynn JL: Activated B cells in the granulomas of nonhuman primates infected with *Mycobacterium tuberculosis*, The American journal of pathology 2012, 181:508-514

5.1 ABSTRACT

In an attempt to contain *Mycobacterium tuberculosis*, host immune cells form a granuloma as a physical and immunological barrier. To date, the contribution of humoral immunity, including antibodies and specific functions of B cells, on *M. tuberculosis* infection in humans remains largely unknown. However, recent studies in mice have revealed that humoral immunity can alter *M. tuberculosis* infection outcomes. *M. tuberculosis* infection in cynomolgus macaques recapitulates essentially all aspects of human TB. As a first step to understanding the importance of humoral immunity to control of *M. tuberculosis* infection in primates, we characterized the B cell and plasma cell populations in infected animals and found that B cells are primarily present in clusters within the granuloma. The B cell clusters are in close proximity to $PNAd^+$ cells and

contain cells positive for $Ki67^+$, a proliferation marker. Granuloma B cells also express CXCR5 and has elevated HLA-DR expression. Tissues containing *M. tuberculosis* bacilli had higher levels of *M. tuberculosis* specific IgG, compared to uninvolved tissue from the same monkeys. Plasma cells were detected within the granuloma, and produce mycobacteria-specific antibodies. Together these data demonstrate that B cells are present and actively secreting antibodies specific for *M. tuberculosis* antigens at the site of infection, including lung granulomas and thoracic lymph nodes. These antibodies likely have the capacity to modulate local control of infection in tissues.

5.2 INTRODUCTION

Mycobacterium tuberculosis (*M. tuberculosis*) is an intracellular pathogen that is the causative agent of tuberculosis (TB), an infection that is estimated to affect a third of the world's population. Ten percent of infected individuals develop clinical symptoms of active TB while the remainder develops latent infection, which is clinically asymptomatic but can reactivate to cause active TB[217-219]. In response to *M. tuberculosis* infection, the host immune system forms an organized conglomeration of cells known as a granuloma. Granulomas are crucial in control of mycobacterial pathogens as they function as an immune and physical barrier to prevent widespread bacterial dissemination within the host[220]. Proper control of *M. tuberculosis* requires immune cells within the granuloma to kill internalized bacilli by activating macrophages while simultaneously balancing anti-inflammatory signals to reduce tissue damage. T-cells in particular play a critical role in activating macrophages via the release of interferon gamma (IFNγ) and

tumor necrosis factor (TNF)[83, 221]. The contribution of B cells to control of human *M. tuberculosis* infection and pathology remains unknown.

Upon activation by antigen, mature B cells proliferate and differentiate into plasma cells for the sole purpose of generating antigen specific antibodies. Antibodies can affect host-pathogen interactions by enhancing phagocytosis, antibody dependent cell cytotoxicity, and blocking pathogen-host receptor interactions[50, 52]. Antibody mediated phagocytosis can modify macrophage behavior depending on how the Fc portion of antibodies interact with the Fc receptors expressed on macrophages[206, 222]. B cells also present antigen to T-cells and enhance $CD4^+$ antigen specific T-cell expansion[223, 224]. B cell depletion slows disease progression of what are predominantly T-cell mediated autoimmune conditions such as multiple sclerosis[225, 226] and type 1 diabetes[227] in mice and in humans. These studies reinforce the notion that B cell antigen presentation is capable of mediating further effects on T-cells to drive immune activation in the presence of antigen.

As *M. tuberculosis* is primarily an intracellular bacillus, the contribution of humoral immunity to protection was thought to be minimal. Studies in the late 19th and early 20th centuries on the protective effects of passive immunization yielded conflicting results which have been attributed to antisera preparation[210]. *M. tuberculosis* infection of B cell deficient mice have also yielded variable findings ranging from increased pathology or bacterial burden to no apparent change in disease progression[211, 228]. The inconsistencies in these mouse studies make it difficult to ascertain the role of the humoral response against tuberculosis in humans. However, several studies have indicated that other components of the humoral response such as Fc receptors[216], polymeric Ig receptors[229] and IVIg[230] can affect the outcome of *M. tuberculosis* infection in mice. These studies suggest that B cell responses can confer protection against *M.*

tuberculosis infection either directly or by modulating cellular immune responses such as macrophages, T-cell priming and activation.

In the mouse model of *M. tuberculosis* infection, B cells are present in the lungs, often in aggregates that stain positive for PNA, reminiscent of germinal centers in lymph nodes[211, 231]. Some studies have suggested that granulomas may also function as tertiary germinal centers where the T-cell population is continuously activated via antigen presentation by B cells. B cell aggregates have been identified in human lung tissue from TB patients[212, 232]. However, how these B cell aggregates function in the context of *M. tuberculosis* control is still unknown.

This study aims to determine the characteristics of B cells and plasma cells within the granulomas of *M. tuberculosis*-infected cynomolgus macaques. The NHP model of *M. tuberculosis* infection has been shown to mimic all aspects of human tuberculosis, particularly in granuloma type and structure.

5.3 MATERIALS AND METHODS:

5.3.1 Experimental animals

Samples from 14 adult (>4 years of age) cynomolgus macaques (*Macaca fascicularis*) (Covance, Alice, TX; USA Valley Biosystems, West Sacramento, CA) experimentally infected with *M. tuberculosis* for other studies were obtained for the B cell studies described here. All animals were housed under BSL-3 conditions. These studies followed all animal experimentation guidelines and all experimental manipulations and protocols were approved by the University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee.

5.3.2 *M. tuberculosis* infection

Cynomolgus macaques were infected with 25-200 CFU Erdman strain *M. tuberculosis* via intra-bronchial instillation as previously described[42, 43]. Infection was confirmed by conversion of negative to positive tuberculin skin test and elevated PBMC responses to mycobacterial antigens from baseline in lymphocyte proliferation (LPA) and ELISPOT assays[43, 233]. The tissues used for the studies described here were samples from *M. tuberculosis* infected control monkeys involved with other TB studies.

5.3.3 Necropsy procedures

Monkeys were maximally bled prior to necropsy and euthanized using pentobarbital and phenytoin (Beuthanasia; Schering-Plough, Kenilworth, NJ). Gross pathologic findings were described by a board-certified veterinary pathologist (EK) and were classified as previously described. Representative sections of each tissue were placed in formalin for histologic analysis or homogenized into single-cell suspensions for immunologic studies, flow cytometric analysis, and bacterial burden, as previously described[42, 43, 233]. Bone marrow was also obtained from the sternum by flushing exposed bone marrow with tissue grade 1xPBS (Lonza, Walkersville, MD) using a 20 gauge needle (BD, Franklin Lakes, NJ). Red blood cells were lysed using the BD Pharm Lyse buffer (BD Biosciences, San Diego, CA) according to manufacturer's instructions prior to cell enumeration. Tissue homogenates from numerous necropsy samples were serially diluted and plated on 7H11 media (BD, Sparks, MD) and CFUs were enumerated on day 21. Lung samples with culturable *M. tuberculosis* were classed as 'involved' tissue (Inv) while samples with no cultivable bacteria were 'non-involved' (Unv). Lymph node tissues were

classified based on anatomical location with thoracic lymph nodes being lung-draining (dLN) and other lymph nodes being peripheral (pLN).

5.3.4 Histologic analysis and immunohistochemistry

Tissue sections were embedded with paraffin and stained with haemotoxylin and eosin (H&E). These sections were then reviewed microscopically by a veterinary pathologist (EK) with specific emphasis on granuloma characteristics as described previously[233]. Serial sections were used for immunohistochemistry staining of cell markers. Briefly, slides were processed in antigen retrieval buffer (Tris Base, 0.05% Tween 20, pH9) prior to staining. Antibodies used for staining were anti-human CD20 (rabbit polyclonal, Neomarkers, Fremont, CA), anti-human CD3 (rabbit polyclonal, Dako, Carpinteria, CA), anti-human Ki67 (rabbit polyclonal, Neomarkers, Fremont, CA), anti-mouse peripheral node addressin (PNAd) (clone MECA-79, Biolegend, San Diego, CA), anti-rhesus CXCR5 (mouse monoclonal, NIH Nonhuman Primate Reagent Resource, Boston, MA), anti-human CD138 (clone MI5, Neomarkers, Fremont, CA) and biotinylated peanut agglutinin (PNA) (Vector, Burlingame, CA). Cell nuclei were stained with DRAQ5 (Biostatus Limited, Shepshed, UK). Slides were preserved using Prolong Gold with DAPI (Invitrogen, Carlsbad, CA) prior to being visualized with an Olympus Fluorview 500 upright confocal laser scanning microscope (Model BXL21).

For plasma cell detection, fresh tissues were fixed in freshly prepared 4% paraformaldehyde for 6 hours at 37°C, washed with 1xPBS (Lonza, Walkersville, MD), and dehydrated in sucrose solution (30% sucrose, 1xPBS) overnight at 4°C. The tissue was then frozen with cold isobutanol in liquid nitrogen. The frozen tissue was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) and 8µm sections were cut using a cryostat

microtome (Microm International, model HM505N) at -20°C. Sections were placed on gelatin coated slides and air dried overnight at 37°C. The slides were then stained directly as described above.

5.3.5 Flow cytometry analysis of surface markers and quantification of B cells within tissue

Single cell suspensions were derived from granulomatous lung, normal lung, pulmonary draining lymph nodes and peripheral lymph nodes. Cells from these tissue samples were stained for T-cells using anti-human CD3 (clone SP34-2, BD Biosciences), B cells using anti-human CD20 (clone 2H7, eBioscience) and T-cell costimulatory molecules CD86 (clone 2331, BD Pharmingen) and HLA-DR (clone LN3, eBioscience). Lymphocytes were identified based on size (FSC) and granularity (SSC). B cells and T-cells were further identified based on $CD20^+$ and $CD3^+$ expression, respectively. Further characterization for HLA-DR and CD86 were carried out within the $CD20^+$ population. B cell numbers within tissue were calculated by multiplying the percentage of $CD20^+$, percentage of lymphocyte population from flow cytometry, with total cell numbers obtained from tissue homogenate and normalizing to tissue weight.

5.3.6 Intracellular cytokine staining of B cells within tissue

Single cell suspensions derived from granulomatous lung and pulmonary draining lymph node were stimulated with RPMI (Lonza, Walkersville, MD) supplemented with 1% L-glutamine and 1% HEPES (Sigma, St. Louis, MO) containing *M. tuberculosis* CFP10 and ESAT6 proteins (BEI Resources, Manassas, VA) along with Brefeldin A (BD Biosciences), all at a final concentration

of 1µg/ml for 4 hours. After staining for CD20 as described above, cells were then fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) and finally washed with BD Perm/Wash buffer (BD Biosciences). Cells were then stained using anti-human antibodies against IL2 (clone MQ1-17H12, BD Biosciences), IL6 (clone MQ2-6A3, BD Biosciences), IL10 (clone JES3-9D7, eBiosciences), IL17 (clone eBio64CAP17, eBiosciences), TNFα (clone MAb11, eBiosciences) and IFNγ (clone B27, BD Biosciences). All cytokine producing B cells were identified from the $CD20^{+}$ gate.

5.3.7 IgG ELISA

Supernatants obtained from tissue homogenates (granulomatous lung, normal lung, pulmonary draining lymph nodes and peripheral lymph nodes) after necropsy were used to quantify total and *M. tuberculosis* specific IgG. Tissue culture grade 96 well flat bottom plates (Costar, Corning, NY) were coated with 2µg of *M. tuberculosis* culture filtrate protein (CFP) or mouse anti-primate IgG (clone 8F1, NIH Nonhuman Primate Reagent Resource, Boston, MA) dissolved in tissue grade PBS (Lonza, Walkersville, MD) and incubated overnight at 4°C. 1% bovine serum albumin (BSA) in PBS was used to block the plates either at room temperature for 2 hours or overnight at 4°C. Samples were plated in duplicate and serial dilutions were performed at 1:2 with 1% BSA in PBS. Standard curves were generated using purified cynomolgus macaque serum IgG. Horseradish-peroxidase conjugated mouse anti-primate IgG antibody (clone 1B3, NIH Nonhuman Primate Reagent Resource) was used as the detection agent, diluted at 1:5000. 3, 3', 5, 5'-tetramethylbenzidine hydrochloride (Sigma, St. Louis, MO) was used as the chromogenic substrate according to manufacturer's instructions. All incubations were performed for 1 hour at 37°C unless otherwise stated. All washes were performed with 1x PBS.

5.3.8 Plasma cell ELISPOT

96 well ELISPOT plates (Upstate Millipore, Billerica, MA) were coated with *M. tuberculosis* CFP, ESAT6 protein (BEI Resources, Manassas, VA) and mouse anti-primate IgG (clone 8F1, NIH Nonhuman Primate Reagent Resource, Boston, MA) and blocked as described above. Cells from tissues obtained during necropsy (bone marrow, granulomatous lung, normal lung, pulmonary draining lymph nodes and peripheral lymph nodes) were resuspended to $1x10^6$/ml in RPMI (Lonza, Walkersville, MD) supplemented with 1% L-glutamine and 1% HEPES (Sigma, St. Louis, MO). $1.5x10^5$ cells (150µl) were added into each well and were incubated for 16-20 hours at 37°C. Horseradish-peroxidase conjugated mouse anti-primate IgG antibody (clone 1B3, NIH Nonhuman Primate Reagent Resource, Boston, MA) was used as the detection agent, diluted at 1:2500 and incubated for 1 hour at 37°C. Each well was developed using 100µl of 3-Amino-9-ethyl-carbazole (AEC) prepared according to manufacturer's instructions (Vector, Burlingame, CA). All washes were performed with 1x PBS.

5.3.9 Data analysis

Flow cytometry data was analyzed with the FlowJo software package (Tree Star, Ashland, OR). Data were analyzed using Prism 5 (Graphpad Software, San Diego, CA). Fluorescent whole granuloma images are composites of 20 to 40 images taken at 20x magnification and assembled with Adobe Photoshop 7 (Adobe, San Jose, CA). Statistical comparisons were performed using Mann-Whitney test with $p<0.05$ considered statistically significant. Paired samples were analyzed using the Wilcoxon matched-pairs signed rank test with the same threshold, $p<0.05$ as being statistically significant.

5.4 RESULTS

5.4.1 Lung granulomas of NHPs contain aggregates of B cells reminiscent of germinal centers

Serial sections of NHP lung granulomas were characterized histologically then stained for CD3, to identify T cells, CD20 for B cells, and DRAQ5 for cell nuclei. Scattered cells and aggregates of $CD20^+$ cells were observed within the lymphocyte cuff of lung granulomas. $CD3^+$ T-cells assumed a more ubiquitous distribution within the granuloma compared to the clustered appearance of $CD20^+$ B cells and were observed to be closely associated within the B cell clusters. However, $CD3^+$ T-cells were also present closer to the center of the granuloma, which was devoid of $CD20^+$ cells (Fig 6.).

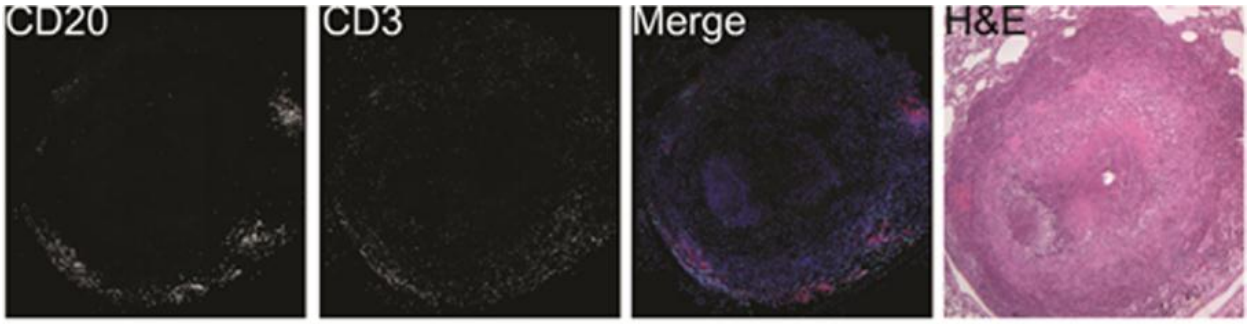

Figure 6. *$CD20^+$ B cells are present within the granuloma as clusters.* A solid granuloma with some caseum and a small mineralized focus was stained for CD3 (green), CD20 (red) and nuclei (blue) to show the distribution of immune cells and with respect to granuloma morphology (H&E). These images show that $CD20^+$ B cells and $CD3^+$ T-cells are found primarily within the lymphocytic cuff (yellow boxes in merged image). B cells generally form clusters but are also present as discrete cells, while T-cells are usually dispersed (top row). All images are composites of 20 to 40 images taken at 20x or 40x magnification.

We hypothesized that the B cell clusters within the granulomas may be similar to ectopic germinal centers. To determine whether the $CD20^+$ clusters have tertiary germinal center characteristics[212], we stained for PNA expression (germinal center B cells)[187, 234], anti-human Ki67 (proliferation) and PNAd (high endothelial venules) [Fig. 7A]. Within a mature germinal

center, Ki67^{+} cells tend to cluster at the center of the B cell follicle to delineate actively proliferating cells (Fig. 7B). Ki67^{+} nuclei were detected within the lymphocytic cuff, indicating that active cellular proliferation was occurring. Cells with Ki67^{+} nuclei were observed within the CD20^{+} B cell clusters in the granuloma but occurred only as isolated cells rather than a distinct cluster (Fig. 7C) compared to mature B cell germinal centers.

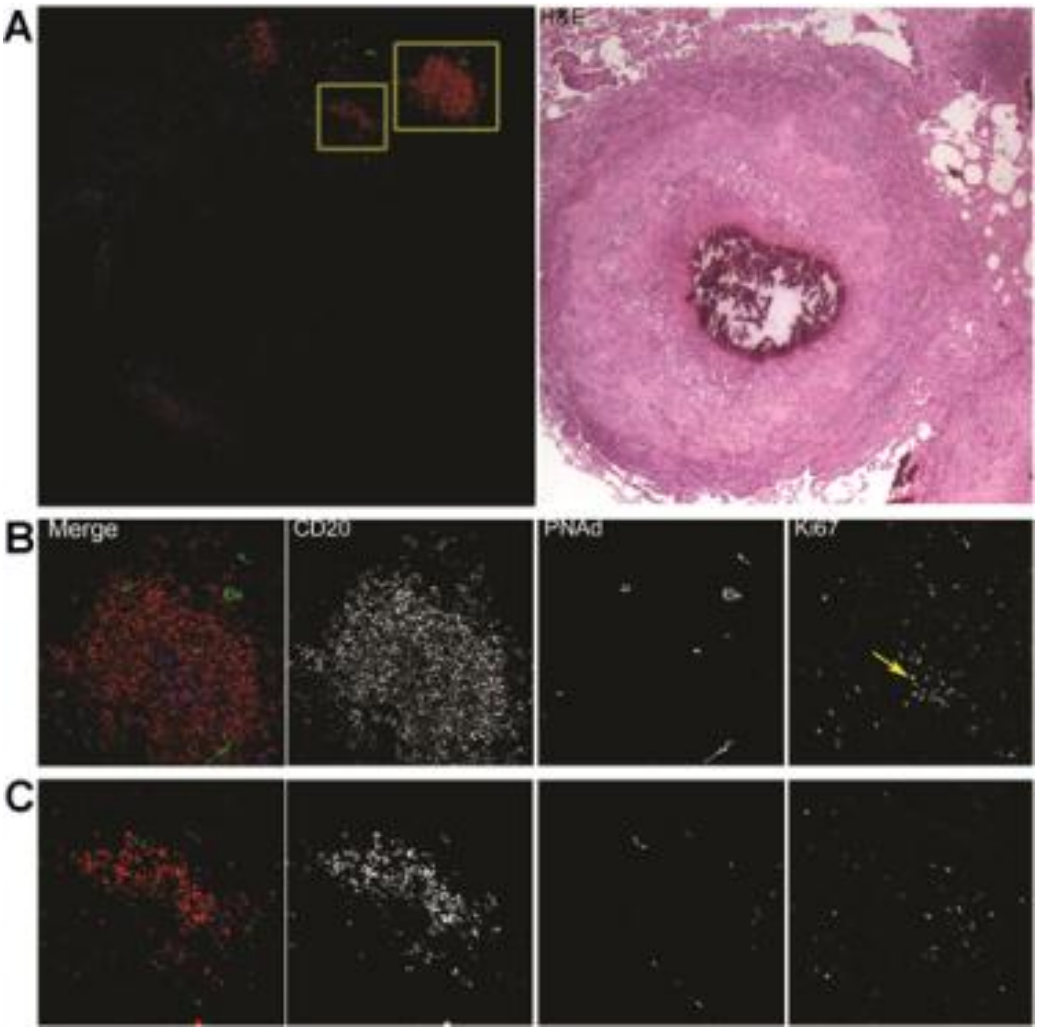

Figure 7. *CD20^{+} B cell clusters within macaque granulomas have germinal center characteristics.* **A)** A caseous granuloma section with a mineralized core (H&E) was stained for CD20 (red), PNAd (green) and Ki67 (blue) to identify B cells, high endothelial venules and proliferating cells, respectively. Two CD20^{+} clusters were imaged in detail for germinal center markers (yellow boxes, top left). **B)** This image depicts a mature BALT cluster exhibiting prominent Ki67 staining within CD20^{+} cells in close association with high endothelial venules (yellow arrow), reminiscent of B cell follicles within lymph nodes. **C)** This image depicts B cell clusters within the lymphocytic cuff of the granuloma, containing some Ki67^{+} nuclei with some sporadic PNAd staining

PNAd is important for lymphocyte chemotaxis and positive expression is associated with the presence of high endothelial venules which is indicative of germinal centers (Fig. 7C). $PNAd^+$ cells were detected in close proximity to $CD20^+$ cells within the lymphocytic cuff of the granuloma (Fig. 7C).

Despite PNA being widely used in identifying germinal centers in the mouse models, no PNA staining was noted within the $CD20^+$ clusters of NHP granulomas, but strong PNA staining was noted within the macrophage layers of the granuloma. Very weak PNA staining was present within the B cell follicles of NHP lymph nodes (data not shown). PNA has also been described to stain human monocytes, macrophages and plasma cells[228] (and unpublished data). These findings suggest that PNA expression is very different in primates compared to mice, and thus we excluded this as a germinal center marker. Thus, these data support that the $CD20^+$ clusters in granulomas display some prominent characteristics of ectopic germinal centers. Further characterization of the B cells within the granuloma was performed to assess cellular activation.

5.4.2 5.4.2. $CD20^+$ cell clusters within lung granulomas contain activated B cells

Flow cytometry was performed on single cell suspensions of lung and lymph node tissues to assess B cell numbers within infected tissue. Lymphocytes were identified based on size and granularity while B cells were further isolated based on $CD20^+$ expression (Fig. 8A). $CD20^+$ cells were found to be present at higher numbers within *M. tuberculosis*-infected lung compared to randomized normal lung samples on a per gram basis ($p<0.005$). No difference in $CD20^+$ cell numbers were observed between lung draining (thoracic) lymph nodes (dLN) and peripheral lymph nodes (pLN) (Fig 8B).

