DISSERTATION

T CELL INDEPENDENT MECHANISMS FOR PROTECTION AGAINST *MYCOBACTERIUM TUBERCULOSIS* INFECTION

Submitted by

Thomas Bickett

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Doctoral Committee:

Advisor: Angelo Izzo

Steven Dow
Jennifer MacLean
Richard Bowen
Lucas Argueso

ABSTRACT

T CELL INDEPENDENT MECHANISMS FOR PROTECTION AGAINST *MYCOBACTERIUM TUBERCULOSIS* INFECTION

The live attenuated *Mycobacterium bovis* strain Bacille Calmette Guérin (BCG) is a potent innate immune stimulator. Innate Immunity provides the host with the ability to immediately respond to invasion by pathogens and can be utilized through the use of molecular adjuvants to trigger specific innate mechanisms leading to adaptive immunity. In the C57BL/6 mouse model of tuberculosis, BCG stimulated immunity causes a significant reduction of *M. tuberculosis* burden after pulmonary infection. Our studies indicate that BCG induced protection against pulmonary *M. tuberculosis* through early monocyte recruitment is present as early as 7 days after vaccination. This protection showed longevity, as it did not wane when mice were infected 30 days post vaccination. As BCG induced mycobacterial killing after 7 days, we sought to identify the contribution of different innate immune components to better understand mechanisms required for mycobacterial killing. When BCG was administered through subcutaneous inoculation, we found that there was significant monocyte recruitment in the lungs within 7 days after vaccination. Further studies revealed that killing of mycobacterium is dependent on BCG being viable and is monocyte derived, independent of trained innate immunity, highlighting a novel mechanism for killing *M. tuberculosis*. With the rise of drug resistant strains of *Mycobacterium tuberculosis*, new vaccine development is paramount. A better understanding of the BCG vaccine will hopefully lead to the development of a more effective alternative.

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DEDICATION

“A life is made up of a great number of small incidents and a small number of great ones”

-Roald Dahl

I dedicate this thesis to my wife Talia for all of her love and support and to my children, PJ and our unborn baby.

TABLE OF CONTENTS

Chapter I

Literature Review

Tuberculosis: a History

Throughout recorded history tuberculosis has plagued mankind as an incurable, deadly disease that today has earned itself the title of one of the most successful bacterial pathogens in humans. Evidence of tuberculosis infection has been dated back to ancient Egypt[1] and there is some suggestion that it has been around since the stone age[2]. These ancient discoveries are usually determined by the presence of spinal tuberculosis, or Potts disease, on human remains[3]. In recent discoveries of tuberculosis alongside ancient civilizations, PCR and other molecular methods have been used to confirm that *Mycobacterium tuberculosis*, or at least a close ancestor, infected human populations 9000 years ago[2]. Ancient Egyptian mummies have multiple characteristics congruent with tuberculosis infection[4] and a papyrus scroll, named the Ebers papyrus, details a consumptive disease alongside cervical cysts that is theorized to be tuberculosis. This papyrus also details treatment as lancing of the cyst along with applying a ground mixture of blood, honey, salt, and various fruits and vegetables[4].

Although tuberculosis disease is known to have spread amongst ancient populations on the continents of Europe and Africa, it was a mystery for a time at how the disease made its way to the "new world" or the Americas. Recent genotyping evidence of *M. tuberculosis* strains has suggested that seals are actually responsible for bringing the mycobacterium from Africa to modern day Peru and spreading it along the coasts of the Americas[5]. As populations of people grew and began to interact more with one another, *M. tuberculosis* evolved alongside humans to be a very successful bacterial pathogen. Many ancient Greek writings tell of a common consumptive disease called phthisis which is presumed to be tuberculosis. Moving into the 19th century tuberculosis became known as the white plague due to the gaunt, pale appearance that

came with the disease[4]. It was not until 1882 that *M. tuberculosis* was discovered by Robert Koch to be the etiological agent behind tuberculosis finally classifying it as a bacterial infection[6].

Mycobacterium tuberculosis characteristics

The species *M. tuberculosis* falls under the genus *Mycobacterium* in the family Mycobacteriaceae of the order Actinomycetales. Its most defining property comes from its staining properties in that it is not able to be classified as gram negative or positive due to the presence of a unique cell wall[4]. The cell wall of mycobacterium is partitioned into two segments, the upper and lower segments. The lower segment is called the cell wall core and is comprised of the peptidoglycan attached to arabinogalactan which is also linked to mycolic acids. The upper segment is rich in lipids such as peptidoglycolipids, trehalose dimycolate (cord factor), sulpholipids, Phthiocerol dimycocerosate (DIM) with proteins dispersed in between such as phosphatidylinositol mannosides (PIMs), lipomannan (LM), and lipoaribinoannan (LAM)[7]. This lipid rich outer membrane, especially DIM, give mycobacterium a waxy coating that contributes to its rigidity and the inability to stain it with gram stain[4]. The cell wall core is very effective at preventing entry of antimicrobials while the outer lipids aid in resisting the host immune system during infection and are involved in disease pathogenesis[7]. With these unique properties mycobacterium must be identified through the use of the acid fast stain[4]. Research conducted on the cell wall and molecules associated with the bacterial membrane of *M. tuberculosis* is mostly performed to aid development of drugs, and will be discussed later.