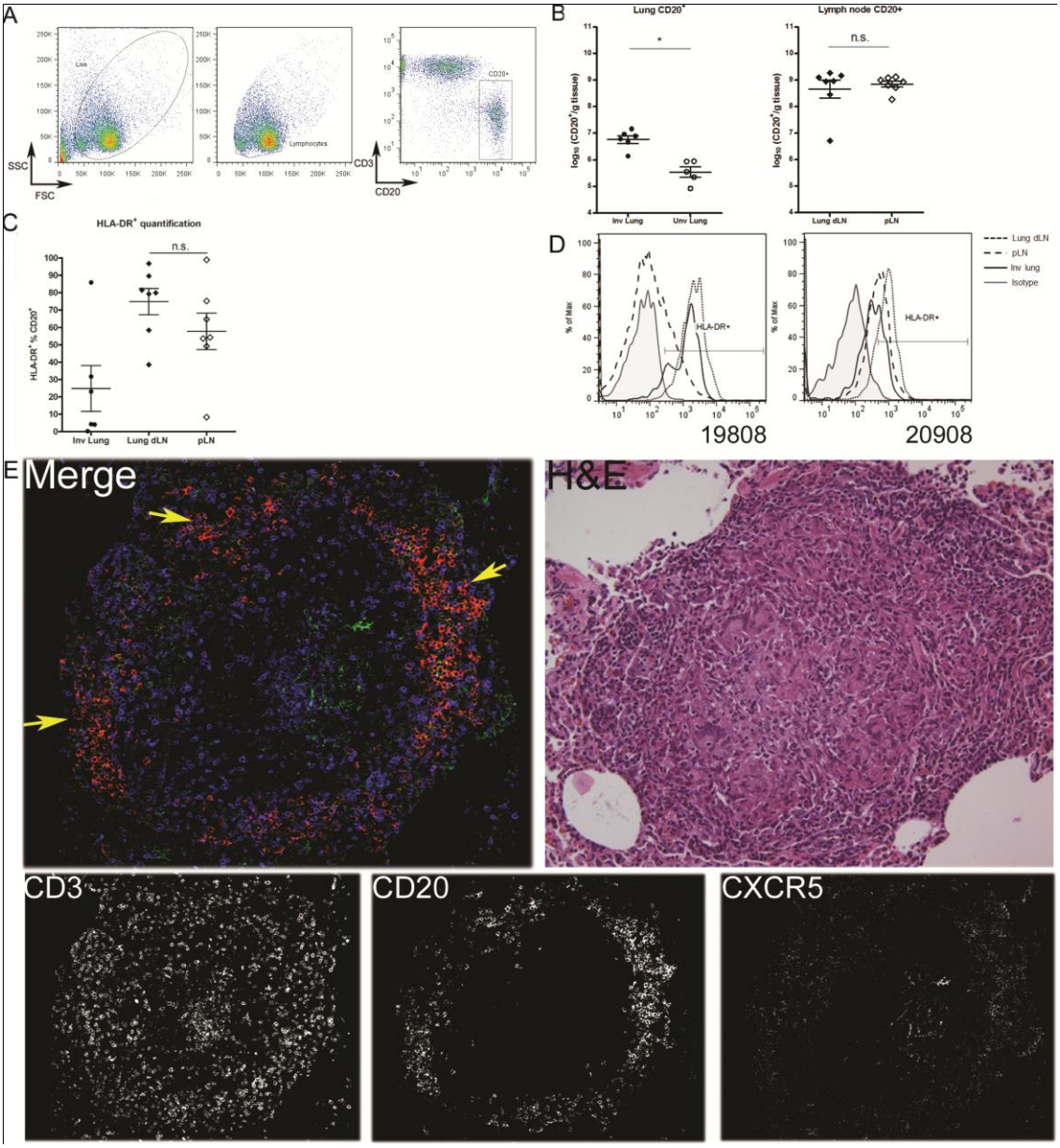

Figure 8. *Mycobacteria-containing tissue contains activated B cells.* **A)** Lymphocytes were gated based on size (FSC) and granularity (SSC). B cells were further identified based on $CD20^+$ expression. The FACS plot shown depicts the cellular profile of a lymph node sample. **B)** B cell numbers in lung and lymph node samples were expressed as B cells per gram tissue. Each point represents one tissue sample from one macaque. Error bars denote S.E.M. **C)** HLA-DR expression by $CD20^+$ cells from lung draining lymph nodes (dLN) although slightly elevated, were not statistically significant compared to peripheral lymph nodes (pLN), n=7. Error bars represent S.E.M. **D)** Analysis of tissues from 2 representative macaques (19808 and 20908) however, showed that HLA-DR expression is elevated within $CD20^+$ cells of lung dLN compared to pLN of the same macaque. $CD20^+$ from involved lung samples also stained positive for HLA-DR. Normal lung lacks sufficient B cell numbers for analysis. **E)** Staining for CD3 (blue), CD20 (red) and CXCR5 (green) was done to determine if B cell clusters within the granuloma behave as ectopic germinal centers. CXCR5 was used to identify lymphocytes that are actively homing towards lymphoid tissue to identify germinal center reactions. Most of the B cell clusters stained positive for CXCR5 (yellow arrows), indicating that the $CD20^+$ cells are activated and are capable of homing towards lymphoid tissue.

Increased HLA-DR expression on B cells was used to identify activated B cells within infected tissue on the premise that activated B cells have enhanced antigen presentation capacity. The frequency of $CD20^+$ cells from thoracic or peripheral lymph nodes expressing HLA-DR was similar (Fig. 8C). However, HLA-DR expression was higher on CD20+ cells from thoracic compared to peripheral lymph nodes within individual animals, 19808 and 20908 (Fig. 8D). Although HLA-DR was demonstrated on cells from involved lung tissue, it was not possible to obtain similar data from "uninvolved" lung, as there were insufficient B cells present within those tissues to perform meaningful analysis. No appreciable difference in CD86 expression could be seen within the $CD20^+$ cells from involved lung samples compared to uninvolved lung tissue (data not shown).

The chemokine receptor, CXCR5 was used to identify activated lymphocytes that respond to trafficking signals into lymphoid tissue. CXCR5 expression was observed on $CD20^+$ B cell clusters within the granuloma. However, very little co-localization of CD3 and CXCR5 was observed within the lymphocytic cuff of the granuloma, (Fig. 8E).

5.4.3 Plasma cells and the antibody responses to M. tuberculosis specific antigen within involved tissues

As the B cell clusters within the granuloma share many germinal center characteristics, the presence of plasma cells would further support the hypothesis that B cells are differentiating *in situ* of the granuloma. Plasma cells were identified within the granuloma based on morphology ("clock-faced" nuclei, higher cytoplasm to nuclei ratio) from H&E stained granuloma sections (Fig. 9.). CD138 (a plasma cell marker in humans) staining was performed on PFA fixed frozen granuloma sections but failed to detect any $CD138^+$ plasma cells (data not shown). NHP lymph

node samples do contain cells stained positive with CD138 within the B cell follicles (data not shown).

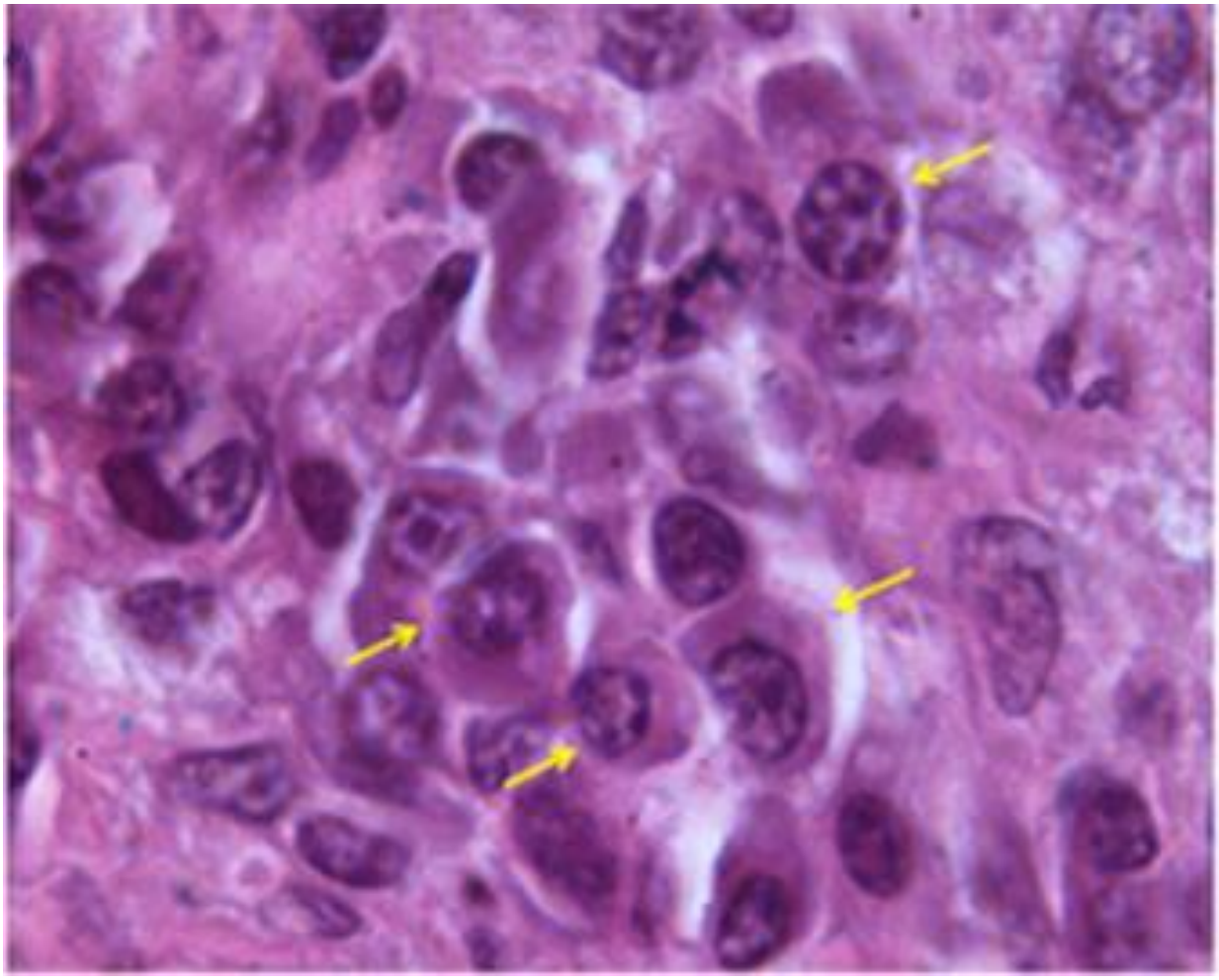

Figure 9. *Granulomas contain plasma cells.* Plasma cells with classical histologic features including clock-faced nuclei, perinuclear huff and higher cytoplasm to nuclei ratio, were identified within macaque granulomas (yellow arrows).

As another measure of plasma cells, a short-term IgG ELISPOT was used to assess CFP and ESAT6 specific plasma cells within lymph node, lung and sternum bone marrow samples from 7 infected NHPs. Antigen specific plasma cells for mycobacterial antigens [culture filtrate proteins (CFP) and ESAT6] within involved lung samples and for CFP within lung dLN were higher on average compared to uninvolved lung samples and pLN, respectively, despite not achieving statistical significance. However, lung dLN did contain statistically higher numbers of

ESAT6 specific plasma cells compared to pLN, $p<0.05$ (Fig. 10A). Numbers of plasma cells specific for CFP and ESAT6 were comparable to each other within sternum bone marrow samples (Fig. 10B).

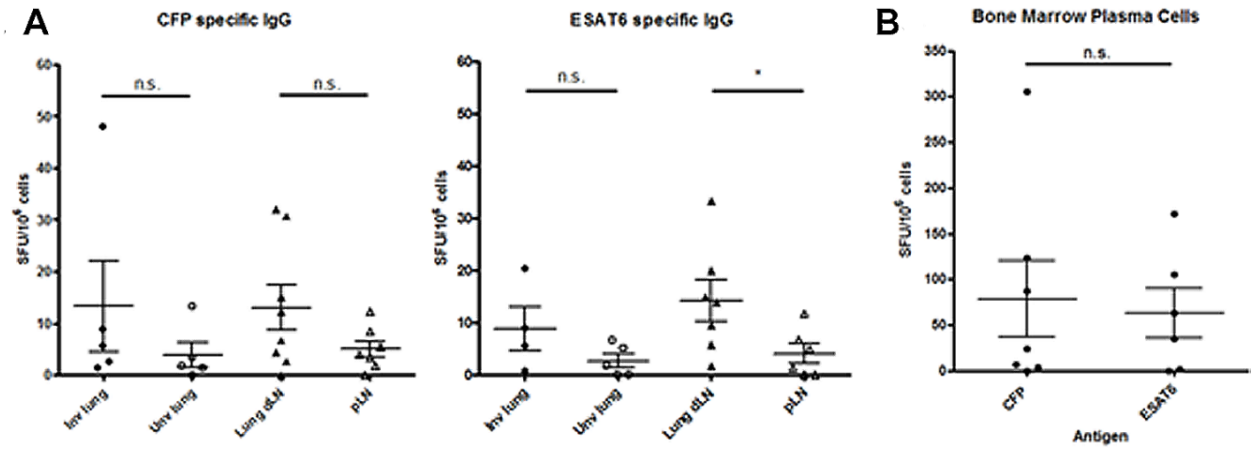

Figure 10. *Plasma cells within the granuloma secrete mycobacteria-specific IgG.* **A)** Lung and lymph node samples were used in a plasma cell ELISPOT assay to quantitatively assess numbers of IgG secreting plasma cells specific for CFP and ESAT6, n=7. Each point represents one sample from one macaque. Some groups have less than 7 points because insufficient cells were obtained to perform the ELISPOT. Significantly more ESAT6 specific plasma cells were detected within lung dLN compared to pLN (*$p<0.05$). ESAT6 specific IgG plasma cells within lung samples appear to be higher within inv lung but were not statistically significant. **B)** Both CFP and ESAT6 IgG specific plasma cells within bone marrow from the sternum were similar. Error bar denotes S.E.M.

To quantitate antibody levels within tissue samples, lung and lymph node supernatants from 14 NHP were assayed by ELISA for CFP specific IgG antibodies. Pairwise analysis of involved and uninvolved lung samples from the same NHP also showed an increase in CFP specific IgG amounts within involved lung supernatants, $p<0.05$. The same increase was observed within lung dLN supernatants compared to pLN samples, $p<0.01$ (Fig. 11.). Total IgG levels were comparable within both lung and lymph node groups (data not shown).

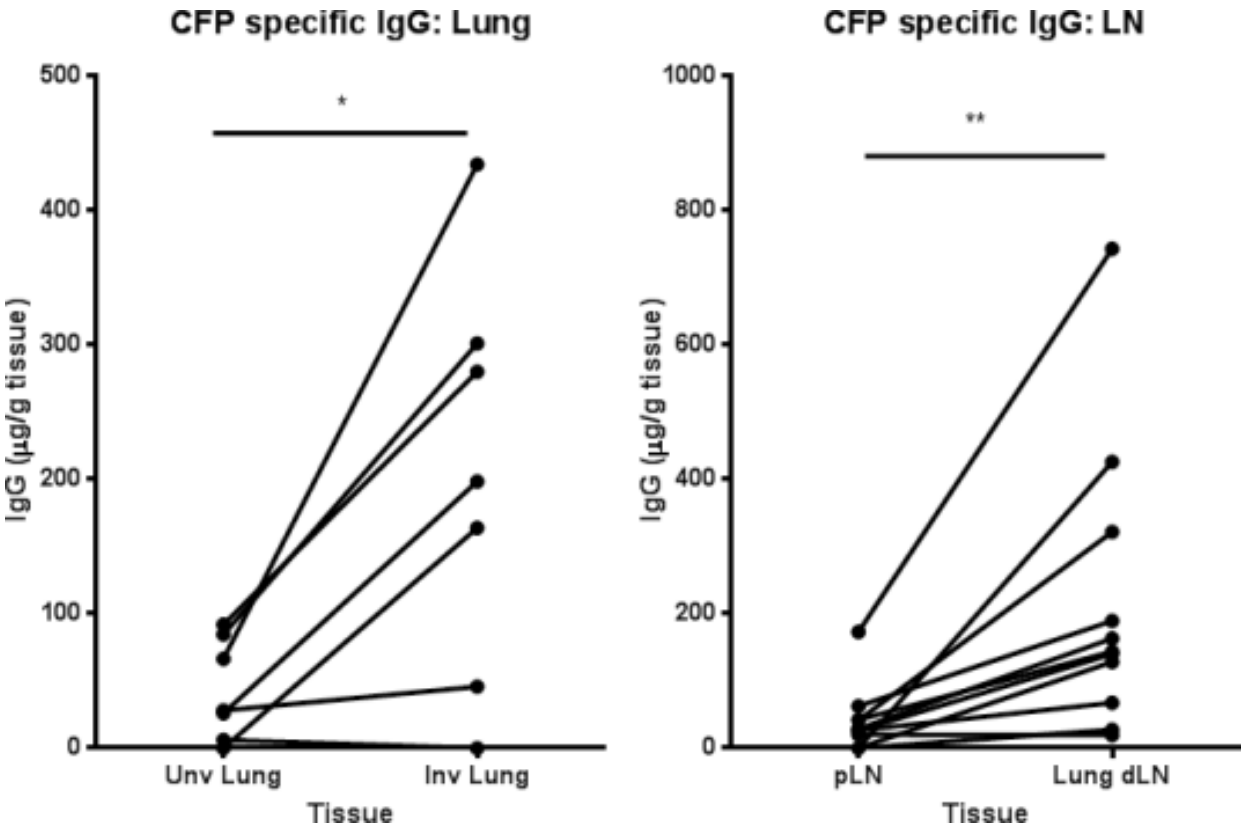

Figure 11. *Granulomas contain more CFP specific IgG.* A pairwise comparison of CFP-specific IgG in tissue supernatants was done using ELISA. Each data point represents a single sample from one macaque (n=14). Each line represents a paired sample of uninvolved lung (unv) and involved lung (inv) or pLN and lung dLN. Involved lung or lung dLN contained higher levels of CFP-specific IgG when compared to uninvolved lung (*$p<0.05$) and pLN (**$p<0.01$), respectively. Error bars denote S.E.M.

5.4.4 $CD20^+$ cells within the granuloma produce an assortment of cytokines

The capacity of B cells to produce cytokines after encounter *M. tuberculosis* antigen was also assessed by flow cytometry. B cells from lung granulomas or lung dLN were stimulated with ESAT6 and CFP10 protein and stained for IL2, IL6, IL17, IL10, TNF and IFNγ. Cells were deemed positive for cytokine after comparison with media stimulated control samples (Fig. 12.).

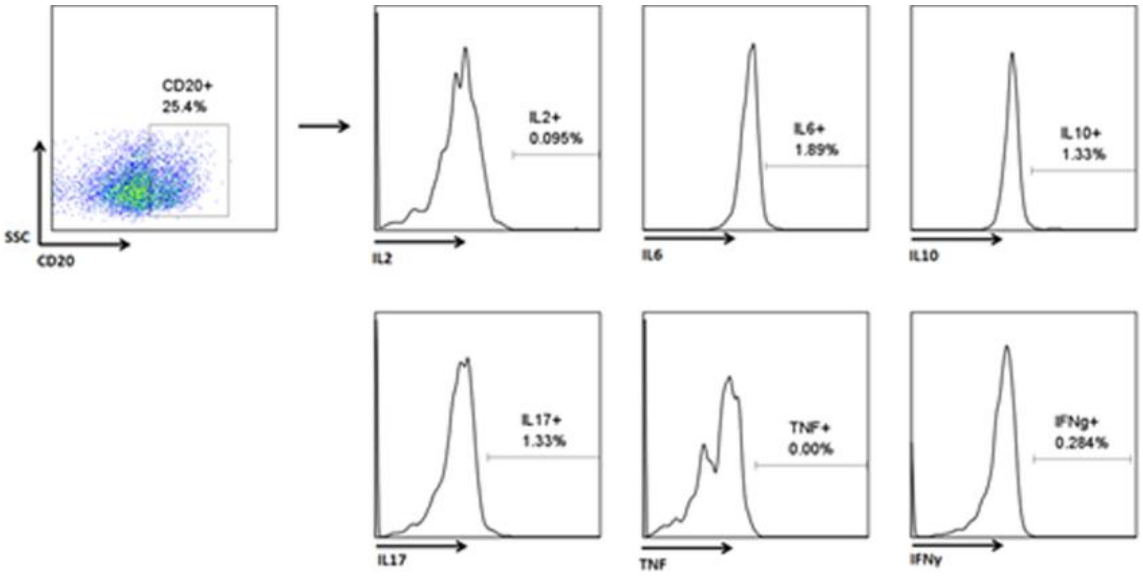

Figure 12. *Gating strategy to identify CD20$^+$ cells that produce IL2, IL6, IL10, IL17, TNF and IFNγ.*

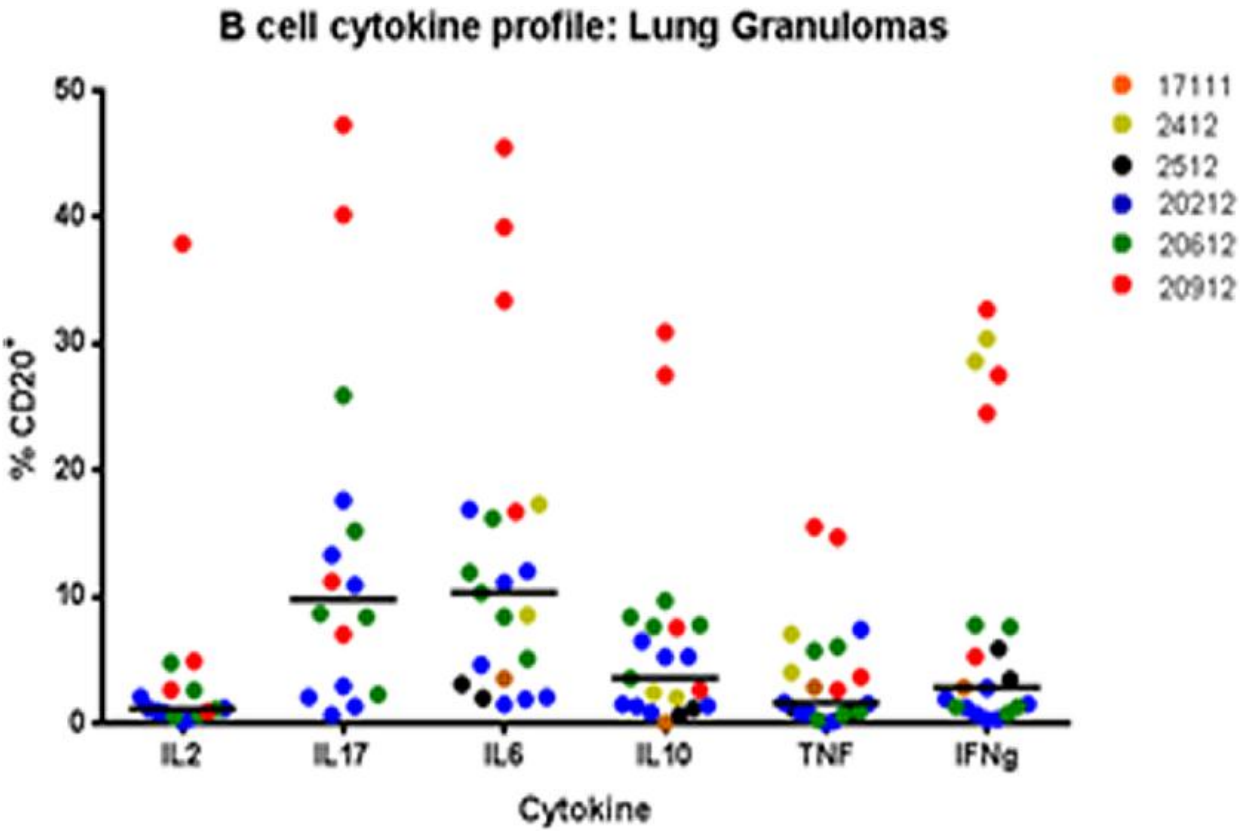

Figure 13. *B cells are capable of producing cytokine within sites of infection.* Primary cytokines produced by B cells are IL10, IL6 and IL17. Lung granulomas from 6 animals are depicted in this figure and differentiated by color. Each point denotes one sample

The major cytokines being produced by B cells within the granuloma are IL17 and IL6, both pro-inflammatory cytokines capable of encouraging TH17 T-cell development and neutrophil recruitment. A modest percentage of B cells are also producing TNF and IFNγ, around 1.6% and 2.5%, respectively. Approximately 3.5% of B cells are also producing IL10, a cytokine associated with anti-inflammatory activity which strongly suggests that B cells are also capable of regulating inflammation within the granuloma. IL2 does not appear to be a major cytokine being generated by B cells (Fig. 13.).

Multifunctional cytokine analysis revealed that B cell cytokine secretion of particular cytokines fall into distinct patterns. B cells either secrete IL6 or IL17 or both cytokines together. A very small subset of B cells secretes only IL10 alone but a larger proportion of IL10 secretion occurs with both IL6 and IL17 (Fig. 14A). When TNF and IFNγ secretion is included in the study, the same cytokine secretion behavior is observed within B cells. IL6 appears to be the main cytokine being produced and is associated with both pro-inflammatory cytokine secretion (TNF and IFNγ in various combinations) and anti-inflammatory IL10. IL10 secretion seems to only be secreted either alone, or in some combination with IL6 or any other cytokine. A small percentage of B cells (a median of around 1%) was observed to secrete TNF and IFNγ alone (Fig. 14B).