M. tuberculosis is classified as highly aerobic and requires oxygen to grow and as such is primarily a respiratory infection in mammals. This is why *M. tuberculosis* prefers the upper lobe of the lung. It has high oxygen pressure and a slightly delayed immune response compared to the rest of the lung[8,9]. During infection the mycobacterium resides primarily intracellularly and has evolved a unique arsenal of tactics to thrive in this environment. To better understand how the mycobacterium can survive different conditions, changes of gene

expression have been characterized during the life cycle of the mycobacterium and in differing environments it may encounter during infection. Through activating different parts of its genome, *M. tuberculosis* is able to alter its metabolism so that it can thrive in oxygen rich environments, or lay dormant during periods such as latent infection[10,11]. The ability of *M. tuberculosis* to almost shut down and lay dormant for years before reactivating to active disease is what makes it such a difficult pathogen to protect against and is ultimately responsible for its success in human populations. Many studies have centered around understanding the regulatory mechanisms that are used by the mycobacterium to survive in such conditions, and they have identified the restriction of a few biosynthetic activities that allow this lifestyle[12,13]. The method of metabolic revival of the mycobacterium has also been under scrutiny as a potential method of treating latent infection and preventing relapse into active infection. A few key pathways have been identified[14] but this aspect of mycobacterial metabolism is still largely unknown.

The hallmark of *M. tuberculosis* infection is the granuloma. Granuloma formation occurs as a result of the unique infection cycle of the organism. When the mycobacterium is inhaled into the lung the first immune cells it will come into contact with are macrophages. The bacilli are then endocytosed by macrophages and sequestered in a phagosome[15]. Normally the phagosome would be able to fuse with a lysosome, resulting in the production of free oxygen and nitrogen radicals that can kill bacterial pathogens. Reactive oxygen and nitrogen can interfere with a variety of metabolically important molecules such as nucleic acids, proteins, lipids, and carbohydrates[16]. However, *M. tuberculosis* has evolved special mechanisms for surviving these processes namely in detoxifying the toxic chemicals before harm is done[17–20], or in repairing the damage before it becomes lethal[21–23]. In addition, it is suggested that *M. tuberculosis* has a unique mechanism of phagolysosome escape, allowing it to escape the phagosome and persist in the cytoplasm of the macrophage[24,25]. This is mediated through the

Esx-1 secretion system[26–28]. There is also some indication that *M. tuberculosis* can reside long term in a vacuole[29]. Regardless, it is able to survive the intracellular environment of a macrophage which progresses to granuloma formation.

Granuloma formation begins with the interaction between the mycobacterium and the cellular environment of the lung. Initially, pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) are key to instigate the inflammation that brings about the formation of a granuloma[30,31]. Without TNF-α, granulomas show very delayed onset and do not develop normally[32]. After initiation of the immune response by dendritic cells, CD4 T cells begin to contain the accelerated growth of the mycobacterium by supplying IFNγ [33]. Once mycobacterial growth slows, the CD8 T cell response becomes important for its cytotoxic activity and additional IFNγ production[34,35]. During this period of slowed mycobacterial growth it is believed that the population of bacteria is sustained by non-replicating bacilli, but evidence for this is lacking[36]. Namely, in the mouse model, this stage of infection has a curious lack of increasing mycobacterial burden in the lungs as defined by PCR and identification of bacterial genome equivalents[37]. Latency in growth of mycobacterium allows for the beginnings of granuloma formation. The surrounding tissue becomes increasingly vascularized to promote inflammation[38] which allows for the increased recruitment of immune cells, and most importantly macrophages. At the infection site, macrophage cells differentiate into a variety of subclasses. Macrophages are critical for granuloma formation because of their response mechanisms in inflammation, and also tissue repair[39–41]. The structure then becomes more stratified with identifiable layers of different cells and the layer right outside of the macrophages develops into a fibrous cuff. The cell arrangement is depicted in **Figure 1.1**. Lymphocytes then begin to form “follicle-like centers” that surround infected macrophages and dendritic cells surrounding a necrotic center in which the live mycobacteria are trapped[42]. At this point the infection is deemed latent and the bacteria, while alive, poses no more threat to the host as it is contained

by the immune system. However if the immune system should be compromised in any way, the infection is poised to regain its active form and spread. Additionally, effective granuloma formation requires that macrophages remain in contact with T cells, or lack of macrophage activation may lead to uncontrolled mycobacterial growth[43].