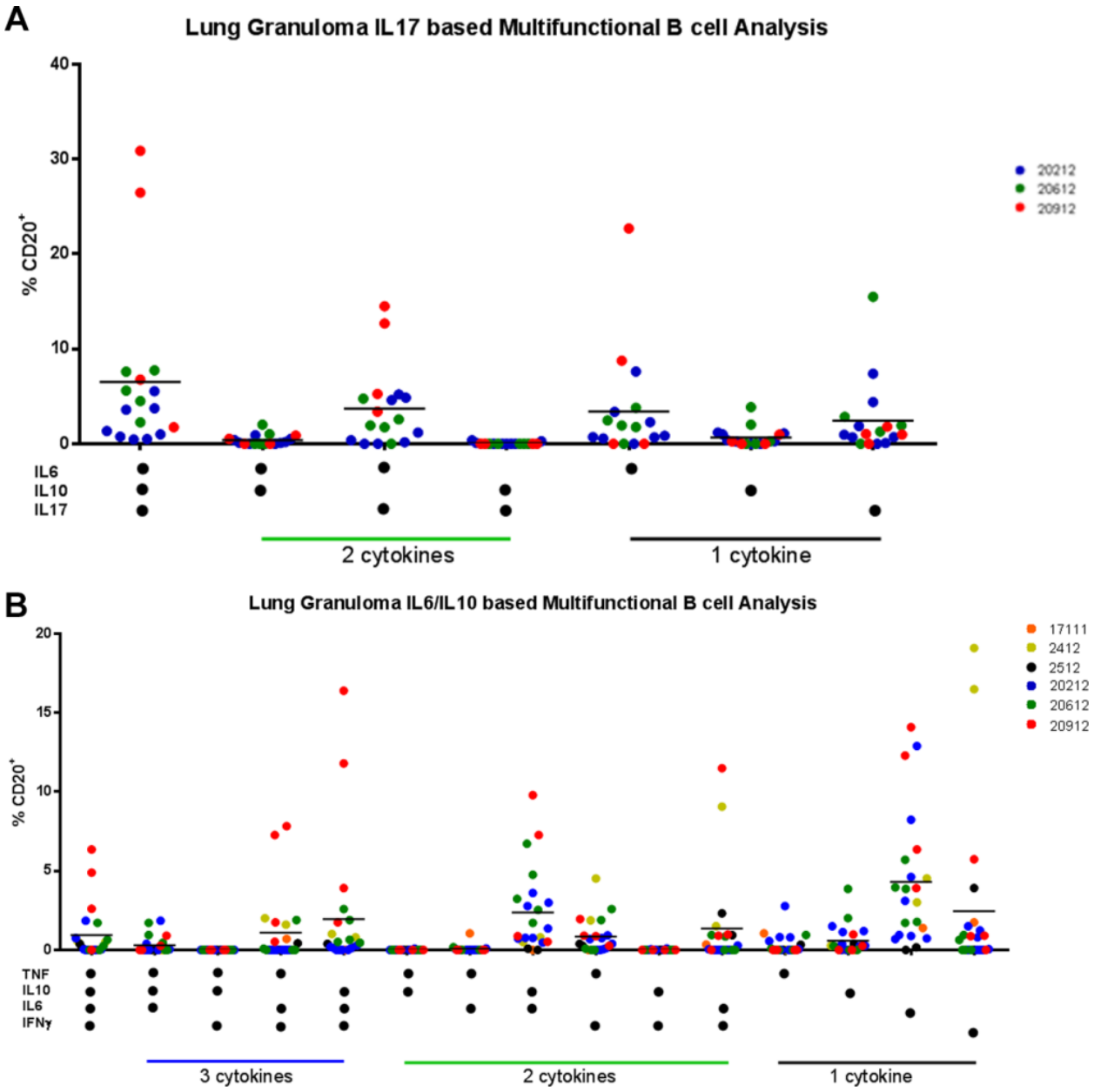

Figure 14. *Multifunctional analysis of cytokine combinations produced by B cells within the granuloma.* **A)** Multifunctional B cell analysis for IL6, IL10 and IL17 for 3 animals as differentiated by color. **B)** Multifunctional B cell analysis for IL6, IL10 and TNF and IFNγ for 6 animals as differentiated by color. Each point denotes one sample.

5.5 DISCUSSION

The importance of B cells in control of human *M. tuberculosis* infection remains unclear, although numerous studies on antibody responses in humans have been published[210, 235, 236]. Here we assessed the presence of B cells and antibodies in the granulomas of *M. tuberculosis*-infected macaques, using a model that accurately recapitulates both the pathology (including granuloma types) and infection outcomes seen in humans infected with *M. tuberculosis*. B cells have been demonstrated to contribute to control of *M. tuberculosis* in mouse models[211] and being reminiscent of germinal centers of lymphoid tissues based on cell organization and chemokine response[231]. However, the structure of murine granulomas is not similar to granulomas seen in humans. We demonstrate that B cells are present as a significant population within NHP lung granulomas, and are organized into discrete clusters with germinal center characteristics and numerous plasma cells and activated B cells.

Several studies have suggested that B cells within the granuloma are highly reminiscent of germinal centers based on surface marker expression[212, 213]. Ectopic germinal center formation or lymphoid neogenesis has been closely associated with chronic inflammation in autoimmune and infectious disease settings[187] and was considered a more efficient way of presenting antigen to drive cellular activation at lesion sites. In the case of the NHP granuloma, the B cell clusters may be positioned within the lymphocytic cuff evenly, analogous to the black spots on a white soccer ball. This may help drive antigen presentation for T-cell activation given the similarities of granuloma B cell clusters to lymph node germinal centers and the increased HLA-DR expression present on involved lung B cells.

The findings of B cells within the granuloma producing IL17 and IL6 combined with the germinal cluster distribution of B cells within the granuloma indicate strongly that B cell clusters

are capable of providing a microenvironment that encourages T-cell activation. The cytokine data in this study also support findings in both humans and mice infected with *M. tuberculosis* that implicate B cells as possible regulators for neutrophil recruitment[213, 215], although the exact mechanism of control is still unknown. Further functional analysis may determine the ability of B cells from granulomas to present mycobacterial antigens and activate T-cell responses, as well as the relative efficiency of granuloma B cells for these processes compared to peripheral B cells mechanism of neutrophil recruitment beyond IL17 production.

The cytokine secretion profile of B cells suggests that the predominant cytokine produced is IL6, either alone or in various combinations with other cytokines. This fits the observation in other fields on the pro- and anti-inflammatory nature of IL6 such as the ability to induce T_h17 or T_{reg} differentiation[237] and also attest to the plastic nature of B cells in modulating inflammation[165]. IL6 production by B cells feature prominently in T cell dominant autoimmune conditions such as multiple sclerosis[164, 238] where IL6 produced by B cell is suspected to amplify T cell responses via interactions within the lymph node. However, with the observation that B cells are forming ectopic germinal centers and the predominant cytokine produced by B cells within the granuloma is IL6, it is very likely that these tertiary lymphoid structures within the granuloma serve as a means to enhance T cell responses within the granuloma. One caveat is that IL6 producing B cells contribute significantly to pathology in certain cases of autoimmunity[238]. It remains unknown the extent of contribution provided by B cell cytokine production in tuberculosis control or induction of pathology.

IL17 is the other another major cytokine being produced by B cells aside from IL6. This is in line with observations in human granulomas where B cell clusters within the granuloma are associated with elevated levels of IL17 and IL22[213]. However, in B cell depleted mice infected

with *M. tuberculosis*, elevated neutrophils were noted along with increased lung inflammation which suggests that there is a defect in mechanisms that regulate neutrophil recruitment and activation, of which IL17 and IL22 are involved. IL17 is invariably tied to T_h17 cells which in the case of *M. tuberculosis* infected mice, can contribute towards the recruitment of T_h1 T cells essential for control of tuberculosis[239]. The specific contribution by B cells in terms of IL17 mediated immune response in primate tuberculosis is still unknown but may be closely related in neutrophil recruitment and function based on evidence from the mouse models[208, 211, 215] and observations in human clinical tuberculosis cases[208, 211, 213, 215].

The B cell cytokine secretion profile also suggests that activated B cells can be separated into distinct subsets based on cytokine secretion patterns. As mentioned, IL6 is predominantly secreted by B cells either alone or in combination with other cytokines as discussed above. IL10 however, appears to either be secreted alone or in the presence of multiple pro-inflammatory cytokines such as IL6 and IL17 or IFNγ and TNF. This suggests that IL10 secretion can be used to identify a subset of regulatory B cells or after B cells have acquired multifunctional cytokine secretion capacity, indicating prolonged activation. The idea of distinct B cell effector function has been proposed in mouse models of B cell activation[164]. Within the granuloma, B cells receive cytokine cues from T cells and could possibly differentiate into distinct effector and regulatory B cell populations based on their respective cytokine secretion profiles. Thus, the contribution of B cells in granuloma function may be significant both in terms of containing the bacilli and reducing tissue damage.

The increased amount of antigen specific IgG at the site of infection lends support to the notion that antibody may modulate the host-pathogen interactions in the granuloma. Antigen specific IgG secreting plasma cells present within lung granulomas suggest that substantial

antibody production is occurring. However, a much greater increase of both antigen specific IgG and plasma cells was noted within lung draining lymph nodes which indicates that *M. tuberculosis* specific plasma cells are being generated and possibly retained within lung draining lymph nodes. These lymph nodes are often infected, and usually contain *M. tuberculosis*-specific T cells[42, 43]. It is very possible that the plasma cells in involved lung tissues are derived from activated B cells that are recruited to the granulomas. Upon fully differentiating into plasma cells, these terminally differentiated B cells likely gain CD138 expression and migrate back to the bone marrow. This would explain the absence of $CD138^+$ cells within the granuloma. Although B cell clusters within the granuloma share many characteristics similar to lymph node germinal centers, granuloma B cell clusters contain far fewer B cells, making it less likely to detect mature $CD138^+$ expressing cells.

The role of antibody remains unclear in tuberculosis. Historically, passive immunization and serum therapy has yielded conflicting results in human patients. Studies in humans have mostly been confined to using *M. tuberculosis* specific antibodies as indicators of disease states by measuring serum IgG specific to *M. tuberculosis* antigens[235, 236, 240]. However, there may be a role for antibodies at the site of infection. One proposed hypothesis is that antibody-coated (extracellular) *M. tuberculosis* bacilli engage Fcγ receptors (FcγR) rather than just DC-SIGN and complement receptors, resulting in increased macrophage activation[50, 241]. These changes are suspected to be mediated by crosslinking of Fcγ receptors expressed on macrophages and neutrophils by immune complexes of IgG and *M. tuberculosis* bacilli. Data from *M. tuberculosis* infected Fcγ receptor knockout mice also suggest that antibody mediated events are involved in the anti-tuberculosis response[216]. Given that macrophage activation is essential for control of *M. tuberculosis*, antibodies may play a role in regulating macrophage function within the

granuloma. However, antibody mediated phagocytosis may not necessarily be protective, as demonstrated in dengue fever or Coxsackie B[222]. In rabbits infected with *M. tuberculosis,* increased B cell activation was observed to be associated with failure to contain the disease[242]. Antibody dependent enhancement of infection may also play a role in exacerbating the course of infection for *M. tuberculosis*.

Our findings here demonstrate that activated B cells, plasma cells, and antibodies are enriched within the granuloma, and have some characteristics of germinal centers. Further studies will focus on defining the protective, pathologic or immune modulatory roles for these cells in tuberculosis.

5.6 ACKNOWLEDGEMENTS

This study was supported by the Bill and Melinda Gates Foundation and NIH RO1 HL68526 and RO1 HL075845 (JLF). Anti-primate specific reagents were kindly supplied by Dr. Keith Reimann (Beth Israel Deaconess Medical Center, Harvard University). Peanut agglutinin lectin was kindly supplied by Dr. Geetha Chalasani (University of Pittsburgh). The authors gratefully acknowledge Dr. Edwin Klein and Dr. Chris Janssen for performing necropsies and histologic analyses, and technical assistance provided by Mark Rodgers, Catherine Cochran, Melanie O'Malley, Jamie Tomko, Dan Filmore, Carolyn Bigbee, Matthew Bigbee and Paul Johnston. The authors also thank Dr. John Chan (Albert Einstein College of Medicine, NY) for intellectual discussions.

6.0 THE EFFECTS OF B CELL DEPLETION IN *MYCOBACTERIUM TUBERCULOSIS* INFECTED CYNOMOLGUS MACAQUES

6.1 ABSTRACT

The role of B-cells and antibody in the control of *M. tuberculosis* remains largely unexplored. Although recent studies in mice have shown that components of humoral immunity can affect *M. tuberculosis* pathology and inflammation, the role of these components in human and non-human primate infections remain unknown. The cynomolgus macaque model of *M. tuberculosis* infection closely mirrors the infectious outcomes in human tuberculosis. To begin to address the contribution of the humoral immune system in control of tuberculosis, we used anti-CD20 antibody (Rituximab) to deplete B cells within a cohort of six cynomolgus macaques prior to *M. tuberculosis* infection and monitored them for ten weeks post infection. Although B cell follicles and *M. tuberculosis* specific antibodies were markedly diminished, no discernible differences in overall pathology or bacterial burden were observed between Rituximab treated animals and saline controls at 10 weeks post infection. Cytokine levels within lung granulomas as assessed by IFNγ ELISPOT, FACs and ELISA were also comparable between Rituximab treated and saline control groups. Taken together, our data suggests that both B cells and antigen specific antibody are not required for control of early *M. tuberculosis* infection.

6.2 INTRODUCTION

The relative contribution of B cells and antibody towards the control of tuberculosis in humans or non-human primates remain relatively unknown. Data from B cell or FcγR-depleted mouse models of tuberculosis suggest that components of the humoral immune system do play a role in regulating disease pathology. This is evident from the excessive inflammation and neutrophilia found within lungs of *M. tuberculosis* infected μMT mice (no B cells)[211] and Fc common γ chain deficient mice (no functional activating FcγR signaling)[216]. Passive transfer of infected mice sera into *M. tuberculosis* infected μMT mice is capable of reducing both lung inflammation and reversing neutrophilia[211, 215]. Furthermore, bacilli coated with *M. tuberculosis* antigen specific antibody were noted to induce greater killing by cultured macrophages *in vitro*[222], suggesting that antibody mediated signaling is capable of enhancing the killing capacity of macrophages against internalized bacilli.

This study aims to explore the effects of B cell depletion in the cynomolgus macaque model (*Macaca fascicularis*) of tuberculosis. B cell depletion in non-human primates can be achieved by the administration of anti-human CD20 chimeric monoclonal antibody, Rituximab[243]. Rituximab is in clinical use for the treatment of certain B cell lymphomas and autoimmunity conditions such as lupus, rheumatoid arthritis and multiple sclerosis. B cells are thought to be depleted via antibody dependent cell cytotoxicity modes of clearance by natural killer cells[244]. Although Rituximab use can predispose patients towards certain infections, the risk associated with its use in susceptibility to *M. tuberculosis* infection or reactivation is unknown[245].

Rituximab has been used in nonhuman primate research, particularly in animal models utilizing simian immunodeficiency virus (SIV) and is thus known to be effective at depleting

nonhuman primate B cells[243]. Although B cells are depleted, Rituximab has not been observed to interfere with the plasma cell compartment, since these cells are not generally $CD20^{+}$. Hence, the antibody component of the humoral response would not be affected by Rituximab although further antigen specific antibody generation would be impaired. This study was undertaken to help elucidate the contribution of functional B cells and antigen specific antibodies on the control of tuberculosis. Such findings would allow for potential enhancement of future vaccine development or development of novel treatment modalities involving B cells or antibody.

6.3 MATERIALS AND METHODS

6.3.1 Experimental animals and B-cell depletion

12 adult (>4 years of age) cynomolgus macaques (*Macaca fascicularis*) (Covance, Alice, TX; USA Valley Biosystems, West Sacramento, CA) were obtained for the B cell studies described here. The 12 animals were divided into 6 pairs where 1 animal receives Rituximab and the other receives saline as a control and are infected at the same time.

Rituximab (Genentech, San Francisco, CA) was administered at a dosage of 50 mg/kg over a period of 45 minutes with the first dosage given 2 weeks prior to *M. tuberculosis* infection. Subsequent doses of Rituximab were administered every 3 weeks until the study termination at 10 weeks post infection. Control animals received saline infusion at the same time as the Rituximab counterparts within the pair.

All animals were housed under BSL-3 conditions. These studies followed all animal experimentation guidelines and all experimental manipulations and protocols were approved by the University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee.

6.3.2 *M. tuberculosis* infection

Cynomolgus macaques were infected with a low dose of approximately 4 to 8 CFU Erdman strain *M. tuberculosis* via intra-bronchial instillation as previously described[42, 43]. Infection was confirmed by conversion of negative to positive tuberculin skin test and elevated PBMC responses to mycobacterial antigens from baseline in lymphocyte proliferation (LPA) and ELISPOT assays[43, 233].

6.3.3 Necropsy procedures

Monkeys were maximally bled prior to necropsy and euthanized using pentobarbital and phenytoin (Beuthanasia; Schering-Plough, Kenilworth, NJ). Gross pathologic findings were described by a board-certified veterinary pathologist (EK) and were classified as previously described. Representative sections of each tissue were placed in formalin for histologic analysis or homogenized into single-cell suspensions for immunologic studies, flow cytometric analysis, and bacterial burden, as previously described[42, 43, 233]. Bone marrow was also obtained from the sternum as previously described[214]. A portion of tissue homogenate from numerous necropsy samples were serially diluted and plated on 7H11 media (BD, Sparks, MD) and CFUs were enumerated on day 21, while the rest were filtered using 0.45 micron syringe filter units (Millipore, Darmstadt, Germany) for downstream assays.

6.3.4 Immunologic analysis

Blood was drawn from each animal every 2 weeks starting 2 weeks prior to the first dose of Rituximab administration until necropsy at 10 weeks post infection. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described via percoll gradient centrifugation[42]. Axillary and inguinal lymph nodes were biopsied prior to the first dose of Rituximab administration and then at weeks 4 and 8 post infection.

6.3.5 Flow cytometry

At necropsy, single cell suspensions were derived from lung granulomas, uninvolved lung and thoracic draining lymph nodes. Cells from PBMCs and tissue samples were stained for T-cells using anti-human CD3 (clone SP34-2, BD Biosciences), CD4 (clone L200, BD Biosciences) and CD8 (clone DK25, Dako), B cells using anti-human CD20 (clone 2H7, eBioscience) and CD79a (clone HM47, BD Pharmingen), neutrophils with CD11b (clone ICRF44, BD Pharmingen) and calprotectin (clone MAC387, Thermo Scientific) and co-stimulation marker HLA-DR (clone LN3, eBioscience). Lymphocytes and neutrophils were identified based on size (FSC) and granularity (SSC). B cells and T-cells were further identified based on $CD20^+$ and $CD3^+$ expression, respectively. Neutrophils were identified from CD11b and calprotectin expression.

For intracellular cytokine staining, tissue single cell suspensions were stimulated in RPMI (Lonza, Walkersville, MD) supplemented with 1% L-glutamine and 1% HEPES (Sigma, St. Louis, MO) containing *M. tuberculosis* CFP10 and ESAT6 proteins (BEI Resources, Manassas, VA) along with Brefeldin A (BD Biosciences), all at a final concentration of 1µg/ml for 4 hours. After staining for CD3, CD4, CD8 and CD20 as described above, cells were then

fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) and finally washed with BD Perm/Wash buffer (BD Biosciences). Cells were then stained using anti-human antibodies against IL2 (clone MQ1-17H12, BD Biosciences), IL6 (clone MQ2-6A3, BD Biosciences), IL10 (clone JES3-9D7, eBiosciences), IL17 (clone eBio64CAP17, eBiosciences), TNF (clone MAb11, eBiosciences) and IFNγ (clone B27, BD Biosciences). All cytokine producing cells were identified as described above.

6.3.6 IFNγ and plasma cell ELISPOT

ELISPOT assays were performed as previously described[42] with $1.5x10^5$ PBMC per well using ELISPOT reagents with known cross reactivity against macaque IFNγ (MabTech, Mariemont, OH)[233]. M. tuberculosis antigens used in ELISPOT were peptide pools (overlapping 20 mers; 10μg/ml) from CFP10, ESAT6 and Rv1196 obtained from Sigma-Genosys (Woodlands, TX). Phorbol 12, 13-dibutyrate (PDBu) and ionomycin (50 nM and 10 μM final concentration respectively; Sigma) were used as positive controls. Media-only wells were used as negative controls. Cells in ELISPOT assays were incubated with antigens for two days at 37°C and 5% CO_2 prior to being developed. All conditions were performed in duplicate wells. Plasma cell ELISPOTs were performed as previously described[214] using bone marrow from sternum. $1.5x10^5$ bone marrow cells were added into each well. Plates were read using an ELISPOT plate reader (Cellular Technology LTD, Cleveland, OH). All ELISPOT data were expressed as SFU per 10^6 cells.

6.3.7 Antibody, cytokine and calprotectin ELISA

ESAT6 and Rituximab specific antibodies were quantified via ELISA by coating 96 well plates with 2μg per well of ESAT6 whole protein (BEI Resources, Manassas, VA) or Rituximab (Genentech, San Francisco, CA) dissolved in 1x PBS (Lonza, Walkersville, MD). Undiluted filtered tissue homogenates were incubated for 1 hour at 37°C after blocking for ESAT6 antibody quantification. Serum samples diluted to 1:100 using 1x PBS containing 1% BSA were used to assess for anti-Rituximab antibodies. Mouse anti-primate IgG conjugated to HRP (clone 1B3, NIH Nonhuman Primate Reagent Resource, Boston, MA) were used as the detection antibody at 1:3000 dilution. Plates were developed using 3, 3', 5, 5'-tetramethylbenzidine hydrochloride (Sigma, St. Louis, MO). CFP specific IgG and total IgG present within tissue homogenates were quantified as previously described[214].

Cytokine ELISAs were also performed to quantify for IL6, IL17, TNF and IL10. Anti-human IL10 (Invitrogen, Carlsbad, CA), TNF, IL6, and anti-primate IL17 ELISA kits (MabTech, Mariemont, OH) were performed according to manufacturer's instructions using filtered tissue homogenates obtained at necropsy as samples.

All quantifications for cytokine or antibody amounts were calculated on a per granuloma basis. All data from pertaining to lymph nodes were normalized to the weight of the lymph node in milligrams.

6.3.8 Immunohistochemistry

Tissue sections were embedded with paraffin and stained with haemotoxylin and eosin (H&E). These sections were then reviewed microscopically by a veterinary pathologist (EK) with

specific emphasis on granuloma characteristics as described previously[233]. Paraffin embedded slides of relevant tissue sections were stained as previously described[214] for the presence of T cells (CD3), B cells (CD20) and antigen presenting cells (CD11c). Images were taken at 20x magnification and serial images were used to generate a composite of the tissue section.

6.3.9 PET/CT scans

All PET/CT scans were performed in a biosafety level 3 imaging suite using a hybrid preclinical PET/CT system that includes a micro-PET Focus 220 preclinical PET scanner (Siemens Molecular Solutions, Knoxville, TN) and an 8-slice helical CT scanner (Neurologica Corp., Danvers, MA) as previously described[133].

6.3.10 Data analysis and statistics.

Flow cytometry data was analyzed with the FlowJo software package (Tree Star, Ashland, OR). Data were analyzed using Prism 6 (Graphpad Software, San Diego, CA). All data are shown as medians to account for the variability of the data. Statistical comparisons were performed using Mann-Whitney test with $p<0.05$ considered statistically significant. Paired samples were analyzed using the Wilcoxon matched-pairs signed rank test with the same threshold, $p<0.05$ as being statistically significant. Multivariate analysis and linear correlations were performed using JMP 10 (SAS, Cary, NC).

6.4 RESULTS

6.4.1 Rituximab depletes B cells of non-human primates within the blood and tissue compartments

Rituximab was administered 2 weeks prior to *M. tuberculosis* infection to ensure that B cell numbers were minimal upon infection. Subsequent doses of Rituximab were given every 3 weeks to ensure continued B cell depletion over the course of the study. We confirmed that B cells were successfully depleted within treated animals over the course of infection by following B cell percentages within the peripheral and lymphoid compartments. B cell depletion was successfully achieved within all 6 treated animals with no treatment failure. PBMCs from biweekly blood draws and single cell suspension from monthly peripheral lymph node biopsies were stained with anti-human CD20 and an alternative intracellular marker, CD79a to identify B cells. CD79a was used as an alternative B cell marker to independently confirm B cell depletion in the event that Rituximab interferes with CD20 staining via flow cytometry. Animals given Rituximab had almost no B cells in the peripheral compartment and reduced B cells within lymphoid tissue compared to saline control animals (Fig. 15.).

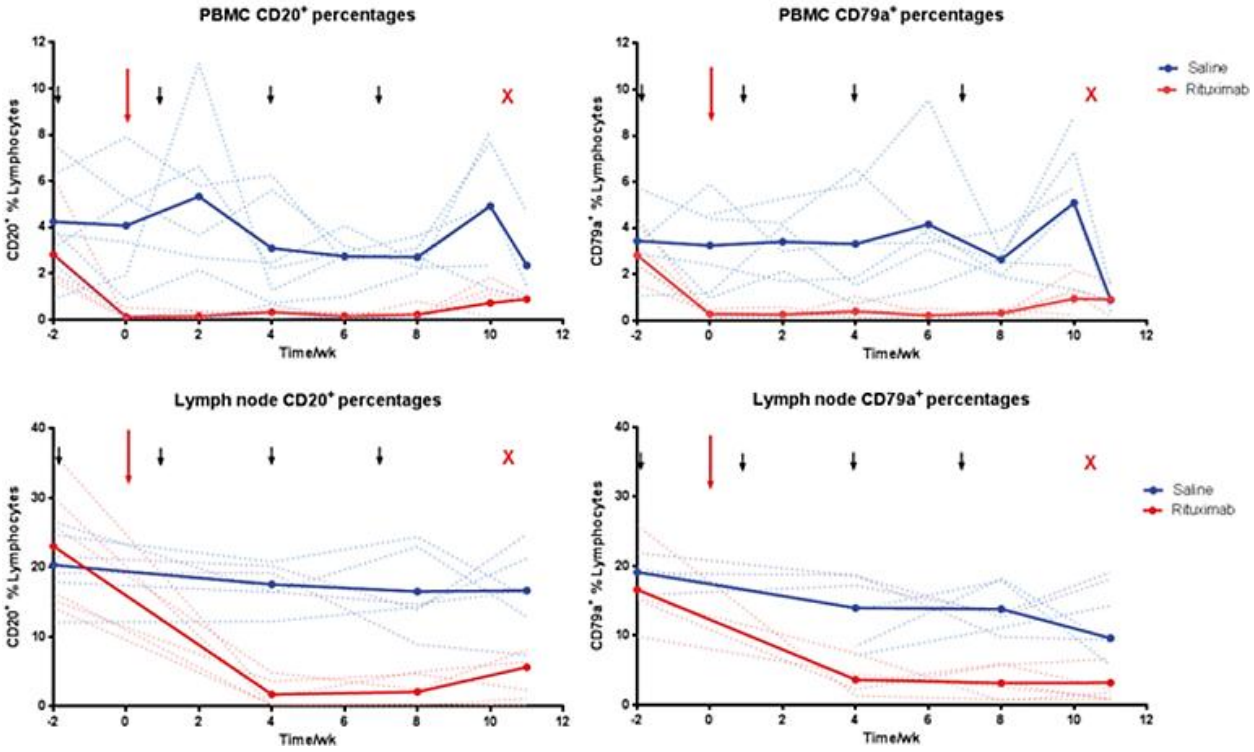

Figure 15. *Confirmation of B cell depletion with Rituximab treatment.* Depletion of $CD20^+$ and $CD79a^+$ cells within the peripheral and lymphoid compartment of treated NHP (red) and saline control NHP (blue) after administration of Rituximab (Black arrows). Infection with *M. tuberculosis* occurs 2 weeks after the first dose of Rituximab (Red arrow). The study runs for a total of 12 weeks with necropsy at 10 weeks post infection (Red X). A total of 12 NHPs are used in the study, 6 per group. The solid line depicts group mean and individual animals are represented by the fainter dotted lines.