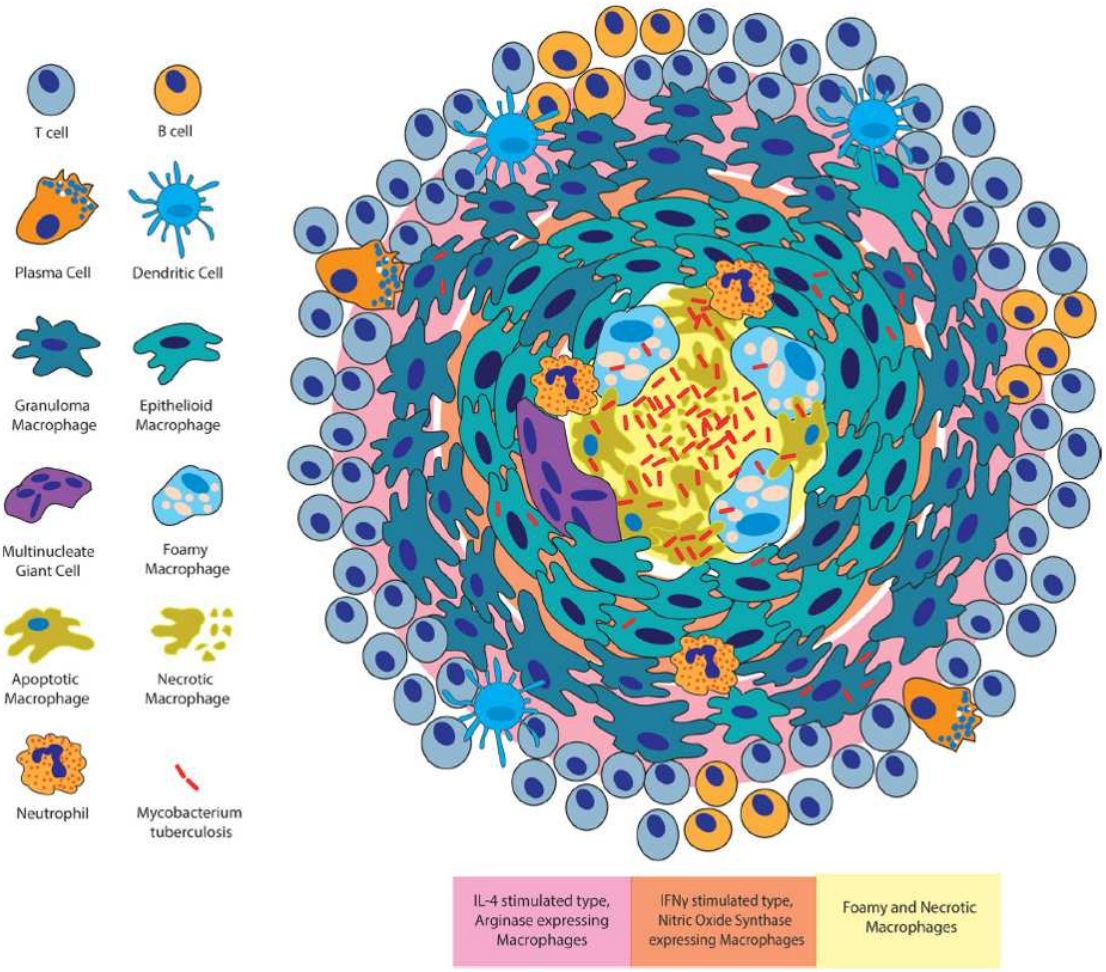

Figure 1.1 Cell arrangement of granuloma as shown by McClean and Tobin (2016)[44].

Infection and Disease: Global Crisis

Today, it is estimated that one third of the world's population is infected with tuberculosis, and even with the application of modern medicine, tuberculosis manages to kill around 1.5 million people every year [45]. Globally tuberculosis incidence rates have remained

steady or decreased since 1990. High income countries such as the United States, Canada, Australia, New Zealand, and those in Western Europe show the lowest incidence of tuberculosis infection in the world; less than 10 cases per 100,000 people. The countries of highest incidence include India (which accounts for 26% of global cases), China, and South Africa[46]. People with HIV/AIDS are at the highest risk of tuberculosis due to prevalence of both diseases in similar regions and the immunological impairment from HIV infection. In 2012, 13% of total tuberculosis cases were estimated to be among HIV positive individuals[47]. Tuberculosis is estimated to have killed 1.3 million people in 2012 with ¼ of those being HIV positive. One-third of the deaths globally from tuberculosis that year came from India and South Africa. In line with global incidence of the disease, global mortality from tuberculosis has been decreasing since 1990[46]. The success of decreasing both incidence and mortality of tuberculosis has come about through the efforts of organizations such as the World Health Organization by disseminating information and increasing treatment success in high incidence areas[47]. It was estimated that in 2011 87% of new cases were treated successfully on a global scale[46]. However, not all new cases of tuberculosis are treatable. Even though global incidence of tuberculosis is on the decline, the cases of multi drug resistant (MDR) and extensively drug resistant (XDR) tuberculosis is on the rise. This is in part due to inadequate treatment methods and the difficulty some patients have in fully completing the drug regimen required[46]. Surprisingly the countries most affected by MDR tuberculosis are in Eastern Europe and Central Asia[47]. It is estimated that 3.6% of newly diagnosed cases and 20.2% of patients previously treated have MDR tuberculosis[46] and that 9.6% of all MDR cases are XDR[47]. One of the main problems with the spread of MDR tuberculosis is accurately diagnosing it to initiate the proper treatment regimen. To combat this, in 2013 the WHO released many guiding policies and new molecular techniques to help correctly diagnose these types of infection early[46].

Current Therapies and Treatments

Even though tuberculosis is considered a treatable disease, the antibiotic regimen required to eliminate infection can be an overwhelming task. In general, the CDC recommends the use of four antibiotics, Isoniazid, rifampin, pyrazinamide, and ethambutol[48]. Recent research on treatment regimens has shown, however, that a new treatment regimen called 3HP can be used. The treatment consists of once weekly isoniazid and rifapentine given for just twelve weeks. This treatment method has been thoroughly tested, and shown to be just as effective as 9 weeks on isoniazid for treating latent tuberculosis infection[49]. Isoniazid and rifampin are still the most used antibiotics in use for treating tuberculosis however. Combination therapies are helpful in this case, as each drug has a unique method of action against *M. tuberculosis*. Isoniazid is most effective at killing rapidly dividing mycobacterium, but can have little to no effect on slow growing bacilli. Isoniazid must be activated by a catalase peroxidase produced by *M. tuberculosis* called KatG. Once activated it forms an isonicotinic radical which can interfere with a variety of essential mycobacterial pathways such as mycolic acid synthesis, and can cause DNA damage to the mycobacterium[50,51]. Rifampin complements isoniazid because it has strong sterilizing activity on less active, even dormant, mycobacteria[52]. Rifampin inhibits bacterial DNA-dependent RNA polymerase which is integral to RNA synthesis[53]. Although effective, these medications are incredibly harsh on the human body and can cause liver damage[48]. In addition to the hepatotoxicity of these drugs and such long treatment protocols it becomes hard for medical professionals to confirm completion of treatment which has led to an increase in antibiotic resistance. Even in the United States the number of drug susceptible and multi drug resistant cases of tuberculosis has been slowly increasing[54]. Treatment for multidrug resistant cases of tuberculosis is even longer and requires the use of even more toxic second line drugs known as fluoroquinalones such as levofloxacin, moxifloxacin, and gatifloxacin[48]. In the early 2000s cases of what became known as extensively