At necropsy, single cell suspension of tissue samples similarly stained with CD20 and CD79a antibodies showed markedly reduced B cell numbers within lung granulomas (median B cell percentage of 0.8% in Rituximab group compared to 6% in saline control animals, between 1 to 9 samples per animal, N of 6 per group) of Rituximab animals. Thoracic lung draining lymph node samples of Rituximab treated animals also showed a similar depletion in B cells (median B cell percentage of 0.6% compared to 10% of saline control animals) compared to animals receiving saline. These data were confirmed with a similar decrease seen with CD79a staining, an alternative pan B cell marker (Fig. 16.).

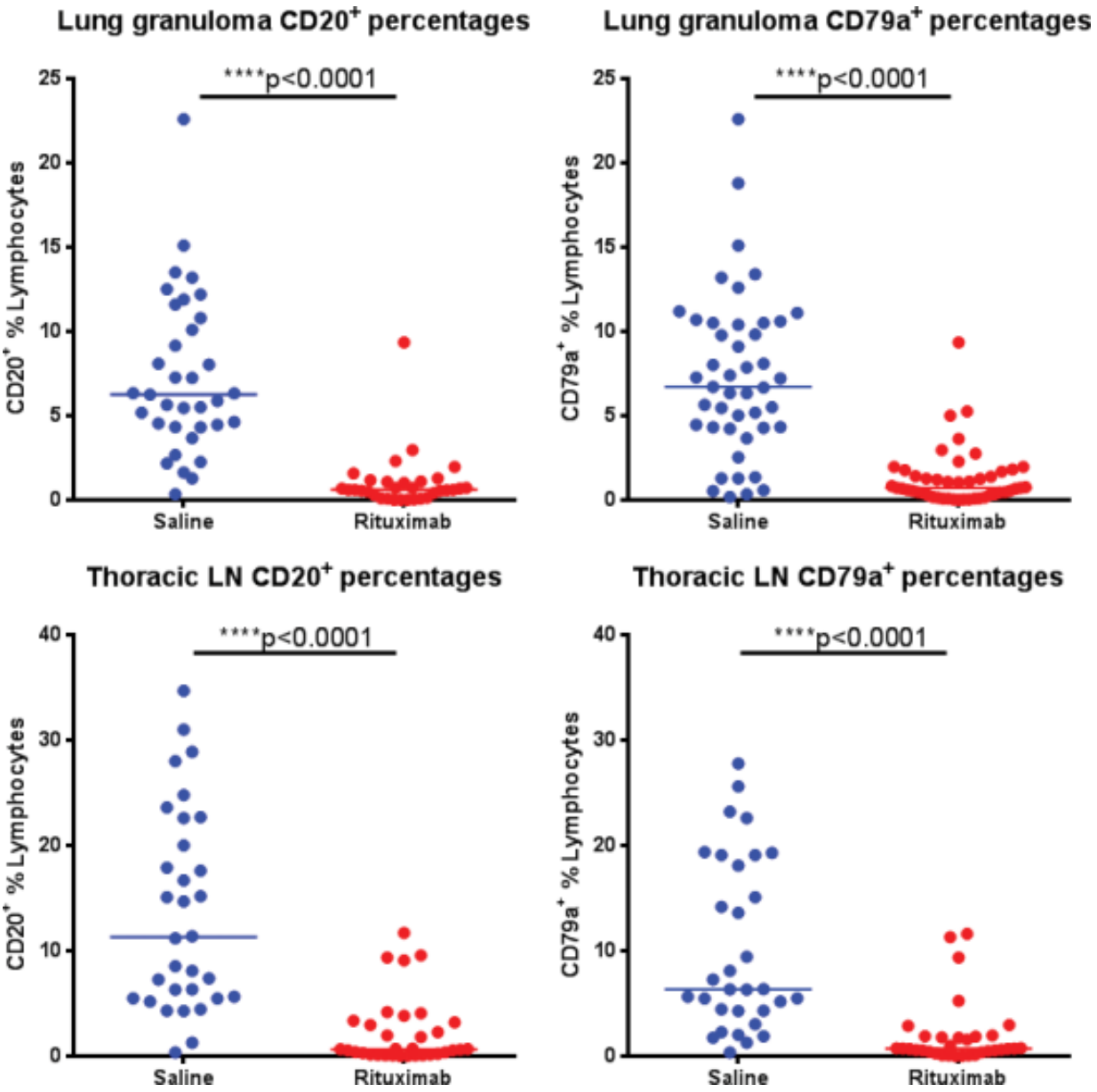

Figure 16. *Confirmation of B cell depletion within lung granulomas and thoracic lymph nodes (LN) of Rituximab treated animals.* Rituximab treatment also depletes B cells within the tissue compartment as seen in samples obtained at necropsy. Rituximab treated animals (red) showed substantial decrease in $CD20^+$ and $CD79a^+$ lymphocyte percentages compared to control animals (blue) within lung granulomas and thoracic lymph nodes.

Immunohistochemistry staining of lung granuloma from Rituximab treated animals showed reduced numbers and size of B cell aggregates compared to granulomas of saline control animals (Fig. 17.). B cell follicles were also similarly reduced within lymph node sections of treated animals as compared to control lymph nodes. This confirms that Rituximab treatment was

successful in reducing the B cell population of treated cynomolgus macaques for the duration of the study.

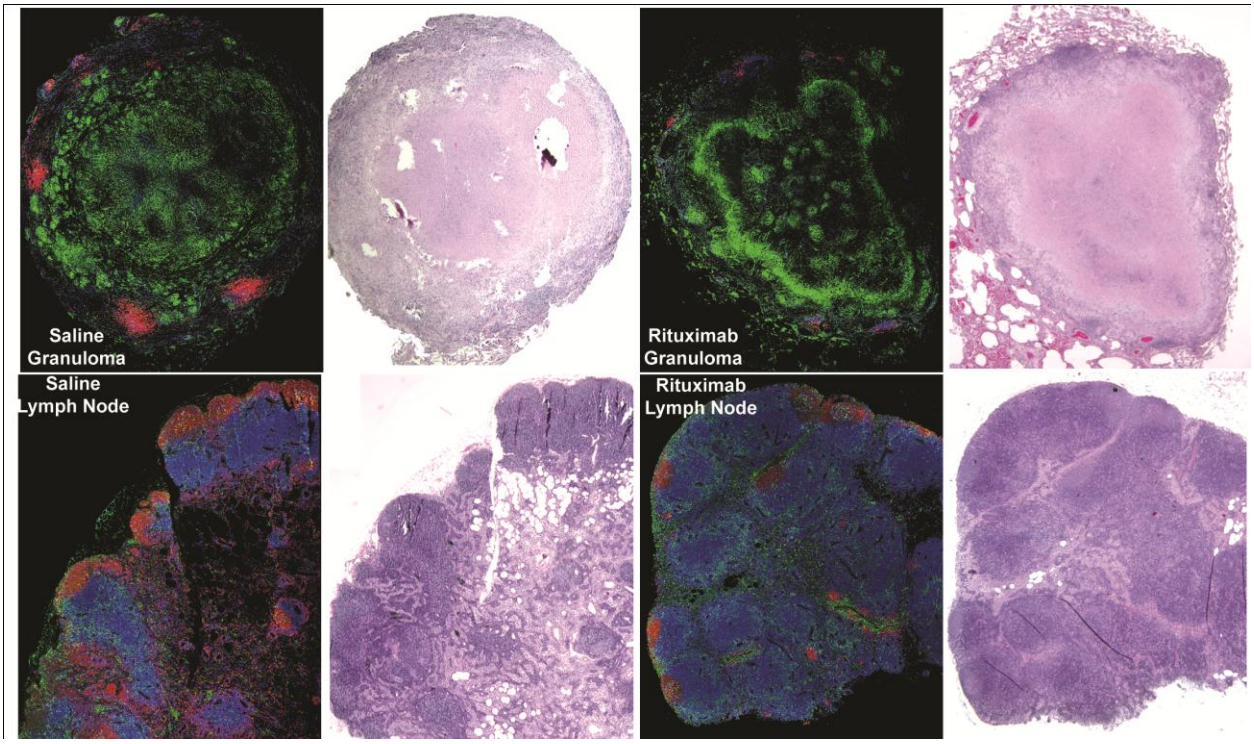

Figure 17. *Confirmation of B cell depletion via immunohistochemistry imaging.* Immunohistochemistry staining of paraffin embedded sections of lung granuloma and lymph node from Rituximab treated animals showed reduced B cell clusters within lung granulomas and B cell follicles within lymph nodes. Samples from treated animals and saline controls were stained with anti-human CD3 (blue) for T cells, CD11c (green) for myeloid or APCs, and CD20 (red) for B cells

6.4.2 B cell depletion is accompanied by a reduction in antibody levels within lung and lymphoid tissue but not serum antibody levels

Tissue homogenates prepared from lung granuloma and thoracic lymph node samples were used to quantify the amount of *M. tuberculosis* specific IgG (CFP and ESAT6) and total IgG present using ELISAs. Lung granulomas of Rituximab treated animals showed significantly lower amounts of IgG specific for the mycobacterial antigen mixture CFP or the ESAT6 protein of *M. tuberculosis* and total IgG (Fig. 6.4.). Similarly, lymph node samples from Rituximab treated

animals had lower amounts of CFP specific antibody and total IgG compared to lymph nodes from saline control animals (Fig. 18.).

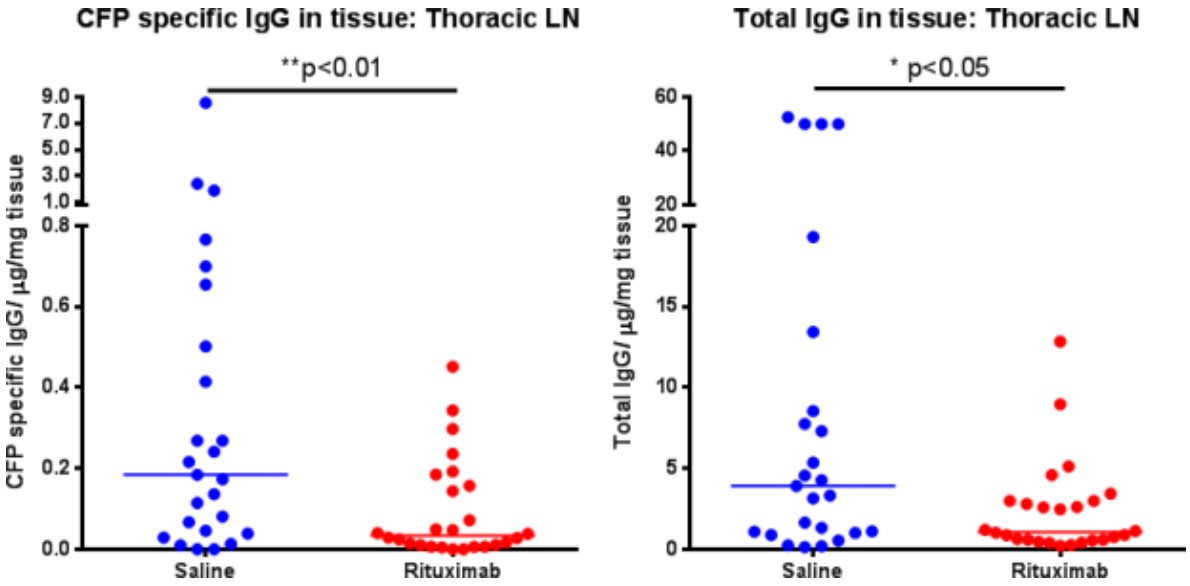

Figure 18 *B cell depletion is accompanied by reduction in thoracic lymph node tissue IgG levels.* CFP specific and total IgG was reduced within lymph node samples of Rituximab treated animals.

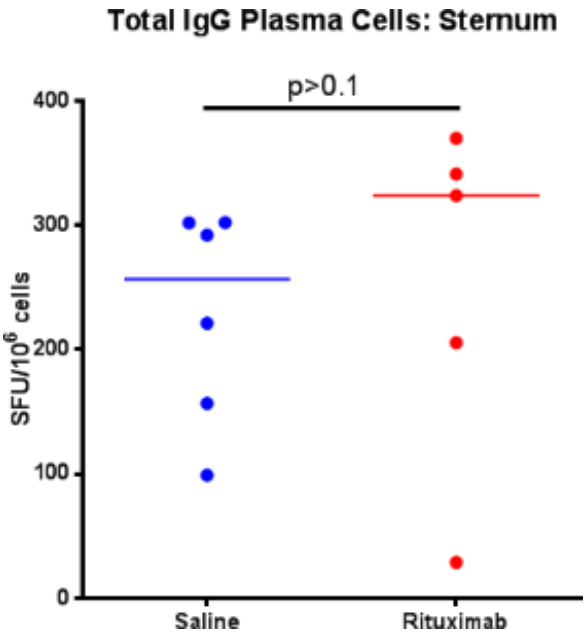

Figure 19. *B cell depletion did not affect plasma cell compartment.* Plasma cell numbers within the bone marrow is unaffected within animals of both groups. Each point represents one tissue sample obtained from a total of 12 animals.

However, there was no difference in numbers of plasma cells present within the bone marrow of the sternum (Fig. 19.), which suggests that Rituximab depletion did not affect the plasma cell compartment at least over the course of the study. Furthermore, quantification of serum samples suggests that levels of CFP specific IgG and total IgG in serum were not different overall between animals from B cell depleted groups versus saline control animals, although significant variation in serum antibody content were noted within individual animals from both groups (Fig. 20.).

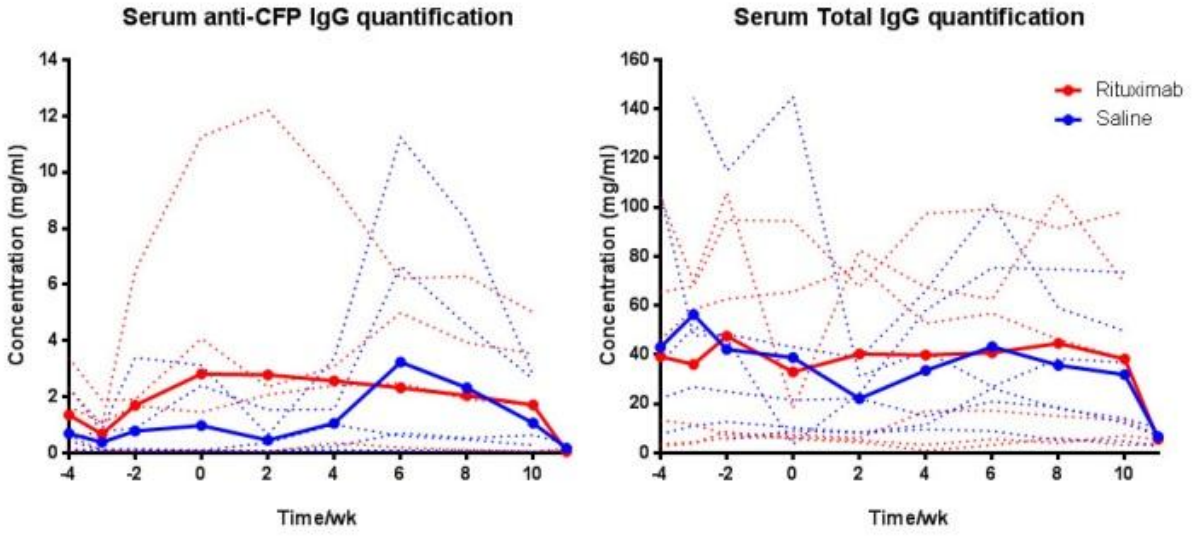

Figure 20. *Serum antibody levels of CFP specific IgG and total IgG were similar to saline control sera.* Serum samples from Rituximab treated animals did not show any differences in the levels of CFP specific IgG or total IgG compared to saline control animals. Dotted lines represent individual animals and solid lines represent group averages with Rituximab animals in red and saline control animals in blue.

Although Rituximab depletes B cells, the process of depletion within the lymphoid tissue was not complete and some B cells were noted to still be present. Quantification of anti-Rituximab antibodies undertaken to ensure that over the course of treatment, anti-Rituximab responses were not generated such that it would interfere with process of B cell depletion. Analysis of serum antibodies specific for Rituximab showed that B cell depleted animals as a group did not mount any notable response to Rituximab since the levels of antibody binding to

Rituximab in the serum is comparable to samples from control group animals. This suggests that the amount of anti-Rituximab antibodies were not different from background as saline control animals did not receive any Rituximab (Fig. 21.).

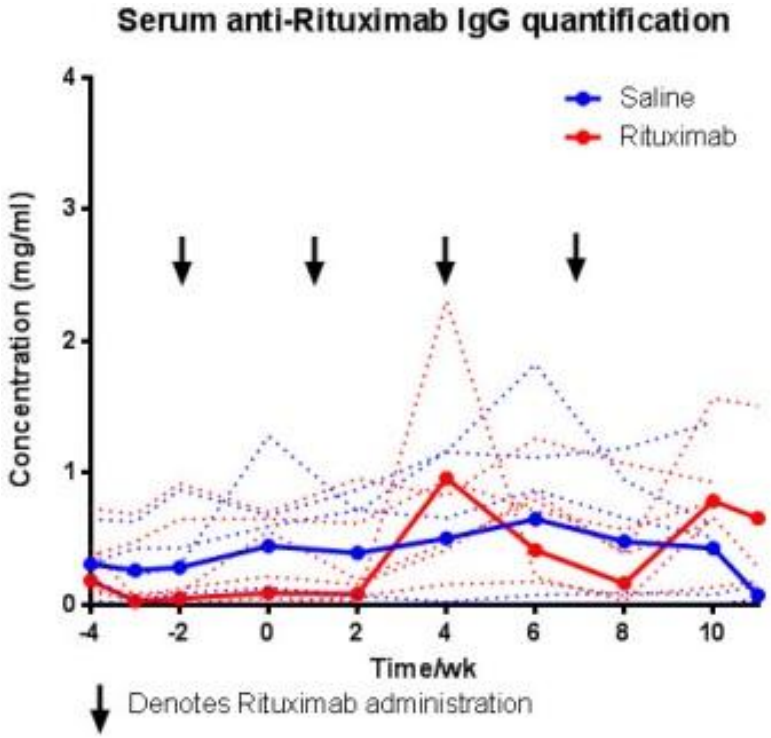

Figure 21. *Anti-Rituximab antibodies were not generated within B cell depleted animals* . Levels of anti-Rituximab antibody within the sera of Rituximab treated animals were not different compared to sera obtained from saline control animals. This suggests that anti-Rituximab antibodies were not being generated within B cell depleted animals.

These data demonstrate that B cell depletion within Rituximab treated animals was also accompanied by a reduction in antibody levels within infected tissue but not in serum. However, existing plasma cells that generate antibody were unaffected. Furthermore, B cell depleted animals are not mounting any responses to Rituximab that would hinder the process of B cell depletion.

6.4.3 Clinical findings of *M. tuberculosis* B cell depleted animals

Prior to necropsy, PET/CT scanning using [^{18}F]-FDG was performed to assess disease progression within the lungs and thoracic lymph nodes and provide a "roadmap" for harvesting lesions at necropsy. Both Rituximab and saline control animals were found to have approximately the same number of granulomas on scan, with a median of 19 granulomas (range of 11 to 24 granulomas per animal) within the Rituximab group and 18 granulomas (range of 11 to 24 granulomas per animal) for saline controls (Fig. 22.).

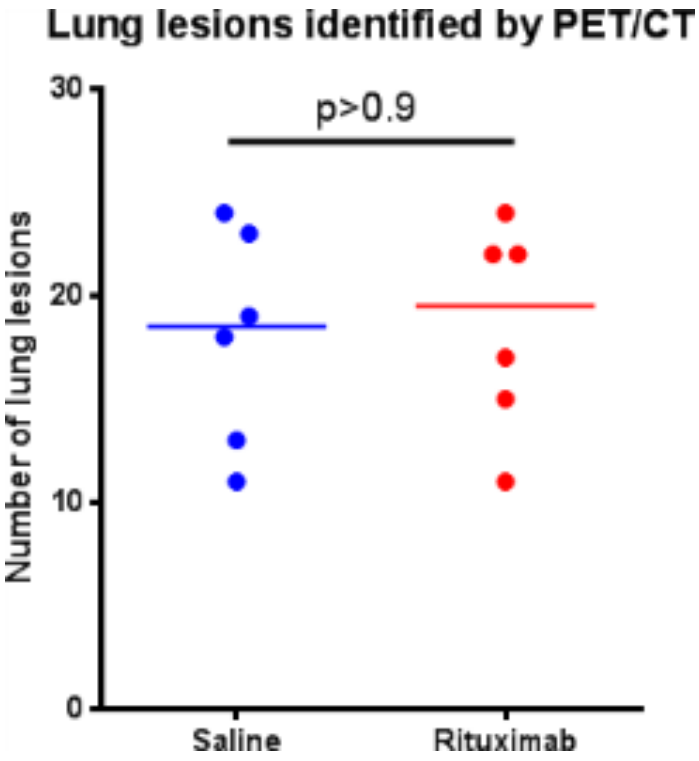

Figure 22. *PET/CT identification of lung lesions.* No differences were seen in terms of granuloma numbers as identified by PET/CT scans.

At necropsy, the gross pathology findings of each animal were used to compile necropsy scores. This score takes into account the pathological findings including s number of granulomas within each lobe, extent of lymph node involvement, disease dissemination within the lungs or

presence of extrapulmonary disease. Animals within the Rituximab group generally displayed the same amount of disease as monkeys within the saline control group, with similar necropsy scores (Fig. 23.). This suggests that disease progression at 3 months post infection was comparable with little difference in terms of gross pathology between B cell depleted animals and control animals.

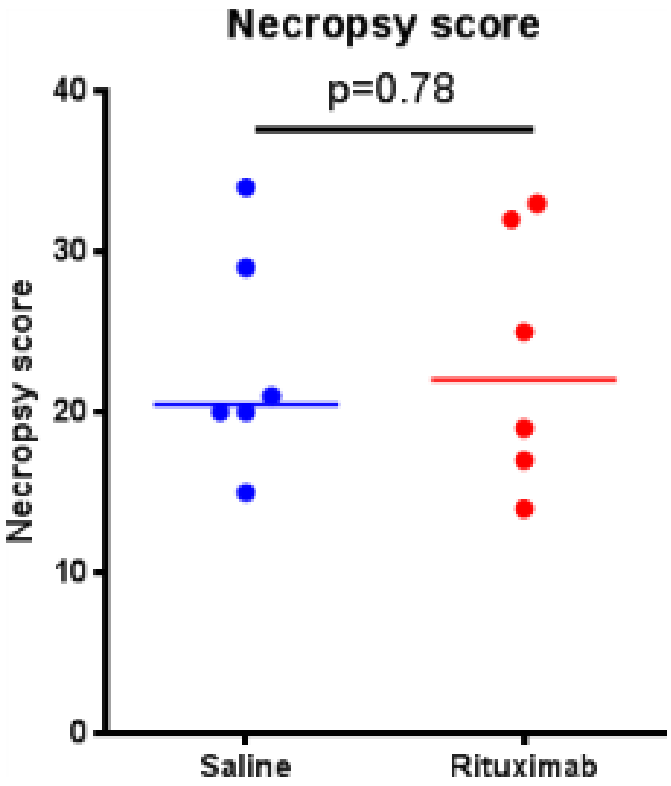

Figure 23. *Necropsy score assessment of animals.* Clinical disease presentation at necropsy, translated as a necropsy score to reflect disease dissemination and general body condition, showed no difference between B cell depleted animals and saline controls.

Previous studies on B cell deficient mice infected with *M. tuberculosis* showed severe neutrophil infiltration into the lungs of μMT mice and elevated IL10 amounts. Granulomas from Rituximab treated animals did not display excessive neutrophilic infiltrate compared to granulomas from control animals. The only noted difference was a marked reduction in the lymphocytic cuff of Rituximab treated granulomas (Fig 24.).

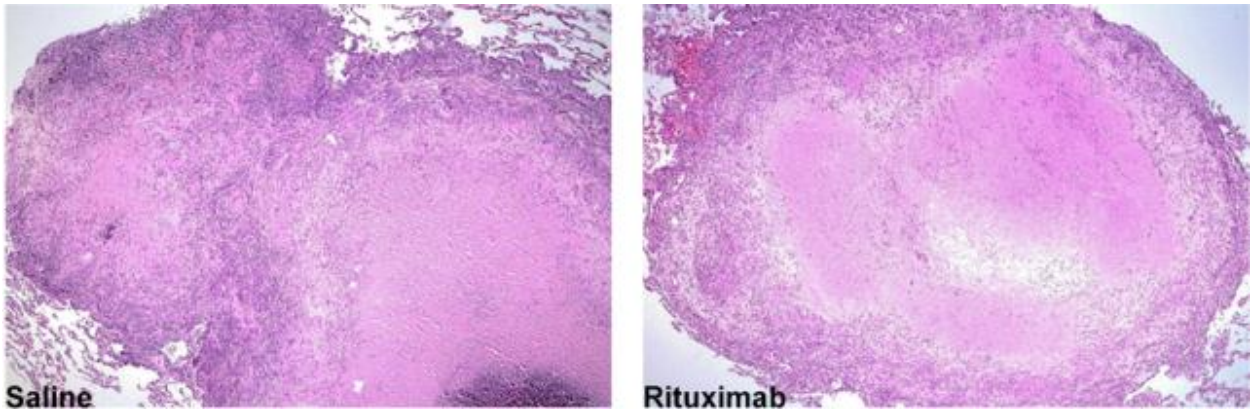

Figure 24. *Histological assessment of lung granuloma slides.* Histological assessment of lung granulomas revealed no overt differences in terms of granuloma appearance aside from a paucity of lymphocytic cells as expected from B cell depleted animals compared to saline controls.

Homogenates from numerous (more than 50) tissue samples from each monkey at necropsy were plated on 7H11 agar to assess bacterial burden. After 3 weeks, the plates were counted to determine bacterial burden for each tissue sample. Bacterial burden for lung granulomas were compiled from each animal to determine the overall bacterial burden within each group. Rituximab animals have a slightly statistically significantly higher overall bacterial burden on a per granuloma basis (Rituximab animals have 0.5 log units higher in bacterial burden compared to saline controls, p=0.01) compared to saline controls. No difference in bacterial burden was seen within the thoracic lymph node samples, with both groups displaying a median bacterial burden around 100 colony forming units (Fig. 25.).

A CFU scoring system was used to assess for overall bacterial burden within an animal, taking into account of all samples including extrapulmonary and non-granulomatous samples. The median CFU score of Rituximab treated animals were not statistically different from the median CFU score of saline controls (saline group median score at 115, Rituximab group median score at 148, p=0.3, Fig. 26.).

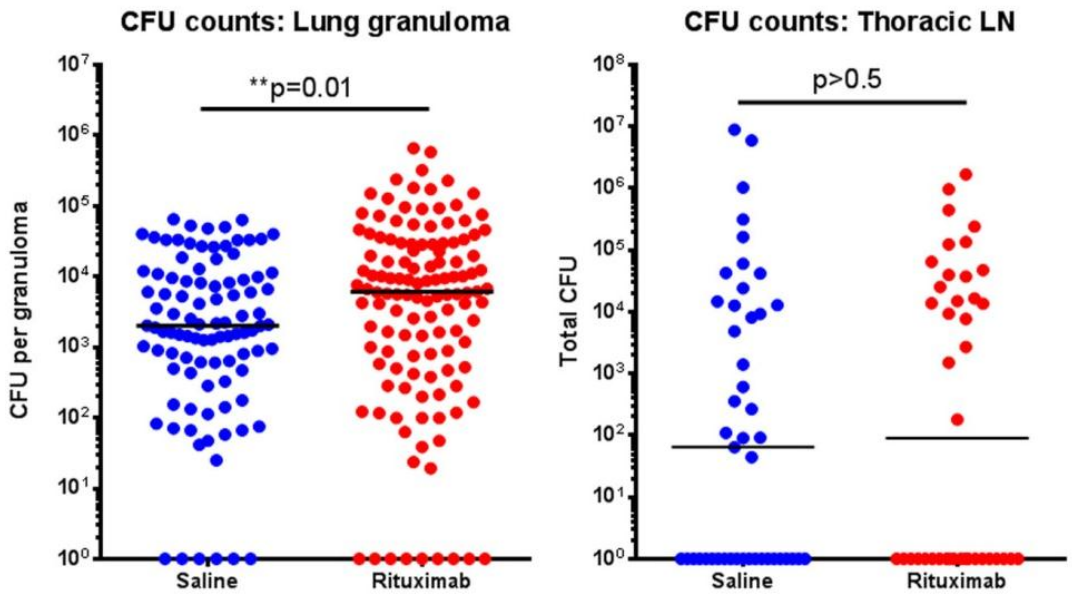

Figure 25. *Bacterial CFU assessment of lung granulomas and thoracic lymph nodes.* Bacterial burden within individual lung granulomas, normalized to per granuloma and from thoracic lymph nodes were performed by plating tissue homogenate on 7H11 agar and read 3 weeks later. A statistically significant higher bacterial CFU was observed within lung granulomas of B cell depleted animals (median difference of 0.5 log units, p=0.01) compared to saline controls. Bacterial burden within the thoracic lymph nodes were comparable within both groups of animals. Each dot represents one sample from one animal with a total of 12 animals.

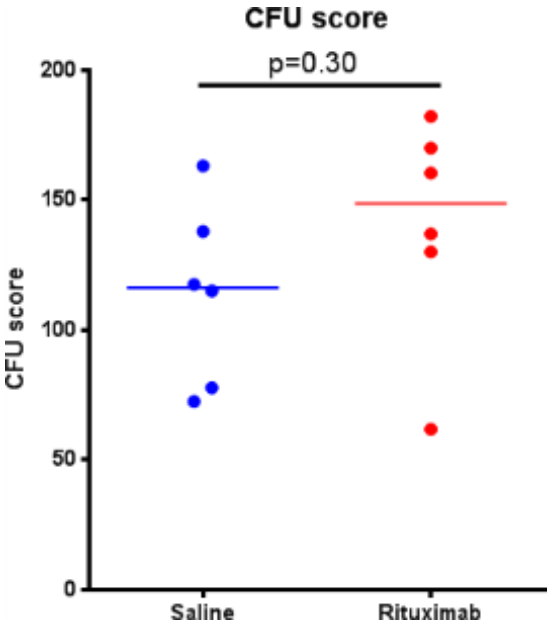

Figure 26. *CFU score of animals to assess overall bacterial burden.* Overall bacterial burden within an animal was assess using a CFU score calculated by taking into account of all bacterial CFU counted in all the collected samples from each animal at necropsy. CFU scores of B cell depleted animals (median score of 148) and saline control animals (median score of 115) were not statistically significant (p=0.30). Each dot represents one animal with a total of 12 animals, 6 per group.

The percentage of sterilized granulomas with no bacterial growth on 7H11 agar were not significantly different in samples from both groups (7.85 percent for saline control versus 5.69 percent in Rituximab samples, p=0.92, Fig. 27.). Lung granuloma samples from individual animals were found to have either no bacteria or up to 10^6 bacterial present (Fig. 28.) which reflects the granuloma variability within individual animals and between animals.

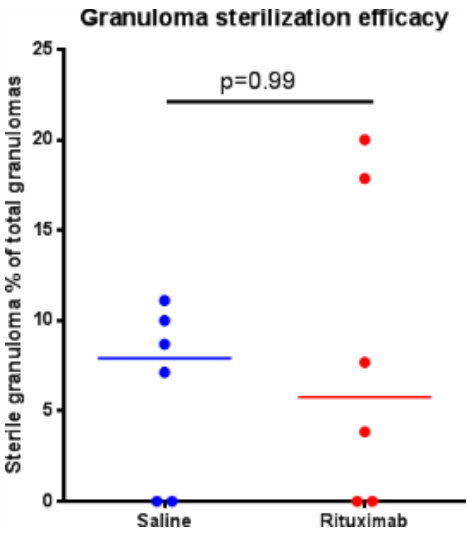

Figure 27. *Granuloma sterilization efficiency assessment.* This figure depicts the percent of granuloma samples that did not have any bacterial CFU on 7H11 agar after 3 weeks of incubation. These samples are termed sterile due to the absence of any *M. tuberculosis* bacteria recovered. Granulomas from saline controls and B cell depleted animals were not statistically different in the percent of sterile granulomas (p=0.99). Each dot represents one animal with a total of 12 animals, 6 per group.

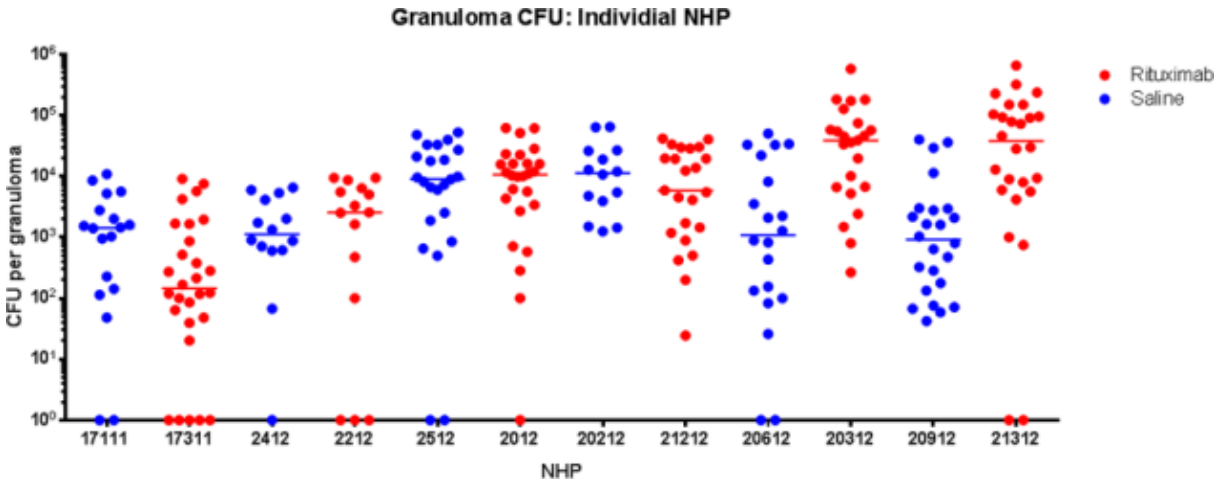

Figure 28. *Variation of granuloma CFU within individual animals.* This graph depicts the range of bacterial burden within lung granulomas of animals from B cell depleted group (red) and saline controls (blue). Each dot represents one sample from the respective animals from a total of 12 animals, 6 per group.

6.4.4 T cell responses within non-human primates were unaffected by B cell depletion

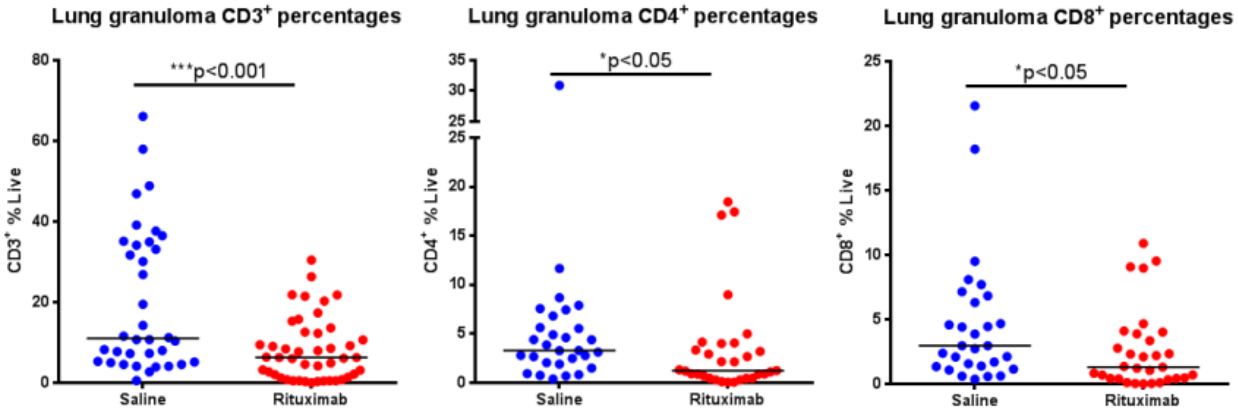

Figure 29. *T cell percentage of live cell assessment.* Median lung granuloma CD3, CD4 and CD8 percentage of lymphocytes were found to be lower ($p<0.001$ for both CD3 and CD4 percentages and $p<0.005$ for CD8 percentages) within B cell depleted animals compared to saline counterparts. Each dot represents one lung granuloma from one animal from a total of 12 animals, 6 per group.

Single cell suspensions from tissue samples obtained at necropsy were analyzed by cell surface and intracellular cytokine staining for flow cytometry to assess for T cell numbers and cytokine production. CD3, CD4 and CD8 percentages were statistically lower within lung granulomas of the Rituximab treated animal ($p<0.05$) for CD3, CD4 and CD8 percentage of lymphocytes, indicating that fewer T cells were present within lung granulomas in the absence of B cells (Fig. 29.). This is confirmed by enumerating actual CD3 counts and normalizing to tissue weight of the respective tissue. Lung granuloma samples from Rituximab treated animals have a lower median of $CD3^+$ cells compared to saline control granulomas (Fig. 30.).

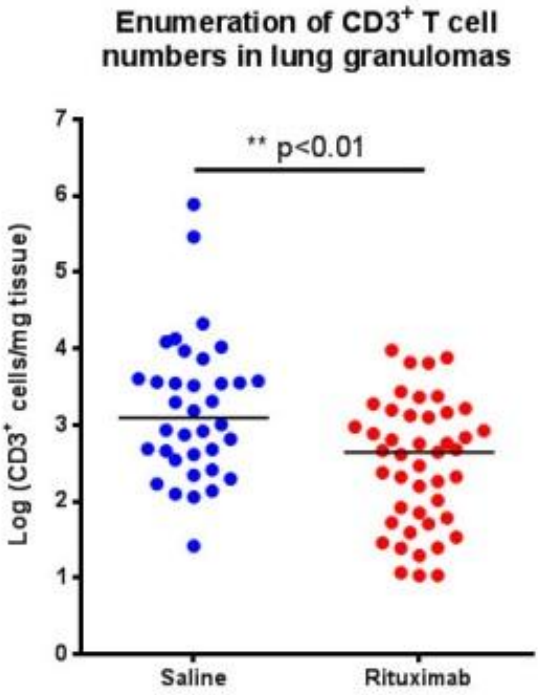

Figure 30. *$CD3^+$ T cell enumeration of granuloma samples from Rituximab treated and saline control animals.* Rituximab treated animals had a lower median of $CD3^+$ T cells within the granuloma (by about 0.5 log units) compared to saline control granulomas, $p<0.01$. Each dot represents on granuloma sample from one animal from a total of 12 animals, 6 per group. $CD3^+$ T cell numbers were normalized to tissue weights expressed in milligrams (mg tissue).

T cell cytokine secretion profiles were also assessed in lung granuloma samples for the percentages of T cells making IL2, IL17, TNF or IFNγ in response to ESAT6 and CFP10 peptides. No difference in T cell cytokine production was observed in lung granulomas, except for IL2, within Rituximab treated animals and saline controls. The percentage of IL2 producing T cells was slightly elevated ($p=0.058$) within samples from B cell depleted animals for lung granulomas (Fig. 31.). A similar pattern was observed in thoracic lymph nodes of Rituximab animals, with only elevation of IL2 producing T cells compared to saline controls.

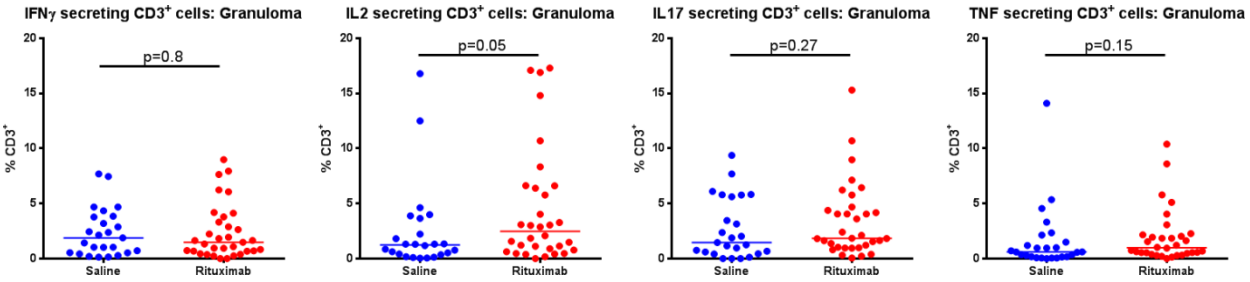

Figure 31. *T cell responses within B cell depleted animals are largely unaffected aside from IL2 production.* Cytokine secretion by CD3 cells as measured by intracellular cytokine FACs showed no difference in IFNγ, TNF or IL17 production. A trend of slightly increased IL2 secretion was observed within B cell depleted animals compared to saline controls (p=0.058). Each dot represents one lung granuloma from one animal from a total of 12 animals, 6 per group.

Enumeration of cytokine secreting $CD3^+$ T cells within the lung granuloma samples were performed to confirm the cytokine secreting T cell percentages within the lung granuloma samples. The numbers of $CD3^+$ T cells secreting IL2, IL17 and TNF were not statistically significant although there was a trend that Rituximab treated animals had lower numbers of cytokine secreting T cells within the granuloma compared to saline controls. However, the number of IFNγ secreting T cells was statistically lower within Rituximab treated granulomas compared to saline controls indicating that less T cells secreting IFNγ was present (Fig. 32.).

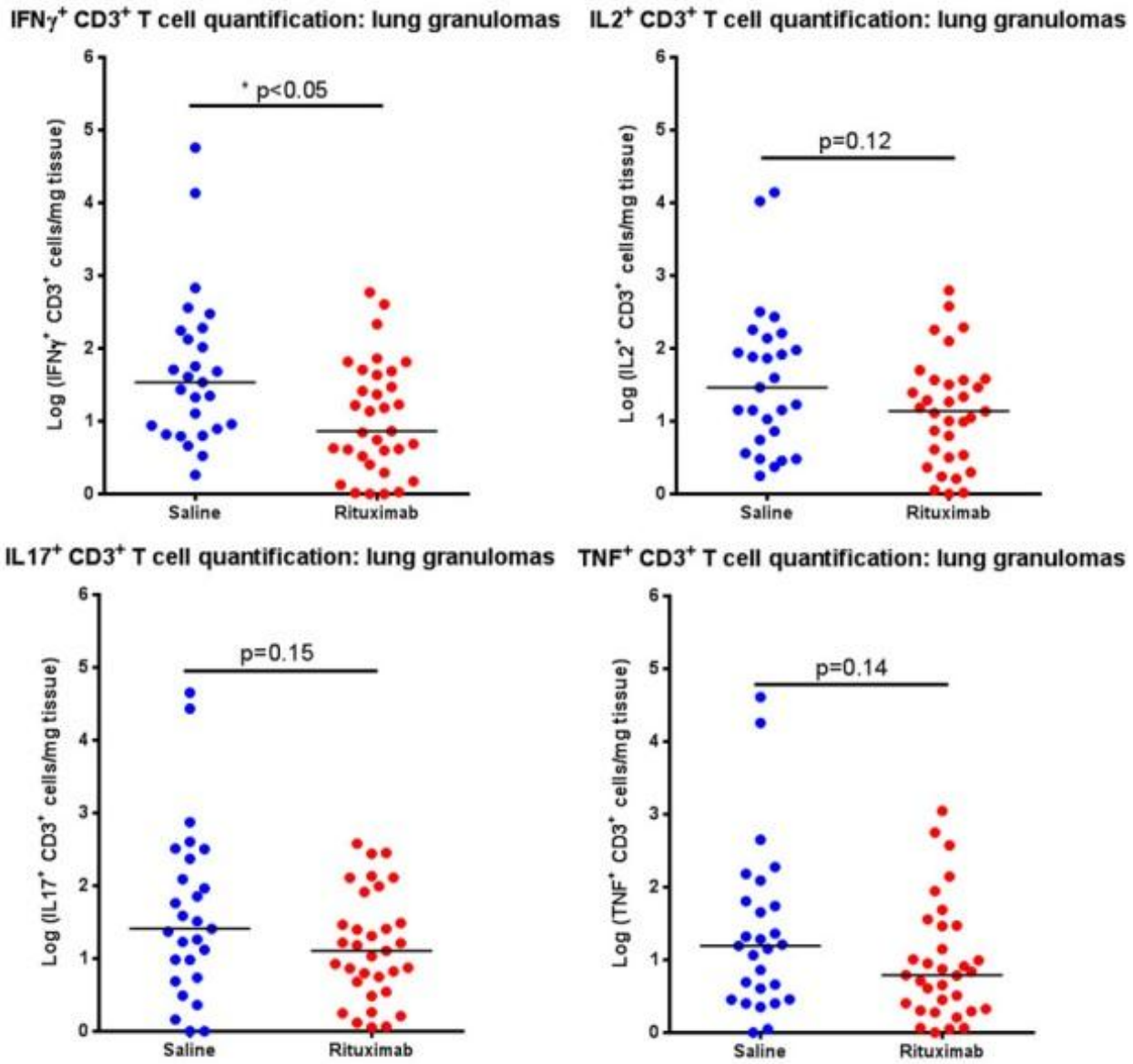

Figure 32. *Cytokine secreting CD3$^+$T cells were enumerated and normalized according to tissue weights.* Although not statistically significant, the number of T cells secreting IL2, IL17 and TNF were slightly lower within Rituximab treated granulomas compared to saline control samples. IFNγ secreting T cells, however, were statistically lower within Rituximab treated granulomas compared to saline controls indicating that there were less IFNγ secreting T cells present at the site of infection within B cell depleted animals. Each dot represents one lung granuloma from one animal from a total of 12 animals, 6 per group.

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6.4.5 Immunological readouts within the granuloma are similar between Rituximab and control groups

To assess for the presence of neutrophils, a calprotectin ELISA was performed as a surrogate readout for neutrophils[246]. Levels of calprotectin per granuloma were not significantly different between Rituximab treated and saline control animals (Fig. 33.), suggesting that neutrophils are obviously impaired in recruitment or function.

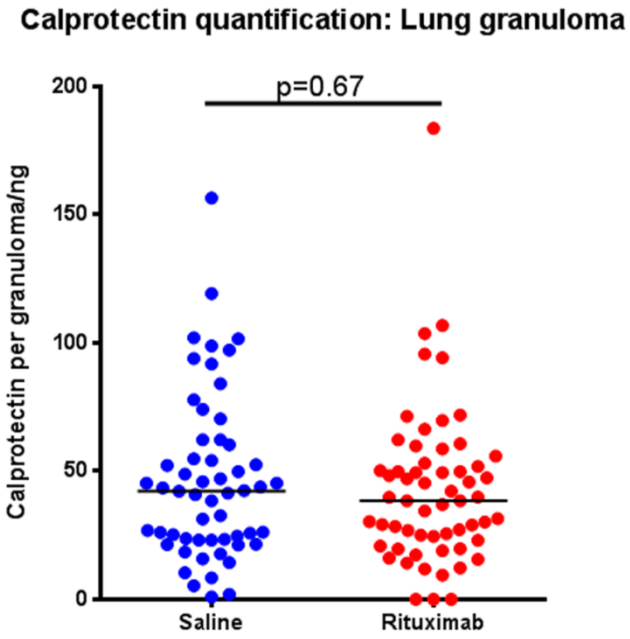

Figure 33. *Quantification of calprotectin protein levels within lung granulomas.* A calprotectin ELISA was performed using lung granuloma homogenates as a means to measure neutrophil presence. No difference was seen between B cell depleted and control animals for calprotectin amounts, $p>0.99$. Each spot represents one lung granuloma sample. 12 animals, 6 per group were represented here.

IFNγ ELISPOT responses to CFP, and ESAT6 and Rv1196 peptides were assessed using lung samples that were culture positive for *M. tuberculosis*. No difference in numbers of IFNγ secreting cells were seen between B cell depleted and control group animals in response to these mycobacterial antigens (Fig. 34.).

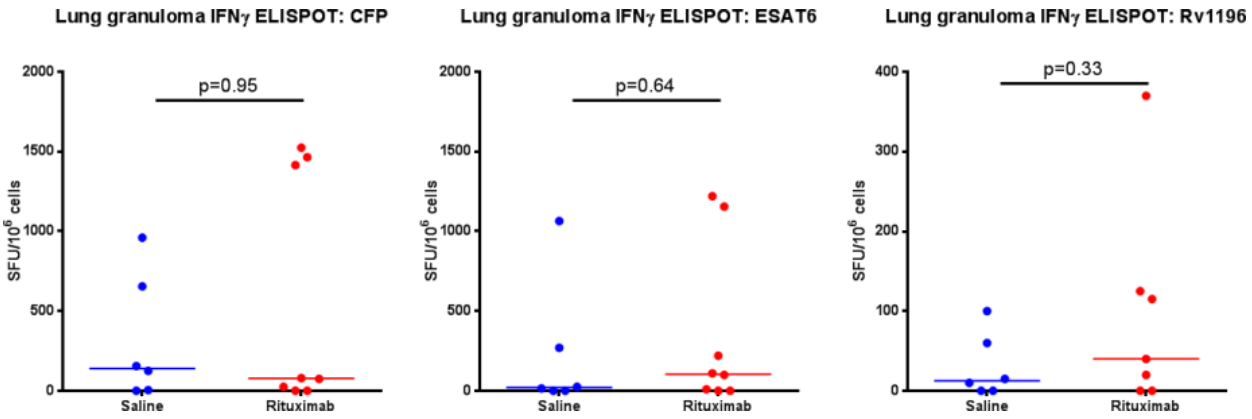

Figure 34. *IFNγ ELISPOT analysis of lung granuloma cells in response to M. tuberculosis antigens.* IFNγ ELISPOTS to assess for cytokine release upon stimulation with CFP, *M. tuberculosis* antigens ESAT6 and Rv1196 showed that responses were comparable within lung granuloma samples from B cell depleted animals and saline control group.