drug-resistant tuberculosis were diagnosed where even the second line drugs were having no effect and led to the classification of a subset of MDR tuberculosis called extensively drug resistant tuberculosis (XDR TB). XDR TB is classified as resistant to all fist line drugs (rifampin and isoniazid), any fluoroquinalone, and at least one of three second line drugs. These cases of XDR TB are especially troublesome in a global context as there is a potential for an epidemic of untreatable tuberculosis that would not only be a major drain on public resources, but would drastically slow the campaign to eliminate tuberculosis[54]. Countries such as South Africa, with a high burden of tuberculosis, are reporting increased numbers of XDR TB cases and with such a high instance of HIV also in the country, XDR TB infection provides an impossible challenge[55]. With treatment options becoming limited in these cases, the only hope for eradicating tuberculosis as a global threat comes in prevention. Currently there is only one vaccine available for *M. tuberculosis*, the attenuated *Mycobacterium bovis* strain Bacille Calmette–Guérin (BCG).

The History and Use of BCG vaccination

Although it is a very ancient disease, it wasn't until 1882 that *M. tuberculosis* was finally discovered as the etiological agent of tuberculosis, and very little was known about how to treat it.[56] Not long after, French scientists Albert Calmette and Camille Guérin, started working on a vaccine at the Pasteur institute in Lille, France. For their research, they began cultivating tuberculous bacteria on media made from glycerin and potatoes. In an effort to reduce the bacterial clumping they added ox bile to their media, and soon discovered that the growing progeny of mycobacterium had decreased in virulence. Starting in 1908, they began passaging an isolate of virulent *M. bovis* cultured from the udder of an infected cow on their special media. After 5 years of passaging, and quite a few unproductive years from limited resources due to the German occupation of Lille during World War I, Calmette and Guerin finally had procured a

strain of *M. bovis* that produced no unwanted sequelae when injected into horses, guinea pigs, rabbits, and cattle. This new vaccine was called Bacille Calmette Guerin, or BCG[56].

The first human BCG vaccine was given orally to an infant born at a hospital in Paris whose mother had died from tuberculosis shortly after birth. After no undesirable effects were observed, more infants were recruited for further trials. With subcutaneous vaccination causing adverse reactions at the site of injection, oral route was continued for application of the vaccine. After three years of cautiously observing the human candidates and finding no complications, the Pasteur Institute began mass production of the vaccine. Soon BCG began to spread around to other countries as well. With its growing use, the new BCG vaccine was met with some opposition by a few scientists. In particular, scientists at the Trudeau Sanatorium, led by Dr. Petroff, were strongly opposed to the BCG vaccine and even claimed that one of the isolates of BCG from Lille had grown a virulent mycobacterium. Calmette and Guérin easily dismissed most of these claims up until what became known as the Lübeck Disaster. In 1930 it was reported that use of the BCG vaccine at a hospital in Lübeck Germany had caused the death of 73 infants and infection of 135 more[56]. Doctors at the hospital claimed that the vaccine they were given was at fault, but further inquiry exposed that it was "entirely probable that there was a mixing of the virulent culture with the vaccine, through some error in handling" [57]. Despite its bad press, the BCG vaccine became widely used likely for the same reasons as today; its ease of production, low cost, and reliable safety[58].

Remarkably, the BCG vaccine first tested by Calmette and Guérin in 1921 remains the only possible option for anyone looking for preventative treatment against tuberculosis. Unfortunately, use of the BCG vaccine has not resulted in success of eliminating tuberculosis as a global threat. Results from multiple studies have concluded that BCG efficacy can range anywhere from 0% to 80% depending on a person's age when vaccinated, which BCG strain used, and even geographic location[59]. With such highly variable results why, then, has an

effective vaccine for tuberculosis not been produced? In 1994 the WHO published a report that showed just how ignored and underrepresented tuberculosis was in worldwide research. While it was a large threat in developing nations, most industrialized countries treated it as a low priority. Compared to other "neglected Infectious diseases" tuberculosis research was last in funding, receiving less than $4 million annually from the NIH in 1992. With MDR TB starting to gain a foothold in developed countries, the WHO urged that more funding be spared to stop the global threat that tuberculosis had become[60]. While most industrialized countries that can support research had been ignoring it, tuberculosis had run rampant through developing nations. In addition, financially it seemed more appropriate to fund research into diseases that were an immediate threat and didn't already have a vaccine. In the past 20 years, research into tuberculosis has increased greatly and vaccine development is on the forefront. Currently there are eleven vaccines in human trials, but most are designed to work with BCG and boost its effectiveness when given at infancy. These vaccines are in the form of viral vectors, whole cell/cell components, and fusion proteins, and will be outlined in the next chapter. It can take many years to show how safe and effective a new vaccine is for use on the public[61]. Until something new passes through clinical trials, BCG remains the focal point tuberculosis vaccination.