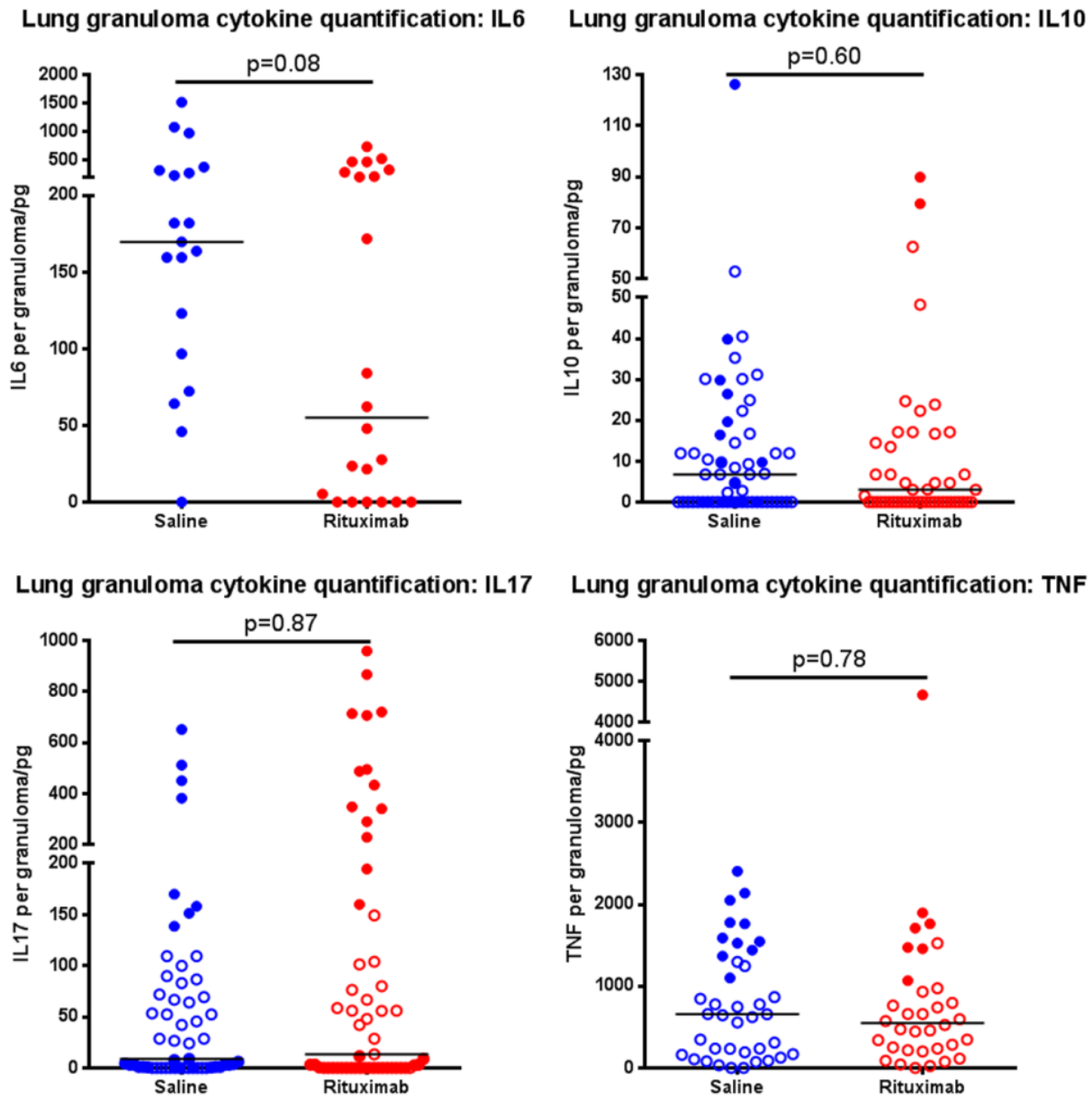

Figure 35. *ELISA data for cytokine quantification within lung granuloma homogenates.* Cytokine ELISAs to assess for IL6, IL10, IL17 and TNF amounts within lung granuloma homogenates of animals of both groups also revealed no significant differences for all cytokines chosen. Each spot represents one lung granuloma sample. 12 animals, 6 per group were represented here. Open circles indicate interpolated sample values that fell below the lowest detectable limit as measured from the standard curves of the respective assays.

Cytokine ELISAs were performed to assess for levels of IL10, IL17 and TNF in an effort to identify any differences in pro- or anti-inflammatory cytokines within granulomas. A great degree of variability was observed in terms of cytokine content within the granuloma

homogenates obtained from one animal and between animals, consistent with other studies we have performed in this model. Granuloma homogenates from Rituximab treated animals were not statistically different in terms of cytokine content for these cytokines compared to granuloma homogenates obtained from animals in the saline control group with the exception of IL6. Median IL6 content within B cell depleted granulomas were almost significantly lower than saline control granuloma homogenates ($p=0.08$), indicating that there is a trend of lower IL6 within the granuloma as a result of B cell depletion (Fig. 35.).

6.4.6 Correlative studies on granuloma cytokine content

Quantification data of cytokines, cell composition and bacterial burden of individual granulomas were used from either B cell depleted or saline control animals to assess for correlations between parameters. This analysis was performed to identify any differences in the correlative interactions between the various cytokine components, T cell percentages, antibody content and bacterial numbers (Table 1.). Within saline control granulomas, IL10 levels positively correlated with IL17 levels ($R^2=0.76$) and IL6 levels positively correlates with bacterial burden ($R^2=0.61$). However, these relationships were different within granulomas from the B cell depleted group where IL6 correlates poorly with bacterial burden ($R^2=-0.08$) [Fig. 36.] and IL10 does not correlate with IL17 ($R^2=-0.23$) [Fig. 37.]. Thus B cell depletion seems to influence the interplay of cytokines, cell percentages and bacterial burden in the granuloma. These differences may hint at changes in cell behavior that could produce significant changes in longer term B cell depletion.

Correlations of parameters: Rituximab

	IL6 (pg)	IL10 (pg)	IL17 (pg)	TNF (pg)	Calprotectin (ng)	CD3 % Live	CFP Ab (ug)	Total IgG (ug)	Log (CFU/gran)
IL6 (pg)	1								
IL10 (pg)	0.4959	1							
IL17 (pg)	-0.3344	-0.2262	1						
TNF (pg)	0.1686	0.4308	-0.2775	1					
Calprotectin (ng)	0.2492	0.3571	-0.1916	0.5447	1				
CD3 % Live	0.2784	0.0417	-0.2043	-0.6249	-0.0274	1			
CFP Ab (ug)	0.429	-0.1223	0.1138	0.4732	0.4098	-0.2353	1		
Total IgG (ug)	0.5367	0.0979	0.0921	0.4686	0.4529	-0.1277	0.9199	1	
Log (CFU/gran)	-0.0836	0.0429	0.3115	-0.1829	0.1678	-0.0692	0.0446	0.0228	1

Correlations of parameters: Saline

	IL6 (pg)	IL10 (pg)	IL17 (pg)	TNF (pg)	Calprotectin (ng)	CD3 % Live	CFP Ab (ug)	Total IgG (ug)	Log (CFU/gran)
IL6 (pg)	1								
IL10 (pg)	-0.125	1							
IL17 (pg)	-0.1417	0.7632	1						
TNF (pg)	0	0.323	-0.0459	1					
Calprotectin (ng)	-0.0289	-0.0243	-0.1677	0.4747	1				
CD3 % Live	0.826	-0.1974	-0.0827	-0.1866	-0.0612	1			
CFP Ab (ug)	0.5003	0.4117	0.3144	0.2517	0.2058	-0.1547	1		
Total IgG (ug)	-0.1911	-0.0537	-0.0913	0.2971	-0.031	-0.0174	-0.021	1	
Log (CFU/gran)	0.6069	0.1826	-0.0707	0.076	0.4371	-0.1734	0.3167	0.5062	1

Table 1. *Analysis of correlations between various cytokines, calprotectin, CFU, T cell composition and antibody content for individual granulomas of the Rituximab treated group and saline controls.* The boxed correlation values were noted to be different within granulomas from saline control animals versus B cell depleted animals. R^2 values were confirmed to be significant following a non-parametric Spearman's ρ analysis (Appendix A, Tables 2. and 3.).

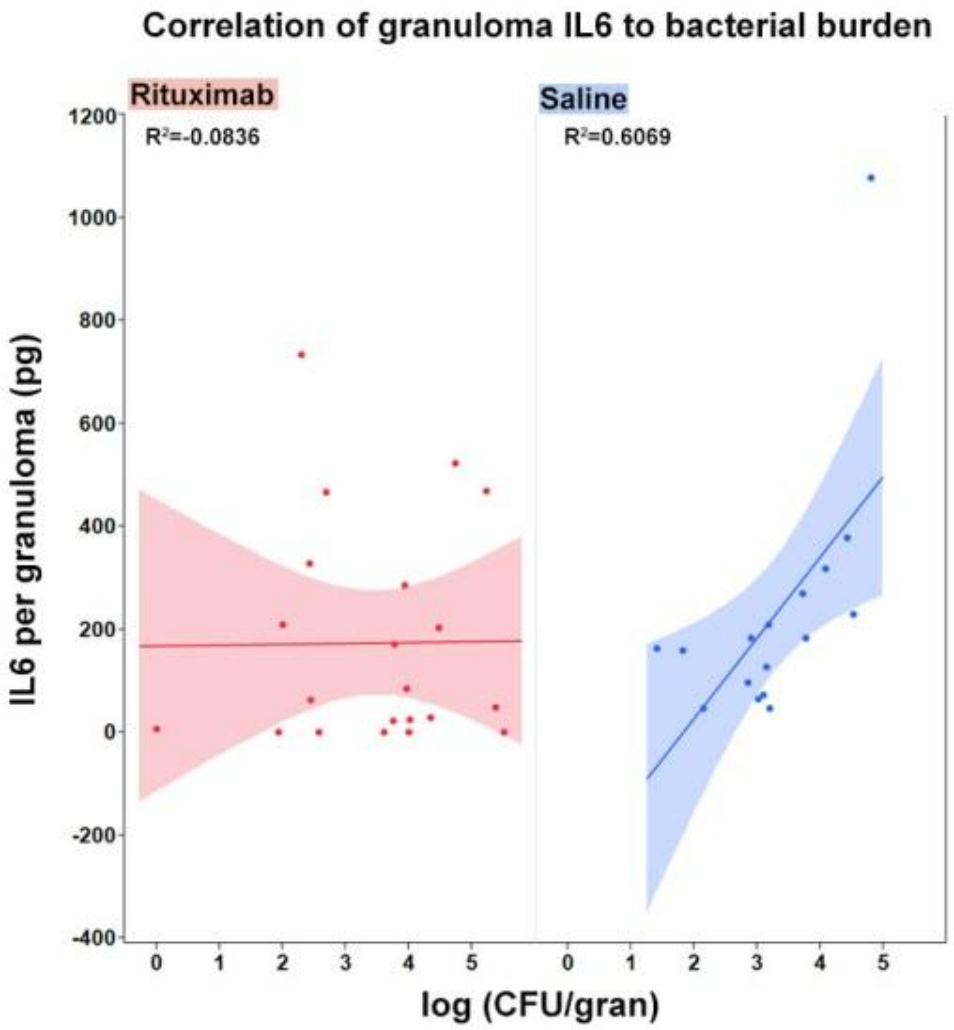

Figure 36. *Linear correlation analysis of granuloma IL6 content to bacterial burden.* The graph depicts the levels of IL6 within a granuloma and its corresponding bacterial burden. A linear correlation was performed to note the relationship between these two variables. R^2 values for Rituximab granulomas were -0.084 compared to 0.607 for saline controls, indicating that the relationship of IL6 and bacterial burden was different from granulomas of B cell depleted animals. Each point represents one granuloma from one animal from a total of 6 animals per group.

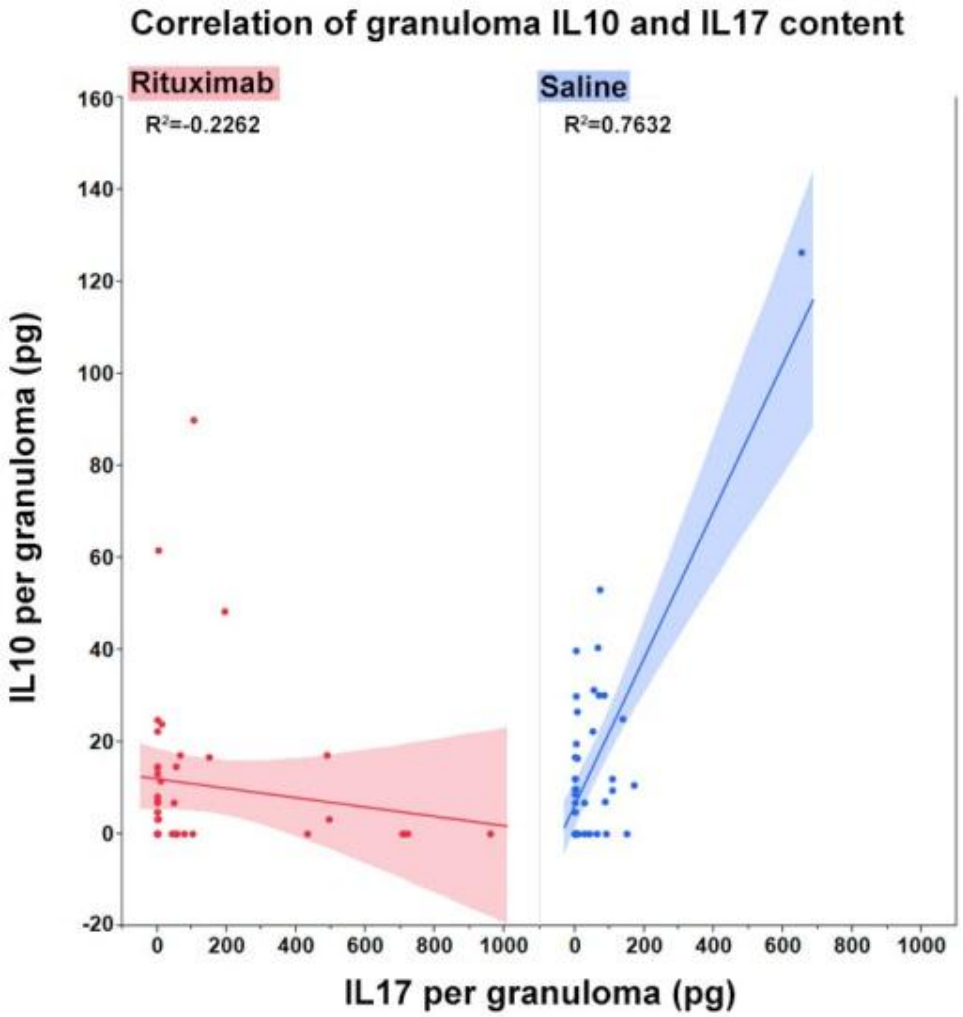

Figure 37. *Linear correlation analysis of granuloma IL10 content to granuloma IL17 content.* The graph depicts the levels of IL6 and IL10 within granulomas from B cell depleted and saline control animals. A linear correlation was performed to note the relationship between these two cytokines. IL10 was noted to be negatively correlated with IL17 within B cell depleted granulomas but being positively correlated with IL17 in saline control granulomas. This suggests that cytokine expression within B cell depleted granulomas is markedly different compared to saline controls. Each point represents one granuloma from one animal from a total of 6 animals per group.

6.5 DISCUSSION

The goal of this study was to determine whether B cell depletion affects the outcome of *M. tuberculosis* infection within non-human primates. B cell depletion was achieved by treatment with repeated administration of anti-CD20 antibody (Rituximab), while control animals were

treated with saline. The choice of saline as a control was made, since administering NHP IVIg could result in serum sickness[230, 247, 248]. The duration of the study was for a total of 12 weeks with 2 initial weeks of B cell depletion prior to infection with *M. tuberculosis*. The infection period of 10 weeks was used due to signs of adverse reactions to Rituximab infusion (anaphylaxis) getting progressively worse in some animals, meaning that B cell depletion states could not be safely maintained and thus necessitates the short study period of 10 weeks post infection. The cause of anaphylaxis is unknown but is unlikely to be due to Rituximab based on the lack of anti-Rituximab antibodies being generated. Furthermore, any B cells that do respond to human antibody epitopes are likely to be depleted anyway with further Rituximab administration. A likely explanation could be that the lysing of B cells during the depletion period releases pro-inflammatory mediators systemically, resulting in anaphylaxis.

This study also allows for the examination of tuberculosis at 10 weeks post infection within non-human primates, which can provide valuable information regarding the acute stage of infection. Indeed, data from lung granuloma bacterial burden from the control animals in this study suggests that the immune system is still actively attempting to reduce lung granuloma bacterial numbers[44, 133]. PET/CT imaging employed in this study also seem to support this idea as lesion numbers and lymph node metabolic activity were comparable from animals from both Rituximab treated and saline control groups. Based on the observations in B cell depleted animals, it appears that B cells may not be playing a significant role in controlling infection at such an early stage. However, with the immune system still actively attempting to reduce bacterial numbers, it is difficult to directly attribute disease outcome with B cell depletion. B cells may very well be contributing towards disease control by more subtle means that is difficult to be measured at 10 weeks post infection. Longer future studies, with serial PET/CT scanning,

would help to resolve B cell contribution to tuberculosis control more clearly as it would permit lesion development to be tracked over the entire course of infection[133].

Within Rituximab treated animals, B cells, antigen specific antibody and total IgG were confirmed to be markedly diminished. The B cell clusters that are reminiscent of germinal centers (Chapter 5) within lung granulomas of animals treated with Rituximab were either reduced in size or absent entirely compared to control granulomas. However, T cell percentages as measured by flow cytometry were also slightly reduced. B cell clusters within the lung granulomas were previously characterized as being areas where antigen presentation occurs judging from the increased expression of MHC class II molecules and proliferation of B cells[214]. Absence of such clusters within the granulomas and reduced B cell follicles within the lymph nodes may thus reduce the efficacy of proliferation, activation, differentiation and trafficking of antigen specific T cells into the granuloma. Furthermore, a trend of increased IL2 production by T cells within Rituximab treated granulomas compared to saline control samples was noted but did not achieve statistical significance ($p=0.05$). This might reflect compensatory mechanisms within T cells to encourage further T cell expansion. Natural killer cells could also be present in higher numbers within Rituximab treated animals. Unfortunately, natural killer cell markers were not included in the panel of study, thus preventing any further possible explanations for the reduction in T cell percentages within B cell depleted animals.

Findings at necropsy in terms of gross pathology, clinical disease manifestation and progression were unremarkable when comparing B cell depleted animals to the saline control counterparts. However, this data may indicate that at this acute stage of infection, the immune system is still in the process of controlling the spread of the bacilli. The most intriguing observation was the increase in median CFU on a per granuloma basis (by 0.5 log units) from B

cell depleted lung granulomas. Although the increase is modest, this does suggest that B cell depleted animal granulomas on average are not as capable as their control granuloma counterparts in controlling bacterial growth, at least at 10 weeks of infection.

Cytokine production by T cells within the lung granulomas does not appear to be different within animals of both groups, at least for the production of IL17 or TNF. A trend of increased percentages of T cells making IL2 was observed within the Rituximab treated animals. Seeing as how both the T cell lymphocyte percentages and numbers were lower within Rituximab treated lung granulomas, the increase in IL2 production might be a way to compensate and induce T cell proliferation within sites of infection. Although the percentage of IFNγ secreting T cells were similar within B cell depleted animals and saline controls, the absolute numbers of IFNγ producing T cells, normalized to tissue weight, was lower within B cell depleted samples compared to controls. With lower numbers of IFNγ T cells, bacterial control within B cell depleted granulomas may be slightly impaired and may contribute towards the increased numbers of bacterial CFU recovered from Rituximab treated animals. The precise mechanism that leads to lower numbers of IFNγ producing T cells are unknown as there could be defects either in the generation or recruitment of IFNγ secreting T cells.

The individual cytokine composition of the lung granulomas was not statistically different when comparing Rituximab treated samples against saline controls at least for IL10, IL17, TNF, and IFN-γ. Calprotectin, a marker of neutrophils, was not different as well within granulomas from either group, indicating that levels of inflammation between granulomas of both groups were comparable. The only notable difference in cytokine levels within the granuloma was IL6 where a trend of lower IL6 granuloma content was observed within Rituximab treated animals ($p=0.08$). This could be attributed to B cell depletion as B cells were

noted to secrete primarily IL6 in their cytokine secretion profile. Depleting B cells appears to result in a reduction in IL6 within infected tissues. These datasets are rather different from findings of B cell knockout mice infected with *M. tuberculosis*, where these mice had increased neutrophilic infiltrate and increased IL10 levels in the lungs[211, 234]. It is very likely that a 10 week low dose infection with *M. tuberculosis* for non-human primates is still in the acute phase unlike the mouse model of infection. Hence, a long term study of 6 months with *M. tuberculosis* infected NHPs undergoing B cell depletion might provide a much more conclusive answer. However, such extended depletion studies are likely not feasible due to adverse reactions to long term repeated administration of rituximab, as with other antibodies in non-human primates[105].

Although the cytokine levels themselves are not different within B cell depleted granulomas and saline control samples, the relationships between cytokines are skewed following B cell depletion. The most interesting observation was the change in the IL10 relationship with IL17 within granulomas of Rituximab treated animals relative to controls (Fig. 37). We demonstrated that B cells were capable of secreting both IL17 and IL10 (Fig. 13). Within control granulomas, B cells organize themselves into aggregates with germinal center features[214] and thus cytokine secretion would presumably have roles in T helper cell differentiation and controlling inflammation. Rituximab treated animals lack B cells and thus, lack the cytokines contributed by B cells. The changes in cytokine correlations might hint at possible modulation of the granuloma cytokine environment. In murine tuberculosis models, an absence of B cells in infected mice results in increased neutrophil infiltration of the lungs and increased bacterial burden at the site of infection. It is possible that the increased IL17 and IL10 content of control granulomas reflect the ability of B cells to recruit neutrophils and activate T cells within the ectopic germinal center clusters to limit bacterial growth while simultaneously

limiting bystander tissue damage via IL10 mediated activity. When B cells are absent, there are less T cells being activated, allowing for increased bacterial growth. This increase in bacterial burden results in the immune cells within the granuloma to start recruiting more neutrophils in an attempt to control bacterial numbers by secreting pro-inflammatory mediators. The increased inflammation in turn results in the increase of IL10 secretion, dampening down inflammatory activity and permitting bacterial growth again. This effectively sets up a vicious cycle of bacterial growth, increased neutrophilia and increased IL10 production as seen in the B cell knock-out mice tuberculosis model where more neutrophils are noted within the lungs along with elevated IL10 levels[211, 215, 234]. Within the B cell depleted animals, only increased bacterial burden is noted and a possible dysfunction in the relative cytokine levels of IL10 and IL17 within the granuloma. However, this study only looked at 10 weeks of infection where the disease is still thought to be in the process of reaching a state of equilibrium with the immune system[44, 133]. A longer B cell depletion study would provide invaluable data in validating this proposed model.

The other observation when assessing relationships between the various immune and bacterial parameters of the granuloma was the correlation of IL6 and bacterial burden (Fig. 34). Within saline control granulomas, IL6 levels positively correlated with granuloma bacterial burden, suggesting that more bacterial brings about more IL6 mediated activity. However, this relationship is absent in B cell depleted granulomas, suggesting that an inflammatory response element to bacterial burden is absent. A lack of pro-inflammatory signaling within these granulomas may explain the higher median bacterial burden among B cell depleted granulomas. IL6 mediated signaling in response to bacterial burden may thus be essential in encouraging pro-inflammatory responses to limit bacterial growth within the granuloma.

This study shows that an absence of B cells did not significantly alter acute *M. tuberculosis* progression and outcome, at least up to 10 weeks. However, there are several observations that hint at possible differences namely the increased CFU within B cell depleted lung granulomas and the altered cytokine correlations. In a more chronic setting, these changes may be significant but for now the contribution of B cells to tuberculosis control is minimal in the acute setting.

6.6 ACKNOWLEDGEMENTS

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7.0 MACROPHAGE BEHAVIOR IN B CELL DEPLETED *MYCOBACTERIUM TUBERCULOSIS* INFECTED CYNOMOLGUS MACAQUES

7.1 ABSTRACT

B cells and antibody are known to be capable of influencing macrophage activation phenotype and regulating inflammation in infection and autoimmunity. In murine *M. tuberculosis* infection models, FcγR mutants were noted to have different susceptibility or resistance to disease depending on various receptor knockouts and point towards the involvement of antibodies in regulating disease progression and inflammation. However, the mechanisms by which B cell and antibody regulating macrophage activation within the non-human primate model of tuberculosis have not been studied. This study aims to elucidate the relationship between B cell and macrophage activation states from non-human primate B cell depleted granulomas by examining the ratio of enzymes arginase 1 and inducible nitric oxide synthase (iNOS). These enzymes are differentially expressed within different macrophages depending on the mode of activation. Although both antibodies and B cells were shown to be depleted previously within Rituximab treated granulomas, the study did not find a difference in the levels of arginase 1 or iNOS expression within the lymphocytic cuff or the epithelioid macrophage layers of Rituximab treated or saline control granulomas. No difference was seen in the ratio of arginase 1 to iNOS within both regions of interest between the granulomas as well. This again reinforces the notion

that the contribution of the humoral immune system towards disease control at least at 10 weeks post infection is not significant.