Continued use of BCG

Today, 157 countries have vaccine programs that include BCG vaccination. In these countries, over 80% of infants are vaccinated with BCG within the first few weeks of life[62]. However, it is well documented that BCG is not a very effective vaccine and any protection established wanes over time[59]. Studies have shown that BCG does induce T and B cell responses[63,64], but it is unclear if this response correlates to protection against *M. tuberculosis* infection as studies have highlighted a disconnect between the magnitude of T cell responses and reduction in mycobacterial burden in animal models[65]. BCG is a potent activator of the

innate immune system, and it has been observed that this can lead to protection against non-mycobacterial diseases[66]. Through these mechanisms, BCG is also used as a successful treatment for bladder cancer[67]. Given all of this information the question arises of should we continue the use of the BCG vaccine? There is some thought that yes we should, given its lack of side effects and potential benefits[68]. If we are to continue use of this vaccine and even expand its use, more research needs to be done on how exactly the BCG vaccine works.

As BCG is still used on millions of humans annually there are many opportunities for studies into its efficacy and effects on the human immune system. Recent studies on the nonspecific effects of BCG have suggested the role in can play in stimulating innate immune memory[69] and this research has been translated to human subjects[70]. However, as mentioned before, BCG is not efficacious under a number of circumstances. Many people have asked, is it worth continuing vaccination even though it disrupts the cheapest and easiest method of diagnosing *M. tuberculosis* exposure? One study tried to answer this question using computer modelling to better understand the impact of discontinuing the use of BCG in an intermediate burden setting of tuberculosis. Their findings indicated that although cases of tuberculosis may increase in numbers, the use of a tuberculin skin test to cheaply and easily detect exposure could improve survival by starting treatment sooner[71]. However, in high burden countries most studies agreed that vaccination in general (including BCG) is necessary as vaccinated groups have increased survival over those not vaccinated. These studies also indicated that vaccine induced protection does not even have to correlate to the disease it is specific for and that non-specific protection from vaccines is also beneficial[72,73]. Given this information, many countries still opt to use the BCG vaccine. Should a booster vaccine be developed, this would help insure a quick and effective dispersal.

Vaccine development; In the Pipeline

With the beginning of the "End TB in my Lifetime" campaign, a call has been made for more vaccine development and increases in research. As a team, different groups are meant to work together on all aspects of vaccine development. From inception of ideas in the lab, to testing in different animal models, and finally to human clinical trials. Ideally, a "pipeline" for fast tracking potential vaccine candidates into animals and successful ones into human trials will expedite the process. Many animal models are used in vaccine pipeline testing and they are outlined as follows.

Animal Models used in Tuberculosis.

Many animal models are used in in the study of tuberculosis infection and the vaccine testing pipeline. The thought process of choosing an animal model for a study is based on the traits of the animal model, namely how well the animal model replicates human infection and immunology, and the availability of reagents/materials for completing the study, with factors such as cost and availability of adequate facilities playing a role. To this end, most tuberculosis research is carried out using the mouse model of tuberculosis infection. Mice are relatively cheap, easy to house, and have a wide variety of readily available reagents/antibodies for analysis after infection. Other models include the guinea pig, and the non-human primate. A brief overview of each animal model is given here.

Mouse Model

The mouse model is the most commonly used model to study tuberculosis infection. There are a wide variety of inbred strains available with varying levels of susceptibility to infection. Susceptibility in the mouse model is defined as death soon after infection coupled with the inability to contain bacterial growth in the lungs leading to a strong inflammatory response[74]. Past studies have ranked the inbred mouse strains based on their median survival time following infection with *M. tuberculosis*[75] and others have noted that of mouse strains, the

A/Sn, C57BL/6, and the BALB/c are categorized as resistant to infection while others are considered more susceptible[74]. Although there exist a number of models, the C57BL/6 model has become the most commonly used mouse strain for vaccine research in tuberculosis[76]. Even though they are widely used, mice are not the best for modeling tuberculosis infection in humans. During the murine infection model, tuberculosis bacilli reside primarily intracellularly in the lungs causing the lung to cultivate an inflammatory, but non-necrotic lesion[77]. During infection in humans, a slightly different pathology occurs where necrotic legions form and a majority are extracellular within the lesion[78]. Analysis of the immune response is much easier than any other animal model due to the diverse accessibility of antibodies for flow cytometry and western blot, along with other immunological tests such as cytometric bead arrays available for purchase and easy completion. In addition there are a wide variety of available genetically deficient mouse strains that aid identifying the roles of various genes to immunity whereas other animal models lack this diverse background. These factors are what make the mouse model critical to the tuberculosis vaccine testing pipeline.

Guinea Pig

Guinea pigs have been used to study tuberculosis infection since the discovery of *M. tuberculosis* as the etiological agent behind the disease by Robert Koch[6]. The guinea pig is an excellent model for human tuberculosis infection for a number of reasons which has helped it retain its place in the pipeline testing of new vaccines for tuberculosis. Firstly, the guinea pig is incredibly susceptible to tuberculosis aerosol infection, requiring only a few mycobacteria to cause disease[79]. Secondly, guinea pig pulmonary physiology parallels humans in that it mimics the response to inflammation in the lung similarly[80]. Finally the guinea pig has been shown to be more immunologically similar to humans than mice[81]. These aspects are very beneficial in testing new vaccines. The guinea pig's high susceptibility to infection means that any vaccine providing protection must induce a strong and effective immune response. In addition to being

a good model for new vaccines, guinea pigs have also proved valuable in testing new antibiotic treatments and will be helpful in the years to come in testing new treatments for drug resistant strains of tuberculosis[82]. Unfortunately the guinea pig model is not an easy model to use for infection for two main reasons. Guinea pigs require special housing and are more expensive than mice making it a difficult model to use if you are not equipped with proper facilities. More importantly there is a severe lack of reagents for studying guinea pig immunology. The number of available guinea pig antibodies or other reagents for completing a full immunological panel is very lacking making the mouse model a better model for any complete analysis.

Non-Human Primate

The non-human primate model of tuberculosis focuses around two different species, the rhesus macaque and the cynomolgus macaque. Rhesus macaques are considered more susceptible to infection and are useful in understanding active infection in humans whereas cynomolgus macaques are considered more resistant and a good model for latent infection[83]. Non-human primate models of tuberculosis infection are a crucial component of the vaccine pipeline as they are the closest animal model to human infection[84]. Unfortunately they are a very expensive model to use, leading to most studying having a limited number of non-human primate subjects. In addition, housing costs and facilities required are also a limiting factor in their use.

Future Vaccine Development

In 2012 AERAS and a similar Dutch research nonprofit, TuBerculosis, released a “strategic blueprint” detailing what they believe to be the most important facets of future vaccine development[85]. In summary they call for increased ingenuity in research areas, a better way to define correlates of immunity for potential tuberculosis vaccines, better cooperation for clinical trials, more rational selection of potential new vaccine candidates, and increased acceptance in

the scientific community and better access to funding. It is also important to recognize that there is a serious lack in variety in which we know how to induce protection through vaccination against *M. tuberculosis*[85]. Future work is needed to address these shortcomings, and the research provided in this thesis attempts to rectify the lack in data surrounding the BCG vaccine.

The research described in this thesis has two main objectives;

1. To better understand the mechanism of action behind the BCG vaccine in the mouse model

Our current understanding of BCG is limited. Through the mouse model to better understand the immune response and induction of mycobacterial killing capability we will hopefully have a more complete picture of how we can utilize it better. By doing this we also aim to help policy makers more fully understand the choices they have to make when deciding to keep or discard vaccine programs such as with BCG.

2. To identify new potential ways of inducing mycobacterial killing through vaccination

Many new potential vaccines for tuberculosis are designed and tested every year. A significant number of these vaccines come as updates or boosters to the BCG vaccine[86]. Without a full understanding of how the BCG vaccine works we cannot update it to its full potential. As an aside, this thesis will also assess the potential that trained innate immunity induced by BCG has in protection against *M. tuberculosis*.

With the research presented here we hope to contribute to a solid foundation for which the strategic blueprint is calling for, and hopefully provide other researchers with knowledge on which to build future vaccines.

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Chapter II

Current Vaccines

New TB vaccines in clinical trials

The process for getting a vaccine approved for human use can be long and arduous. The development pipeline for the majority of vaccines begins with animal model testing and an understanding of the immune response generated by a vaccine. Human clinical trials begin with small studies in healthy individuals to look for adverse side effects. These usually have 20-80 subjects and tests are conducted after vaccination to look at the immune response generated. They will then move into Phase I. Phase I is also very small studies in different populations to determine how to effectively hit target populations. They can also include preliminary dose range studies. Phase IIa is next where more people are recruited (usually 100-300). This is where the dose range is determined and target populations are analyzed for immunological activity. Phase IIb is where the vaccines start to take shape where thousands of people can be recruited. These studies are aimed at demonstrating the efficacy of a vaccine and most importantly safety. They also look at disease prevention in the target population. Phase III trials begin after phase IIb has been concluded. The purpose of Phase III is to demonstrate that the vaccine actually provides protection in the target population and that it is safe over the long term. This whole process can take anywhere from 10-20 years[1]. Below are summarized some of the current vaccines in the various stages of clinical trials.

Viral Vector Vaccines

Ad5 Ag85a

This is a viral vector vaccine being developed at McMaster University and CanSino. As is common with many of newer vaccines, AD5 Ag85 is an adenovirus vector of adenovirus serotype 5 (Ad5) expressing antigen 85A. *Mycobacterium tuberculosis* protein antigen 85A is a

protein secreted by the mycobacterium. It is considered a virulence factor as Ag85A assists intracellular mycobacterial growth and survival once the mycobacterium has been phagocytosed by a macrophage[2,3]. Groups have shown success using this vaccine when given as a mucosal boost to the BCG vaccine in a mouse model[4,5]. Mechanistically, this vaccine increases the number of antigen specific T cells to antigen 85A resulting in more interferon gamma production at the site of infection leading to increased mycobacterial killing, which has been observed in mice[4]. Human trials have concluded that this vaccine does induce strong immune responses in people[6] but there is a worry that previously exposure to adenovirus will induce neutralizing antibodies eliminating its boosting effect[7]. Another similar vaccine, ChAd0x185A, is also in clinical trials which utilizes a chimpanzee adenovirus strain expressing antigen 85A[5].