7.2 INTRODUCTION

Macrophages represent a crucial cell population in the context of tuberculosis as they are the first cells that encounter the pathogen and are essential in eliminating the bacilli, if properly activated. However, should macrophage activation become uncontrollable, disease pathology worsens and allow for *M. tuberculosis* to disseminate. Thus, a balance of pro- and anti-inflammatory inputs to the macrophage is paramount in maintaining proper granuloma function in eliminating the bacilli.

Cytokines such as IFNγ and TNF have proven to be essential in enhancing macrophage killing of ingested *M. tuberculosis* bacilli and overcoming the phagolysosomal fusion blockage induced by the pathogen[84, 85, 103]. Excessive macrophage activation via inflammatory cues such as TNF and IFNγ is detrimental as it exacerbates tissue damage, as observed in the case of chronic disease such as tuberculosis[33, 71].

Inflammatory macrophages can be functionally characterized by assessing the amount of inducible nitric oxide synthase enzyme (iNOS) present. A high level of iNOS is required to generate reactive nitrogen and oxygen radical species from the degradation of arginine. These reactive radical species are one of the primary means by which inflammatory macrophages kill phagocytosed pathogens effectively[72].

Macrophages can also be activated into an alternative pathway that promotes tissue remodeling via the production of IL4[249], and dampening of immune activation via IL10

production[41, 76, 77]. In murine models of tuberculosis, IL10 appears to induce macrophage alternative activation rather than IL4[112, 250]. Such alternative activation is thought to be important in reducing inflammatory damage and promote healing especially in the context of large helmith infections[249]. Macrophages that are activated via this route have been shown to upregulate the levels of arginase, an enzyme that degrades the amino acid arginine into ornithine. Arginase is thought to compete with iNOS for arginine as a substrate, thus decreasing the available pool of arginine for the production of reactive radical species[246, 251]. Macrophage behavior can thus be inferred by determining the relative levels of iNOS and arginase enzymes as arginine metabolism is tied in to microbicidal activity of the macrophage.

The goal of this study is to determine whether the absence of B cells and more importantly antibody affects the pattern of macrophage activation within the granuloma. This was done by examining the relative ratios of arginase to iNOS within the lung granulomas from B cell depleted and saline control animals. Inappropriate macrophage activation in the context of an infection is detrimental as immune cells are not able to effectively contain and eliminate the pathogens[78, 79, 241]. By understanding the factors that dictate macrophage activation, future treatment modalities or vaccines can be developed to direct macrophage activation within the appropriate context to maximize bacterial killing but minimizing tissue pathology.

7.3 MATERIALS AND METHODS

7.3.1 Tissue processing

Granuloma tissues identified at necropsy were flash frozen in liquid nitrogen prior to storage at −80°C. Samples were then homogenized by disrupting the tissue on 40μm cell strainers (BD Falcon, Franklin Lake, NJ) in PBS containing 0.1% Triton X-100 and protease inhibitor mixture (Thermo Fisher Scientific) without EDTA under BSL3 conditions. The homogenate was then filtered through a 0.22μm syringe filter.

7.3.2 iNOS enzyme assay

The iNOS enzyme assay done as described previously[246]. In brief, iNOS activity was determined using a cell lysate based assay that measured nitrite (NO_2^-) via the Griess reaction. Samples were prepared in the presence or absence of N6-(1-iminoethyl)-l- lysine (L-NIL; Cayman Chemical), a selective iNOS inhibitor to isolate the contribution of iNOS from other NOS enzymes.

7.3.3 Arginase enzyme assay

The arginase enzyme assay was performed as described[251]. In brief, arginase activity was assessed by measuring the amount of $^{14}CO_2$ generated from the conversion of L-[guanidino-^{14}C] arginine as a substrate by arginase within the tissue homogenates into [^{14}C] urea. The $^{14}CO_2$ is

generated by adding Jack Bean urease (Sigma, St. Louis, MO) to facilitate the conversion of [^{14}C] urea to $^{14}CO_2$. $^{14}CO_2$ generated is trapped by NaOH soaked grade 3 Whatman paper discs (GE Healthcare, UK).

7.3.4 Immunohistochemistry

Paraffin embedded slides of relevant tissue sections were stained as described[246]. Briefly, sections were stained with mouse anti-human arginase 1 (BD Transduction, Lexington, KY), mouse anti-human CD68 and rabbit anti-human iNOS (Neomarkers, Fremont, CA) antibodies after antigen retrieval processing. Images were taken at 20x magnification and serial images were used to generate a composite of the tissue section.

7.3.5 Data analysis

Data were analyzed using Prism 6 (Graphpad Software, San Diego, CA). All data are shown as medians to account for the variability of the data. Statistical comparisons were performed using Mann-Whitney test with $p<0.05$ considered statistically significant. Paired samples were analyzed using the Wilcoxon matched-pairs signed rank test with the same threshold, $p<0.05$ as being statistically significant.

7.4 RESULTS

7.4.1 Biochemical analysis of lung granulomas

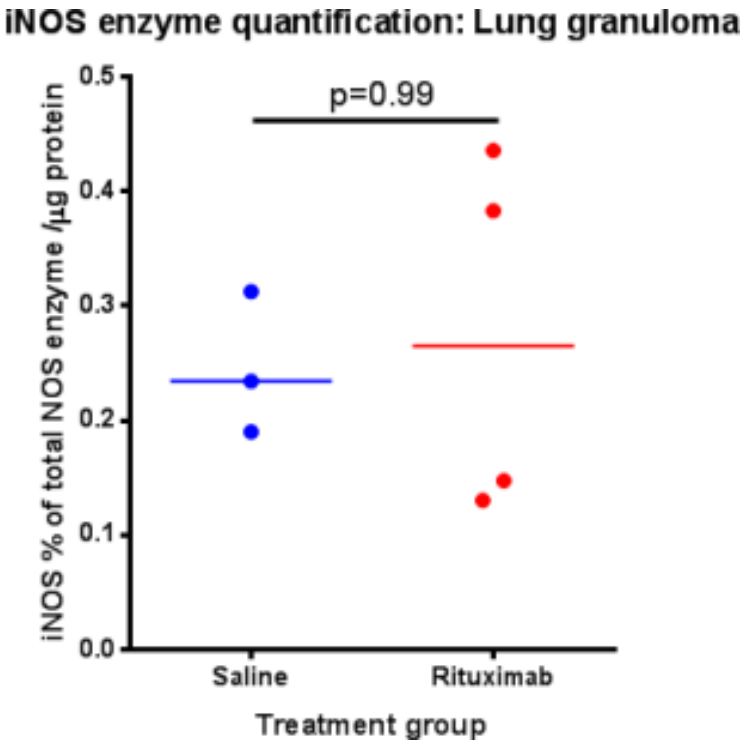

Figure 38. *Biochemical quantification of iNOS enzymes within granuloma samples.* No statistically significant difference in iNOS enzyme quantification was found between granuloma samples of B cell depleted and saline control groups. Each dot represents 1 sample.

Tissue homogenate prepared from freshly frozen lung granulomas obtained at necropsy were used in to assess the activity of iNOS, as a surrogate measurement of classical activation of macrophages, within the total protein content of tissue homogenates obtained at necropsy. The amount of iNOS enzyme activity is quantified by measuring the amount of nitrite produced in the presence or absence of a specific iNOS inhibitor, L-NIL. The iNOS inhibitor is needed to differentiate the activity of iNOS from other related nitric oxide synthase enzymes such as neural nitric oxide synthase (nNOS) and epithelial nitric oxide synthase (eNOS). Data obtained from a

total of 7 granulomas revealed no difference in terms of percent iNOS from total protein between B cell depleted and saline control granulomas (Fig. 38.).

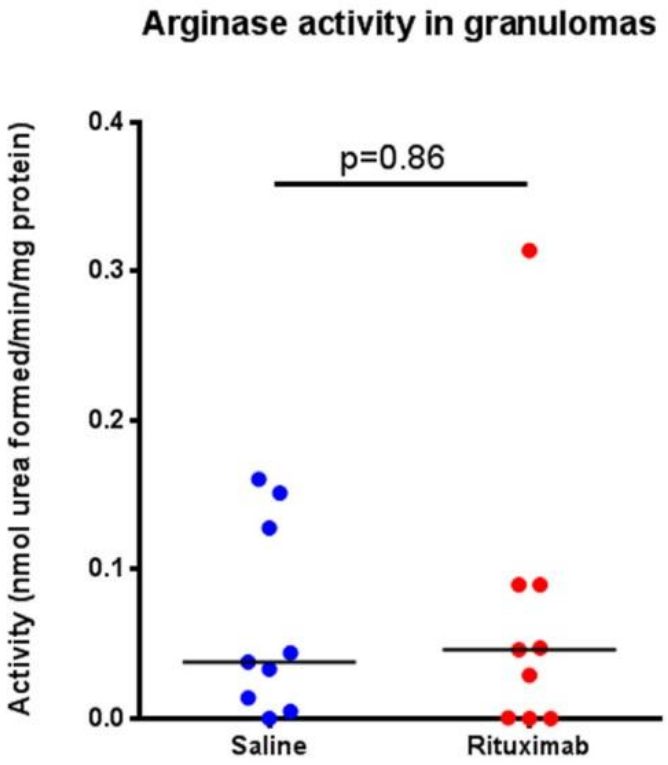

Figure 39. *Biochemical quantification of Arginase 1 enzyme within granuloma samples.* No statistically significant difference in Arginase 1 enzyme amount was found between granuloma samples of B cell depleted and saline control groups. Each dot represents 1 sample.

Arginase 1 activity was performed as a surrogate quantification of alternatively activated macrophages present within the granuloma. Tissue homogenates obtained from frozen lung sections were used to assess for arginase 1 activity by measuring the amount of $^{14}CO_2$ released used in the assay. The arginase 1 activity is calculated by normalizing the activity in the sample to the overall protein content. Analysis of granuloma samples obtained from Rituximab treated or saline control animals revealed no difference in arginase 1 activity between granulomas from both groups (Fig. 39.).

7.4.2 Assessment of enzyme expression patterns for Arg1 and iNOS within the granuloma

Paraffin embedded sections of lung granulomas from B cell depleted and control animals were stained for CD68 to identify macrophages, and enzymes arginase 1 and iNOS (Fig. 40A). CD68 staining was used to locate the epithelioid macrophage layer and approximate the lymphocytic cuff[246]. Pixel intensity analysis was performed to quantify the amount of arginase 1 and iNOS staining present within the different regions of the granuloma (Fig. 40B)[246]. Levels of arginase 1 and iNOS within either the epithelioid macrophages or lymphocytic cuff were not statistically different when comparing B cell depleted granulomas with saline control samples. No differences were seen when comparing the change in ratio of arginase 1 to iNOS between the lymphocytic cuff and the epithelioid macrophage layer of B cell depleted and saline control granulomas (Fig. 40C).

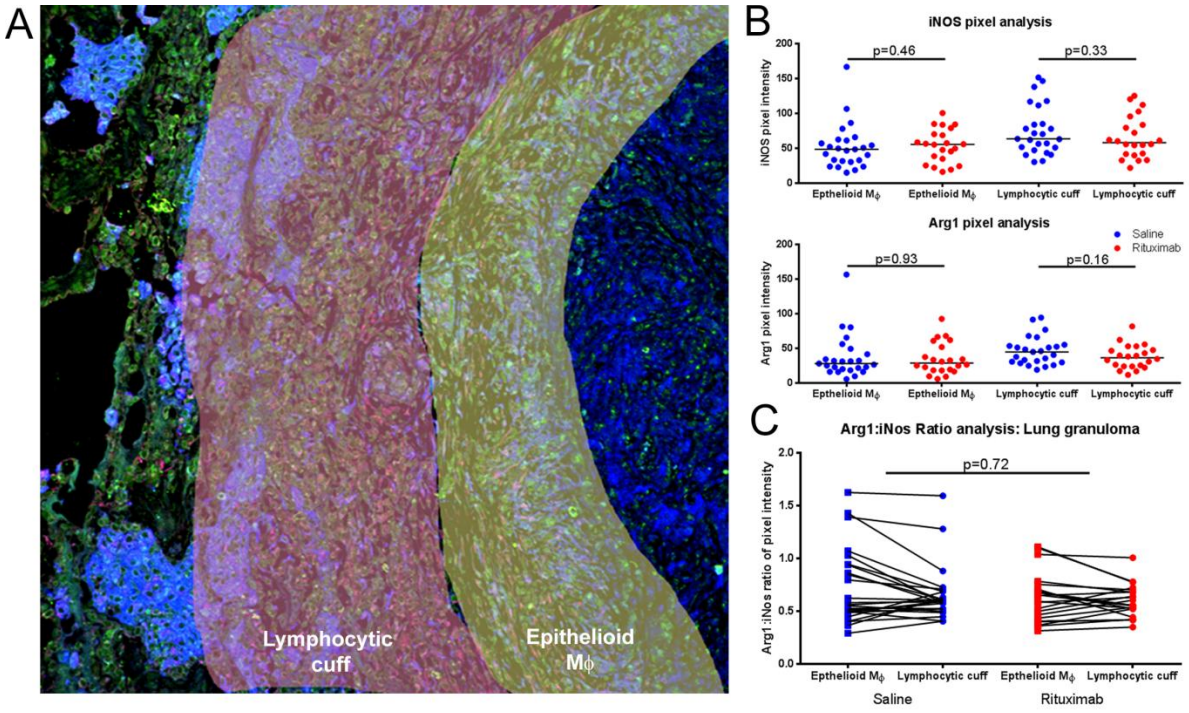

Figure 40. *Histological staining and analysis of NHP granulomas for iNOS and arginase 1 enzyme.* **A)** Staining of lung granulomas from Rituximab treated and saline control granulomas for presence of iNOS (green) and arginase 1(red). CD68 (blue) was used to identify the epithelioid macrophage layer (yellow) and lymphocytic cuff (pink). **B)** Quantification of the amount of iNOS and arginase 1 present using pixel intensity of the regions of interest within the granuloma and comparing the findings of Rituximab treated and saline control samples. **C)** Comparison of the ratio of arginase 1 to iNOS within the epithelioid macrophage and lymphocytic cuff regions of granulomas from Rituximab treated animals and saline controls.

7.5 DISCUSSION

This study was carried out to determine whether there is a relationship between macrophage activation, as measured by the amount of enzymes arginase 1 and iNOS, and B cells, more specifically antibodies. This was done by comparing granulomas obtained from B cell depleted and saline control animals. Antibodies are thought to be capable of influencing macrophage activation based on the size of the immune complex[77, 206, 222]. The goal of this study was to determine if macrophage behavior within different regions of the granuloma would change in the absence of B cells or antibodies that generate immune complexes with *M. tuberculosis* antigens.

The B cell depleted animals were infected for a period of 10 weeks with *M. tuberculosis* and no differences in the outcome of infection were seen between B cell depleted and saline control animals at necropsy. Data from the biochemical analysis of the enzymes arginase 1 and iNOS seem to corroborate with previous observations that disease progression seems to be largely unchanged within the granuloma at 10 weeks post infection. Histological staining for arginase 1 and iNOS also show that macrophage behavior within the granulomas of B cell depleted animals to be very similar to macrophages found within the granulomas of saline control animals suggesting that macrophages are similarly activated.

It is known that antibodies are capable of activating macrophages into different activation states[205, 206] or manipulate the signaling threshold for macrophages via interactions with the Fc receptors[159, 198, 201]. However, the contribution by antibodies towards regulating macrophage activation in tuberculosis is unknown. Although data from the previous chapter showed that *M. tuberculosis* specific antibodies were markedly reduced within B cell depleted granulomas, macrophage behavior and activation, at least as measured by expression of iNOS or arginase, markers for classically and alternatively activated macrophages, did not seem to differ compared to control granulomas. Due to the short period of the study, it is possible that the effects of antibody on macrophage activation states were not noticeable given the chronic nature of tuberculosis. A longer observation period up to around 5 months may provide more clarity on the outcome of infection with B cell depletion. Future vaccination formulations may benefit from elucidating the mechanisms of macrophage activation by antibody.

8.0 IMPLICATIONS OF THESIS FINDINGS

Immunity towards *M. tuberculosis* requires a complex set of interactions between the various players of the immune system to orchestrate bacterial containment within the granuloma. A great deal of effort focused on deciphering how cellular immunity contributes toward tuberculosis control has yielded a greater understanding on the important variables involved in granuloma formation and function[36, 38, 41, 69, 94, 135, 136, 219] and future vaccine development to augment cellular immunity against *M. tuberculosis*[127, 130, 134]. However, less is known regarding the humoral response in tuberculosis. Antibodies may not be effective in neutralizing intracellular pathogens[50, 209, 210] but may play a role in regulating immune cell activation[158, 202, 206, 216]. Furthermore, B cells are recognized as cytokine sources during infection and autoimmunity[164, 238] and thus present a tantalizing target in modulating cellular activation within the granuloma.

8.1 B CELLS FORM ECTOPIC GERMINAL CENTERS WITHIN THE GRANULOMA

Several studies with human granulomas have drawn parallels of the tuberculous granuloma with ectopic lymphoid tissue[212, 213, 231, 232] due to the clustered appearance of B cells. Within infected cynomolgus macaques, B cells were found to be organized in distinct clusters at the lymphocytic cuffs of the granuloma, analogous to the black spots on a white soccer ball (Fig. 41.). These

clusters were also observed to be reminiscent of ectopic lymphoid structures due to the expression of germinal center and cell proliferation markers[214]. These findings suggest that B cells are providing T cell help at the site of infection within the ectopic lymphoid tissue clusters.

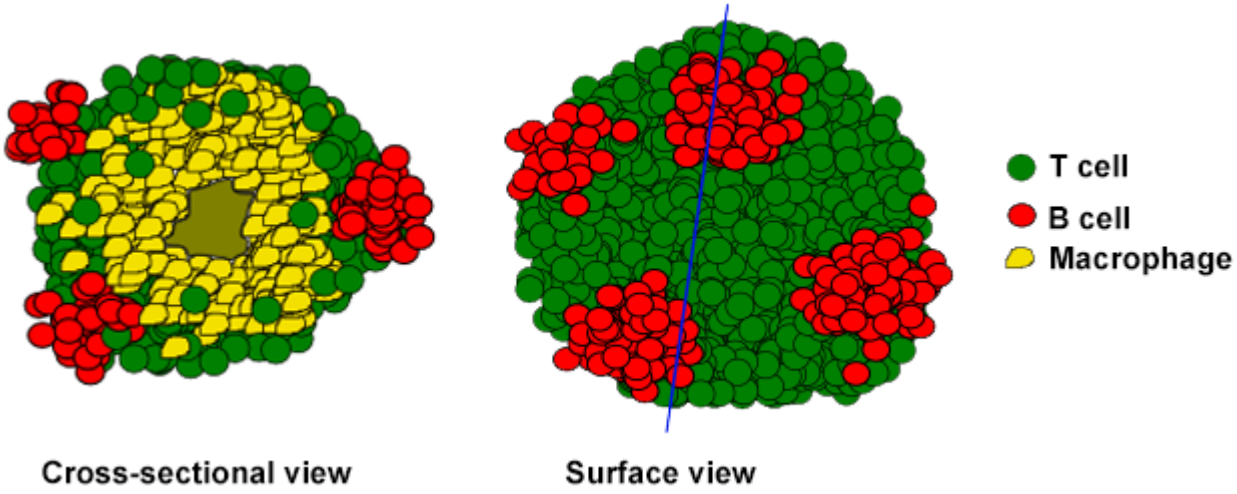

Figure 41. *Cartoon depiction of the soccer ball hypothesis.* This figure illustrates the location of B cell clusters (red) within the lymphocytic cuff and on the surface of a granuloma.

8.2 IMPLICATIONS OF B CELL CYTOKINE PRODUCTION

The cytokine secretion profile of lymphocytes suggests that approximately 20 percent of B cells within the granuloma are mainly producing IL6, IL17 or IL10 in various combinations with a separate, smaller population secreting TNF or IFNγ (Fig. 13. and 14.).

IL6 production by B cells has been noted to a main factor in driving pathology in autoimmunity possibly by driving T cell differentiation into T_h17 cells[165, 226]. Most recently, IL6 expression levels have been identified to be elevated within lungs of vaccinated and *M. tuberculosis* infected cynomolgus macaques[252]. However, in the context of infection, IL6 production by B cells may be essential in providing an impetus for T cell activation. Furthermore, IL6 together with transforming growth factor β (TGFβ) has been noted to restrain

excessive T_h17 mediated pathology[253, 254]. The studies detailed here have established IL6 to be an essential cytokine produced by B cells. Furthermore, IL6 levels within the granuloma appear to be different from control granulomas in the absence of B cells. It is thus possible that B cells are capable of modulating excessive inflammation within the granuloma while encouraging T cell activation to contain the bacilli. However, IL6 knockout mice with *M. tuberculosis* infection only showed a slight delay in the induction of IFNγ but no impairment in bacterial control[255]. Nonetheless, inflammation was not rigorously assessed in these mice studies and the study period was not sufficiently long to look at the effects of IL6 ablation in chronic infection. It would thus be possible to further assess the role of IL6 in tuberculosis control within the murine model due to the genetic tractability of the animal. Understanding the specific contribution of B cell IL6 may shed further light on the role of IL6 in the development of T cell responses to tuberculosis and neutrophil recruitment.

IL17 has been known to provide help to T cells in recruiting IFNγ within the granuloma[256-258]. B cells may be providing an alternative source of IL17 to assist in the recruitment of IFNγ production by T cells, particularly within the ectopic lymphoid tissue clusters. An absence of B cells may result in less efficient T cell recruitment into the granuloma particularly T cells secreting IFNγ, which was noted within the B cell depleted granulomas where there were less IFNγ secreting T cells. IL10 produced by B cells may provide anti-inflammatory cues to dampen down excessive T cell and macrophage activation. However, the interplay between these cytokines in precisely modulating immune activation in response to infection is unknown. The intriguing point to note is that B cells, being organized into lymphocytic clusters, will come into close contact with T cells within the granuloma. Although no significant differences were noted for T cell and macrophage responses during the acute phase

of tuberculosis (10 weeks) in the B cell depletion study, the long term cytokine contribution from B cells toward tuberculosis control remains uncharacterized.

It is thus entirely within the realm of possibility that cytokines being produced by B cells are helping to maintain pro- and anti-inflammatory responses within the granuloma in an attempt to limit bacterial growth and bystander tissue damage. Furthermore, B cells may be coordinating such pro- and anti-inflammatory responses between T cells via possible IL6 mediated T_h17 responses, neutrophil recruitment and bacterial control (Fig. 42.). An absence of B cells means that such a concerted response is not formed, leading to a runaway cycle of uncontrolled bacterial growth and excessive inflammation as seen in the mouse model[211].

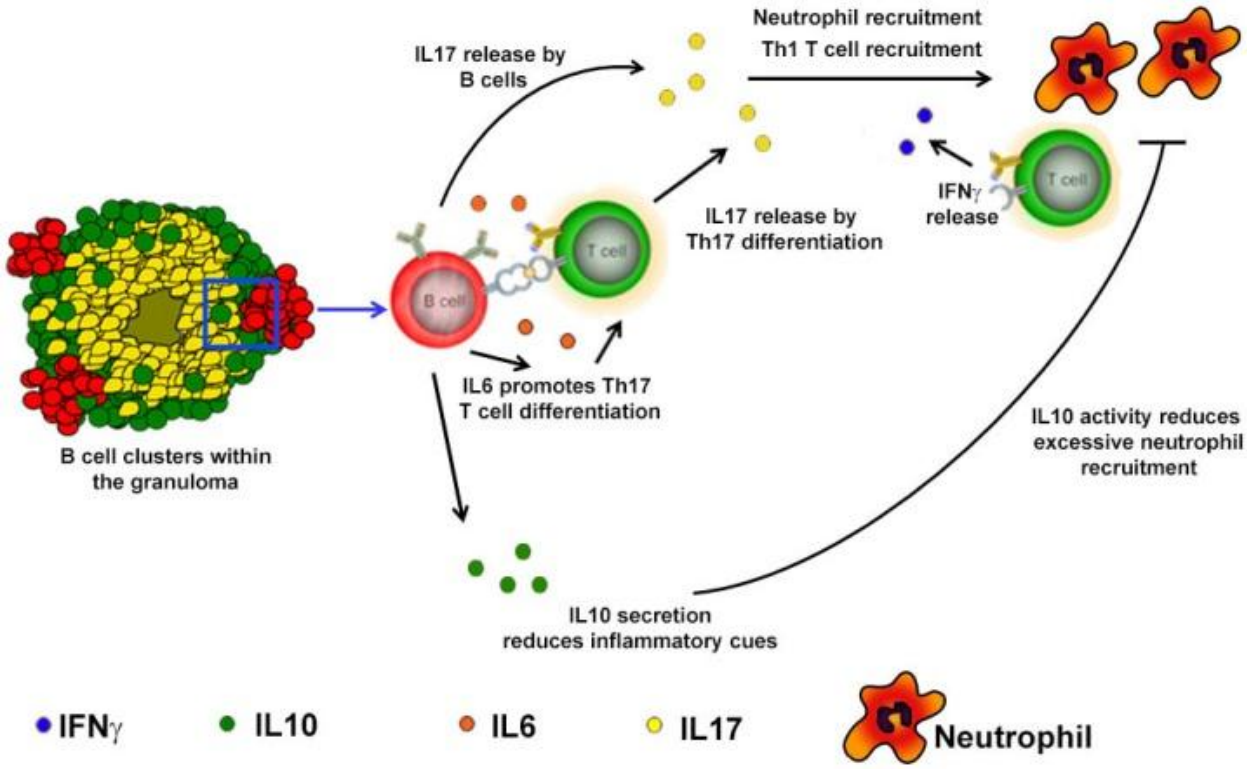

Figure 42. *Cartoon depicting the possible roles of cytokines IL6, IL17 and IL10 produced by B cells within the granuloma.* B cells produce pro-inflammatory cytokines IL6 and IL17 which together with T_h17 T cells recruits neutrophils to assist in bacterial clearance. B cells also produce IL10 to provide a check on excessive inflammatory infiltrate. The germinal center configuration of B cells within the granuloma allows for close contact with T cell that may limit the scope of immune activation by cytokines.