MVA85A

MVA85a is a viral vector vaccine that uses Modified Vaccinia Ankara (MVA). MVA is an attenuated strain of the Chorioallantois vaccinia Ankara (CVA) strain of vaccinia virus that has been passaged over 500 times using primary chicken embryo fibroblasts[8]. This passaging has led to the attenuation of the virus through deletions of parts of the genome[9]. This vaccine was developed as a booster to the BCG vaccine as a way to induce production of more antigen specific T cells important for protection against TB[10]. However, in phase I testing large variability was noticed among different populations[11]. In a phase IIB clinical trial it was shown that MVA85A induced strong and durable T cell responses. Unfortunately these T cell responses were shown to not increase BCG efficacy[12]. Current efforts are still underway to repurpose the MVA85A vaccine, with the most recent trials suggesting that using it as both an aerosol and systemic vaccine may induce potent mucosal and systemic immunity[13]. However, any efficacy against *M. tuberculosis* infection remains to be seen.

TB/FLU-04L

TB/FLU-04L takes an interesting approach in using a recombinant influenza virus strain, A/Puerto Rico/8/34 H1N1, expressing antigen 85A and ESAT6 proteins of *M. tuberculosis*. Although it has not been fully tested for immunogenicity, no adverse effects have been noticed and no influenza infections occurred[14].

Whole Cell/Cellular Component Vaccines

RUTI

RUTI is a new vaccine consisting of cellular fragments of *M. tuberculosis*. In animal models RUTI originally showed the ability to lower bacterial loads and induce potent immune responses[15]. This vaccine is worth mentioning not only because it can induce protection against *M. tuberculosis* infection and so far has tested safe in humans[16], but because it also showed promise as an immunotherapy after infection[17].

MTBVAC

MTBVAC is a live attenuated *Mycobacterium tuberculosis* strain MT103, and is the first vaccine of its kind to enter into safety testing in healthy adult humans. In order to attenuate the strain, MTBVAC is missing two key portions of the MT103 genome, the *phoP* and *fadD26* regions[18]. *phoP* is a transcription factor that induces transcription of up to 2% of the original genome. Part of the region knocked out of production is the ESAT-6 virulence factor and diacyl and polyacyl trehaloses (DAT and PAT), cell wall lipids also contributing to virulence[19–21]. The other region knocked out, fadD26 contributes to the synthesis of phthiocerol dimycocerosates (PDIM) which are a large virulence factor which are part of the cell wall of *M. tuberculosis*[22]. In animal models MTBVAC was determined to be safe and more immunogenic than BCG[18]. In human safety tests, MTBVAC proved to be safe and did not cause infection in healthy adults while inducing

antigen specific responses to key proteins such as CFP10[23]. Continued research on MTBVAC has yielded a further attenuated strain lacking another gene, exported repeated protein or *erp*. This virulence factor contributes to intracellular replication by *M. tuberculosis* and its deletion makes the MTBVAC ERP strain hyper-attenuated and unable to replicate even in SCID mice. This more attenuated version shows promise for use in immunocompromised or HIV infected individuals where live vaccine strains like BCG are not recommended[24]. In clinical trials this vaccine was as immunogenic as BCG, and showed no virulence. MTBVAC was also able to induce strong polyfunctional CD4 central memory cells which supports its continued use in clinical testing[25].

DAR-901

Dar-901 is a whole cell vaccine of heat inactivated *Mycobacterium obuense*, a common environmental non-tuberculous mycobacterium. This vaccine was designed as a booster for those vaccinated with BCG as children, and has shown promise in inducing stronger anti-*M. tuberculosis* immunity than re-vaccination with BCG in the murine model of tuberculosis[26].

M. vaccae

M. vaccae (MV) is a non-pathogenic soil microbe that is found in the same genus as *M. tuberculosis.* In the mouse model, mice treated with heat inactivated MV showed increased protection following challenge with *M. tuberculosis*[27]. MV also showed potential in treating multi drug resistant (MDR) cases of tuberculosis in humans when given in addition to traditional chemotherapy[28,29]. When used as a booster to BCG vaccinated HIV positive individuals in phase III testing, MV tested safe and provided increased protection[30]. Overall this vaccine looks promising at reducing tuberculosis severity and helping as a therapeutic to MDR TB cases.

VPM 1002

VPM 1002 is a recombinant strain of BCG which has been engineered to produce Listeriolysin. Listeriolysin is a protein produced by *Listeria* monocytogenes allowing for phagolysosome escape into the cytosol. In BCG, expression of this protein allows for persistence of BCG and release of bacterial antigens into the cytoplasm to induce apoptosis[31]. This vaccine has shown promise in the mouse model of tuberculosis and can induce stronger CD4 and CD8 T cell responses compared to wild type BCG[31,32]. Because it has shown increased protection over the current BCG vaccine, it has been recommended that this vaccine move into clinical efficacy testing[33].

Fusion Protein Vaccines

ID-93 GLA-SE

ID-93 GLA-SE is a fusion protein vaccine consisting of four proteins, Rv2608, Rv3619, Rv1813, and Rv3620 combined with the adjuvant GLA-SE (glucopyranosyl lipid adjuvant-stable emulsion). In clinical trials this vaccine was able to stimulate durable IgG and T cell response as characterized by antigen specific IFN-γ production. As such, this vaccine candidate was recommended for efficacy testing in further clinical trials[34]. This vaccine has also shown efficacy against the more virulent Beijing family strain of *M. tuberculosis* characterized by production of antigen specific CD4 T cells[35].