8.3 THE EFFECTS OF B CELL DEPLETION IN THE NON-HUMAN PRIMATE MODEL OF INFECTION

The primary limitation in the B cell depletion study was that it was 10 weeks long due to issues with drug interactions within the treated animals. The earlier cohort of Rituximab treated animals developed anaphylactic reactions towards the drug, such that the B cell depleted state could not be safely maintained beyond 10 weeks, necessitating the early termination at 10 weeks post infection.

The general conclusion drawn from the B cell depletion study was that disease progression at 10 weeks remains unchanged between Rituximab treated and saline control groups. Findings at necropsy did not reveal any significant differences in animals from B cell depleted or control groups, both presenting with comparable amounts of disease and dissemination. Tissue pathology did not appear to be different as granulomas from both groups were highly similar in appearance upon standard histological staining (hemotoxylin and eosin). The only notable difference was that the lymphocytic cuff was less prominent within Rituximab treated animals and was attributed to the ongoing B cell depletion.

Immune parameters within the granuloma were also comparable between Rituximab treated animals and saline controls. T cell cytokine production was not found to be significantly different between both group while total cytokine quantification and macrophage function was noted to be similar in general. However, the correlation between the tested cytokines IL10, IL17, IL6 and bacterial burden were noted to be different between the Rituximab treated and saline control granulomas. This difference means that the cytokines present within each individual granuloma from saline control and B cell depleted animals are different and highlights possible points of control by B cells. It is possible the B cells are providing the immunological support via

cytokine signaling within the germinal center to T cells to ensure efficient control of bacterial burden within the granuloma thus leading to proper long term bacterial control that reduces tissue pathology. An absence of B cells abolishes the coordination of various cell functions that would be detrimental in the long term when bacterial numbers are not properly controlled. Indeed, within the 10 weeks of infection, a subset of granulomas within the B cell depleted animals was noted to have higher bacterial burden compared to control granulomas (Fig. 43.).

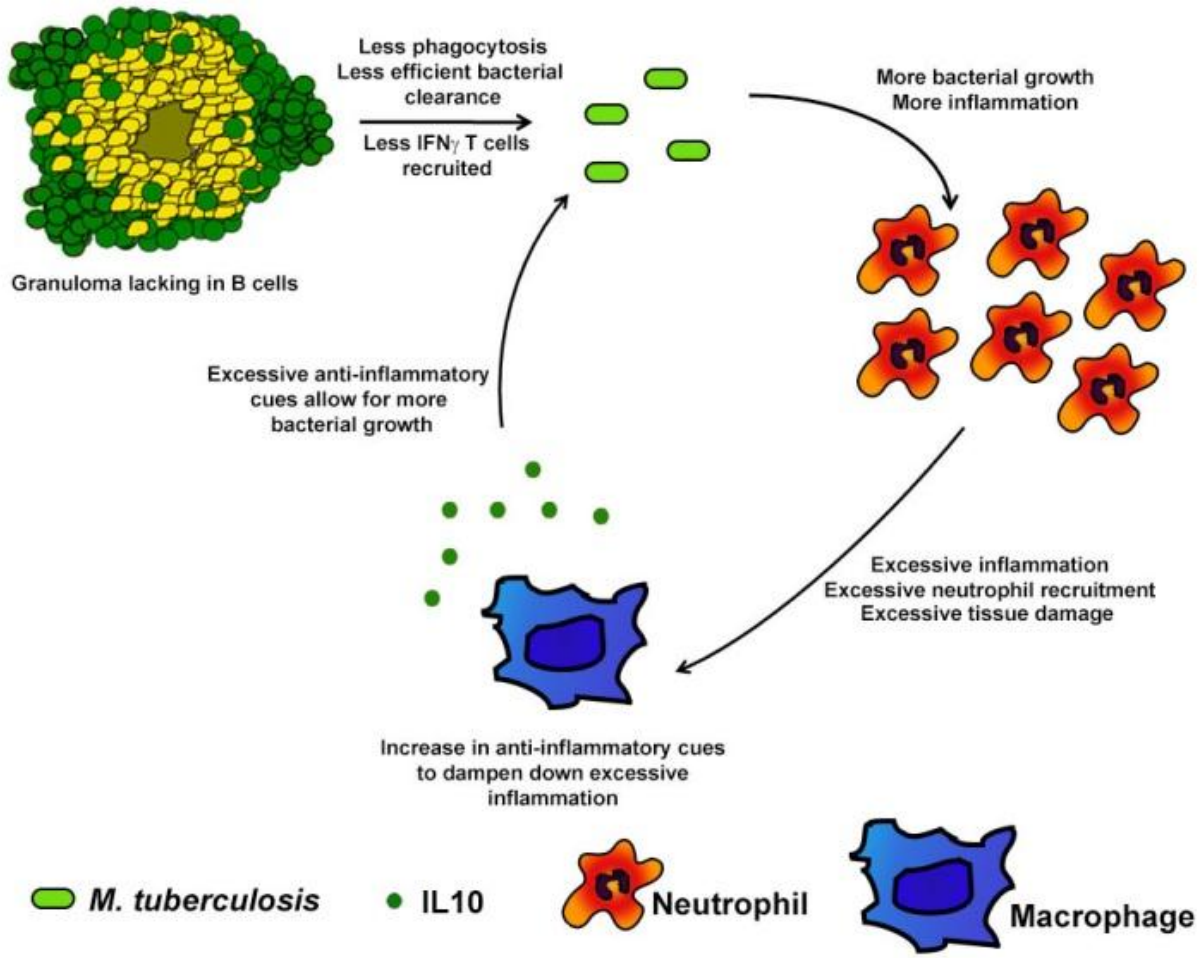

Figure 43. *Cartoon depicting the possible effect of B cell depletion within the granuloma.* The absence of B cells results in a lack of efficient bacterial clearance. The higher bacterial burden results in more inflammatory cues such as increased neutrophil recruitment. The influx of inflammatory cells requires a lot more anti-inflammatory signaling to resolve. The inadequate coordination of pro- and anti-inflammatory activity within a B cell depleted granuloma leads to a vicious cycle of increasing neutrophil infiltrate and elevated IL10 production to reduce inflammation.

B cell depleted animals also did have a statistically higher median bacterial burden per granuloma compared to control animals. This may indicate that B cell depleted granulomas are less able to control *M. tuberculosis* compared to the saline control group granulomas. However, the difference in bacterial burden is not attributed to T cell cytokine secretion and macrophage activation as both parameters were not observed to be significantly different, at least according to readouts used in the above studies (Chapter 6 and 7). The contribution of humoral immunity may be very subtle and involve pathways that were not included in the above studies, such as FcγR receptor expression and function and possible contribution of cytokines.

8.4 FUTURE AVENUES OF STUDY

Bacterial enumeration within infected tissues were done via 7H11 agar plate culture which only takes into account of live bacteria. An alternative method to assess B cell contribution towards bacterial control may be to use chromosomal equivalence unit analyses to determine the total bacterial population originally present within the granuloma, both live and killed. This method may reveal the efficacy of the granuloma in controlling the disease by measuring bacterial killing efficiency.

Longer B cell depletion studies have been proposed to adequately address the role of B cells in controlling tuberculosis within primates. However, another aspect that could be address is delayed B cell responses upon infection. This could be done by depleting the animals of B cells prior to infection and then allowing the B cell population to recover at a predetermined period after infection. This can provide valuable data in how the B cell response is shaped and the implications of a late B cell response on disease control.

One possible aspect of humoral immunity was not pursued due to a lack of specialized reagents for non-human primate use was the characterization of the Fc receptors within the granuloma. Fc receptors, particularly FcγR receptor members were shown to influence disease outcome in *M. tuberculosis* infected mice[216]. Indeed, FcγR are major mediators of macrophage activation[205, 206] and are known to regulate the activity of multiple cell types[52, 201, 202]. With regards to macrophage activation within the granuloma, FcγR receptors are suspected to influence macrophage activation thresholds based on the size of the immune complexes present. Immune complex sizes are, in turn, influenced by the levels of antibody and antigen present (Fig. 44.) within the granuloma. Thus, the interaction between antibody immune complexes and FcγR potentially contribute towards generating the broad spectrum of granuloma microenvironment observed. Unfortunately, the reagents to reliably distinguish activating and inhibitory FcγR in macaques do not exist, and thus these studies cannot be reliably performed in this model at this time.

Another approach in determining the contribution of antibodies and FcγR receptors is to administer intravenous immunoglobulin (IVIg) purified from pooled cynomolgus macaque serum. Human IVIg has been used as an immunosuppressant to treat autoimmune conditions such as lupus and rheumatoid arthritis[247, 248]. IVIg is thought to modulate immune activity by engaging inhibitory FcγR receptors along with recruitment of inhibitory glycans on immune cells particularly macrophages, B cells and neutrophils[248, 259, 260]. By supplementing IVIg into B cell depleted animals, it may be possible to resolve the contribution of B cell cytokine production and antigen presentation from antibody mediated contributions toward control of tuberculosis.

In the B cell depletion study, the level of antigen specific antibody was confirmed to be reduced within the Rituximab treated animals. Despite the difference, no further effects were

seen in terms of granuloma pathology, disease progression, cytokine secretion by T cells or macrophage behavior between B cell depleted and saline control animals. Again, the results show that humoral responses do not seem to be crucial in modulating immunity towards tuberculosis at 10 weeks post infection. However, the longer term effects are still unknown and future studies should take into account longer B cell depletion periods, if possible.

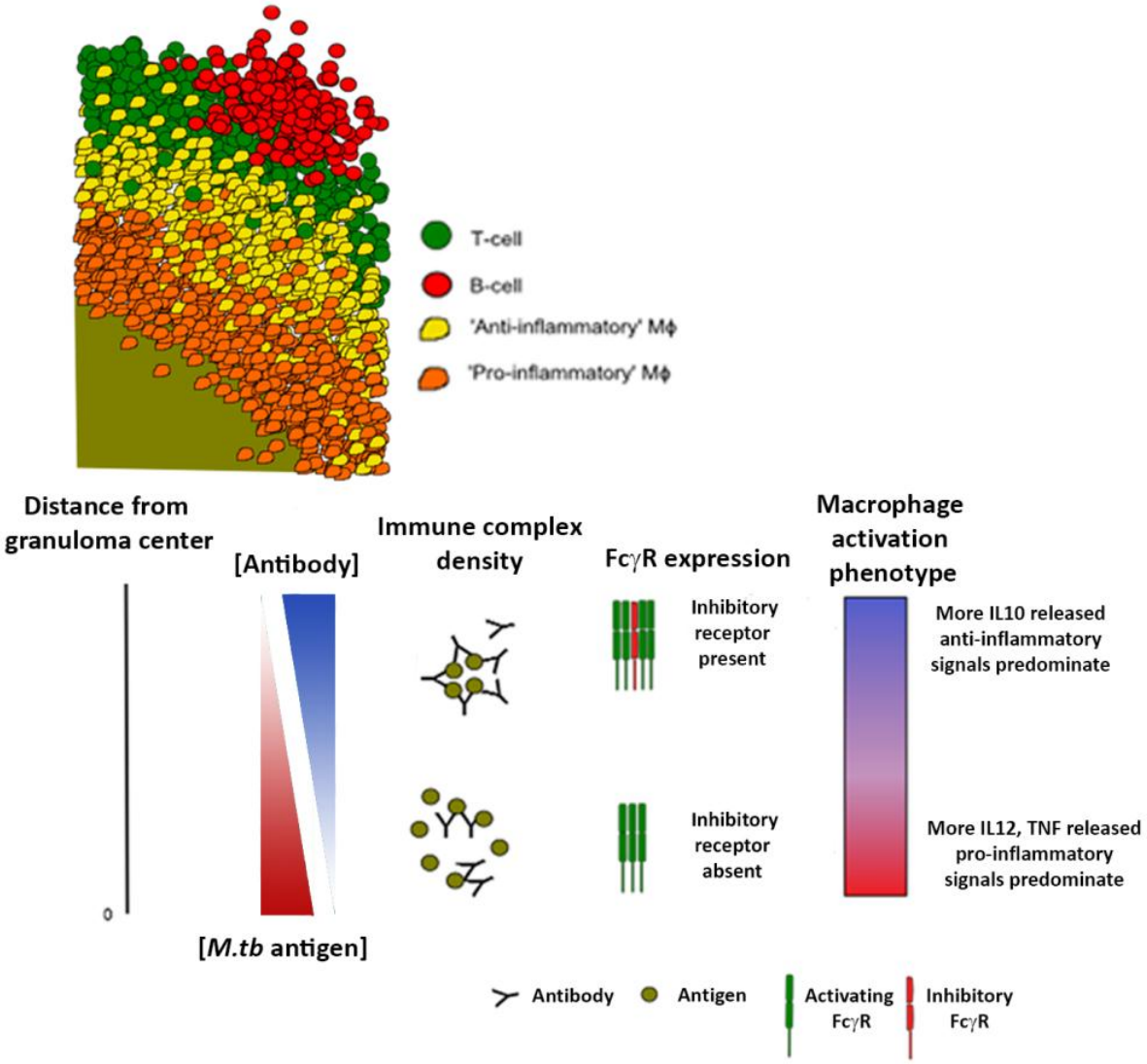

Figure 44. *Cartoon details the proposed model describing the mechanism of macrophage activation being modulated by antibody antigen immune complexes via FcγR recruitment.*

8.5 OVERALL CONCLUSION

It would appear that the contribution of humoral immunity towards the control of *M. tuberculosis* infection within the cynomolgus macaque model of tuberculosis is minimal at 10 weeks post infection. Disease progression is essential unremarkably different within B cell depleted animals compared to saline controls, although bacterial containment is slightly impaired within B cell depleted granulomas. Pathological observations and immune parameters appear to be similar although differences were noted in terms of cytokine composition on the individual granuloma level. Long term contributions by the humoral component of immunity towards tuberculosis control remains to be determined but is suspected to be more significant. By understanding how the humoral components of immunity interact with other immune players within the granuloma, it is possible to enhance future vaccine and novel therapy developments to maximize immune mediated bacterial clearance while minimizing tissue damage.

The studies detailed here indicate that B cells and antibody are not essential for control in the early phase of natural *M. tuberculosis* infection. Nonetheless, the roles of B cells and antibody responses in chronic infection and vaccination models, in particular, are poorly understood. Furthermore, antibody based vaccination against *M. tuberculosis* is just beginning to be explored[261] but has yet to be adequately developed in any animal model system. The outcome of recent human vaccination trials for *M. tuberculosis* has shown that simply inducing higher T cell responses is insufficient for adequate protection against infection[262-264]. Augmenting the natural ability of the host to control infection by antibody production may be a viable alternative since T cell based vaccination strategies have thus far been inadequate in eliciting full vaccine protection against *M. tuberculosis* in any animal model system.

APPENDIX A: SUPPLEMENTARY FIGURES

Non-parametric Spearman's ρ correlation: Saline

Variable	by Variable	Spearman ρ	Prob>|ρ|
IL10 (pg)	IL6 (pg)	-0.2155	0.4227
IL17 (pg)	IL6 (pg)	-0.3504	0.1414
IL17 (pg)	IL10 (pg)	0.3811	0.0128*
TNF (pg)	IL6 (pg)	.	.
TNF (pg)	IL10 (pg)	0.3962	0.0334*
TNF (pg)	IL17 (pg)	0.0791	0.6416
Calprotectin (ng)	IL6 (pg)	0.4133	0.0786
Calprotectin (ng)	IL10 (pg)	0.1272	0.4106
Calprotectin (ng)	IL17 (pg)	-0.0205	0.8821
Calprotectin (ng)	TNF (pg)	0.5264	0.0006*
CD3 % Live	IL6 (pg)	0.3667	0.3317
CD3 % Live	IL10 (pg)	-0.4062	0.0607
CD3 % Live	IL17 (pg)	-0.1128	0.5914
CD3 % Live	TNF (pg)	0.0486	0.8582
CD3 % Live	Calprotectin (ng)	0.1154	0.5828
CFP Ab (ug)	IL6 (pg)	0.5000	0.6667
CFP Ab (ug)	IL10 (pg)	0.1821	0.4172
CFP Ab (ug)	IL17 (pg)	0.2380	0.2627
CFP Ab (ug)	TNF (pg)	0.1871	0.4168
CFP Ab (ug)	Calprotectin (ng)	-0.0461	0.8307
CFP Ab (ug)	CD3 % Live	-0.0211	0.9298
Total IgG (ug)	IL6 (pg)	0.5000	0.6667
Total IgG (ug)	IL10 (pg)	0.2406	0.2807
Total IgG (ug)	IL17 (pg)	0.1526	0.4766
Total IgG (ug)	TNF (pg)	0.3007	0.1853
Total IgG (ug)	Calprotectin (ng)	0.0330	0.8782
Total IgG (ug)	CD3 % Live	0.1459	0.5395
Total IgG (ug)	CFP Ab (ug)	0.7193	<.0001*
Log CFU/gran	IL6 (pg)	0.6588	0.0055*
Log CFU/gran	IL10 (pg)	0.1842	0.1956
Log CFU/gran	IL17 (pg)	0.1227	0.3909
Log CFU/gran	TNF (pg)	0.0591	0.7245
Log CFU/gran	Calprotectin (ng)	0.4896	0.0002*
Log CFU/gran	CD3 % Live	-0.2131	0.3065
Log CFU/gran	CFP Ab (ug)	0.3296	0.0868
Log CFU/gran	Total IgG (ug)	0.5376	0.0032*

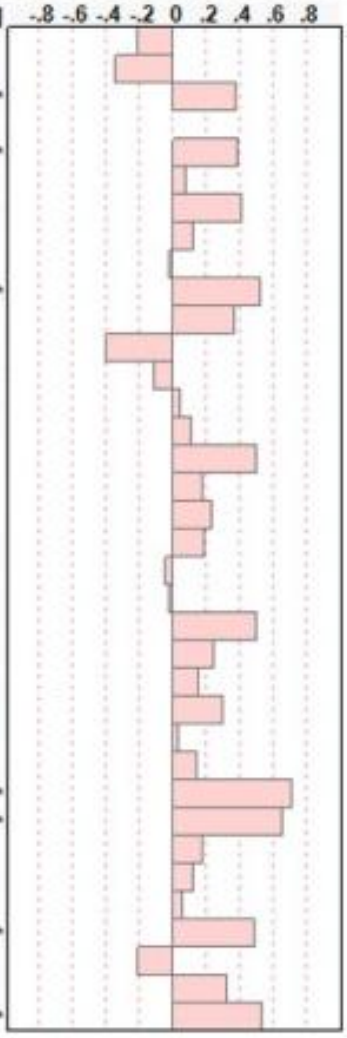

Table 2. *Non-parametric Spearman's ρ correlation of granuloma parameters used in linear correlation analysis of saline control animal samples.*

Non-parametric Spearman's ρ correlation: Rituximab

Variable	by Variable	Spearman ρ	Prob>|ρ|
IL10 (pg)	IL6 (pg)	0.2150	0.3767
IL17 (pg)	IL6 (pg)	0.0114	0.9599
IL17 (pg)	IL10 (pg)	-0.0847	0.5984
TNF (pg)	IL6 (pg)	0.5000	0.6667
TNF (pg)	IL10 (pg)	0.4939	0.0121*
TNF (pg)	IL17 (pg)	-0.3116	0.0643
Calprotectin (ng)	IL6 (pg)	0.0137	0.9518
Calprotectin (ng)	IL10 (pg)	0.5372	0.0004*
Calprotectin (ng)	IL17 (pg)	-0.0606	0.6665
Calprotectin (ng)	TNF (pg)	0.6039	0.0002*
CD3 % Live	IL6 (pg)	0.3805	0.3524
CD3 % Live	IL10 (pg)	0.3245	0.1753
CD3 % Live	IL17 (pg)	-0.0896	0.7154
CD3 % Live	TNF (pg)	0.0022	0.9941
CD3 % Live	Calprotectin (ng)	0.2198	0.3808
CFP Ab (ug)	IL6 (pg)	0.6325	0.3675
CFP Ab (ug)	IL10 (pg)	-0.2710	0.2348
CFP Ab (ug)	IL17 (pg)	0.1683	0.4910
CFP Ab (ug)	TNF (pg)	0.1022	0.6867
CFP Ab (ug)	Calprotectin (ng)	0.1527	0.5326
CFP Ab (ug)	CD3 % Live	-0.1729	0.4791
Total IgG (ug)	IL6 (pg)	0.6325	0.3675
Total IgG (ug)	IL10 (pg)	0.1345	0.5829
Total IgG (ug)	IL17 (pg)	0.3797	0.1202
Total IgG (ug)	TNF (pg)	0.2181	0.4003
Total IgG (ug)	Calprotectin (ng)	0.0681	0.7882
Total IgG (ug)	CD3 % Live	0.3602	0.1421
Total IgG (ug)	CFP Ab (ug)	0.6966	<.0001*
Log CFU/gran	IL6 (pg)	0.0418	0.8571
Log CFU/gran	IL10 (pg)	0.0762	0.5988
Log CFU/gran	IL17 (pg)	0.3365	0.0137*
Log CFU/gran	TNF (pg)	-0.1862	0.2770
Log CFU/gran	Calprotectin (ng)	0.2324	0.1008
Log CFU/gran	CD3 % Live	-0.2472	0.2235
Log CFU/gran	CFP Ab (ug)	0.5538	0.0027*
Log CFU/gran	Total IgG (ug)	0.4708	0.0175*

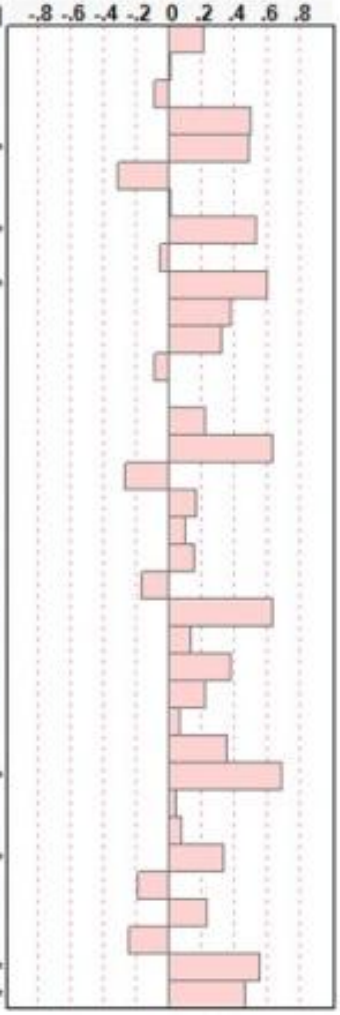

Table 3. *Non-parametric Spearman's ρ correlation of granuloma parameters used in linear correlation analysis of Rituximab treated animal samples.*

APPENDIX B: PUBLICATION RECORD

The role of B cells and humoral immunity in Mycobacterium tuberculosis infection. Kozakiewicz L, **Phuah J**, Flynn J, Chan J. Adv Exp Med Biol. 2013;783:225-50. doi: 10.1007/978-1-4614-6111-1_12. Review. PMID: 23468112

Activated B cells in the granulomas of nonhuman primates infected with Mycobacterium tuberculosis. **Phuah JY**, Mattila JT, Lin PL, Flynn JL. Am J Pathol. 2012 Aug;181(2):508-14. doi: 10.1016/j.ajpath.2012.05.009. Epub 2012 Jun 19. Erratum in: Am J Pathol. 2012 Nov;181(5):1889. PMID: 22721647

Phosphotyrosine-dependent coupling of Tim-3 to T-cell receptor signaling pathways. Lee J, Su EW, Zhu C, Hainline S, **Phuah J**, Moroco JA, Smithgall TE, Kuchroo VK, Kane LP. Mol Cell Biol. 2011 Oct;31(19):3963-74. doi: 10.1128/MCB.05297-11. Epub 2011 Aug 1. PMID: 21807895

Simian immunodeficiency virus-induced changes in T cell cytokine responses in cynomolgus macaques with latent Mycobacterium tuberculosis infection are associated with timing of reactivation. Mattila JT, Diedrich CR, Lin PL, **Phuah J**, Flynn JL. J Immunol. 2011 Mar 15;186(6):3527-37. doi: 10.4049/jimmunol.1003773. Epub 2011 Feb 11. PMID: 21317393

Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. Diedrich CR, Mattila JT, Klein E, Janssen C, **Phuah J**, Sturgeon TJ, Montelaro RC, Lin PL, Flynn JL. PLoS One. 2010 Mar 10;5(3):e9611. doi: 10.1371/journal.pone.0009611. PMID: 20224771

APPENDIX C: REPRINT PERMISSIONS

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