M72/AS01E

M72/AS10E is a fusion protein vaccine employing two antigens produced by *M. tuberculosis*, Mtb 39A and Mtb 32A in addition to the adjuvant AS01. When given as a BCG booster, this vaccine has shown to be more protective than BCG alone in non-human primates and guinea pig models[36]. Part of its success comes from the adjuvant AS01, which contains two immunostimulatory molecules, MPL, and a saponin molecule QS-21, a strong inducer of CD4 T

cell immunity[37]. In human trials this vaccine has shown promise as antigen specific CD4 T cells for the M72 antigen were markedly increased in the treated populations and actually increased further when boosted again[38,39]. However, it is unknown if these populations of T cells will effectively protect against tuberculosis infection.

H56/IC31

H56/IC31 is a fusion protein vaccine formulated with the adjuvant IC31, a TLR9 agonist[40]. It is a trivalent protein consisting of Rv2660, Antigen 85B, and early secretory antigen 6 (ESAT-6), a protein virulence factor secreted by *M. tuberculosis* in early infection[41]. In human trials is has so far proven safe and does induce antigen specific IgG and T-cell responses[42]. In trials with non-human primates H56/IC31 has shown some success at boosting the BCG vaccine and delaying or reducing *M. tuberculosis* infection[43].

The need for future research

It is clear in looking at the current pipeline of vaccines for *M. tuberculosis* that they all share one thing in common. The conventional mechanisms of T cell mediated immunity are the focus of almost all new vaccines. In addition, many of these vaccines are also designed to boost the BCG vaccine. In practicality this is a good approach, as BCG is used in 157 different countries, especially in high TB endemic areas[44]. However, not many alternative vaccine areas have been explore. BCG is a potent activator of the innate immune system[45]. If we are to continue using the BCG vaccine, we need a better understanding on its overall effects on the innate immune system, and all of the mechanisms by which BCG can induce the killing of *M. tuberculosis*.

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Chapter III

Tuberculosis and the Immune Response

Adaptive Immunity

T cells

As noted in Chapter 2, most vaccine development for *M. tuberculosis* is centered on induction of strong T cell responses in the lungs following vaccination. The T cell response in tuberculosis is not only important as a mechanism by which the host fights infection, but also has become the basis behind screening mechanisms to identify infected patients. The PPD skin test for tuberculosis relies on a $CD4^+$ T cell delayed hypersensitivity reaction[1]. Upon infection in the lung, antigen presenting cells (APCs) are responsible for trafficking to the mediastinal lymph node to activate *M. tuberculosis* specific $CD4^+$ T cells[2,3]. Interestingly, in the case of dendritic cell antigen presentation, it is not the original dendritic cell from the lungs that does the T cell activation, but soluble antigens are released in the lymph nodes and taken up by other dendritic cells. This transfer of antigen optimizes the $CD4^+$ T cell priming process[4]. Upon T cell priming and clonal expansion, T cells will begin to circulate and make their way to the lungs, a process that can take up to four weeks. These T cells are part of the Th1 immune response, which is dependent on cytokines such as IL-12 and IFN-γ. IL-12 produced by APCs induces expression of T-bet, which is a transcription factor for many Th1 inducing genes[5]. In addition to its key role in activating macrophages[6], IFN-γ also plays a role in inducing T-bet to help proliferate the Th1 response[7]. Through these pathways, the Th1 response is critical to fighting tuberculosis.

The importance of both $CD4^+$ and $CD8^+$ T cells to survival after *M. tuberculosis* infection has been demonstrated in both the mouse and non-human primate model of tuberculosis[8–11]. However vaccine induced T cells have not been so easily linked to increased protection against *M. tuberculosis*. Especially after BCG vaccination, IFN-γ producing T cells specific to mycobacterial antigen do not always correlate to protection against tuberculosis[12]. Recent studies have identified localization of T cells as a potential cause of these problems. When T

cells are recruited to the lung they are homing to the lung vasculature as opposed to the parenchyma where they will have the most effect[13]. However, T cell homing is only part of the issue. Antigen availability is another concern that the T cell response faces when responding to pathogens such as *M. tuberculosis.* One study found that T cells specific for tuberculosis antigens face different complications when attempting to clear infection; specifically with two common vaccine target antigens Ag85 B and ESAT-6. Ag85B specific T cells show limited control of infection due to low levels of antigen expression during active disease and therefore low levels of T cell activation. Alternatively, ESAT-6 specific T cells were prone to T cell exhaustion, as ESAT-6 is a heavily expressed protein and stimulated continuous high levels of T cell activation[14]. T cell exhaustion represents an interesting hurdle for tuberculosis. Recent advancements in cancer immunotherapies have shown that using checkpoint inhibitors to block T cell exhaustion leads to an increase in efficacy of the immune response[15]. However, it remains to be seen if this will help during tuberculosis[16].

In addition to the Th1 immune response, the Th17 T cell response has demonstrated the ability to enhance the immune response to tuberculosis. The cells of the TH17 immune response are supported by the production of IL-17. Some evidence suggests that TH17 cells promote the formation of a strong memory T cell population after vaccination with BCG[17]. There is also evidence that cells form the Th17 response recruit $CD4^+$ T cells to the infection site and aid mycobacterial clearance[18]. However there have been some conflicting data on the role of the TH17 response. One study has suggested that IL-17 expression in lymphocytes increased the severity of diseases in patients with active tuberculosis[19]. Furthermore, cases of MDR tuberculosis have shown high levels of IL-17 which lead to tissue damage suggesting an immunopathological role for the TH17 immune response[20]. Overall this suggests that the Th17 immune response may be helpful in some cases where it promotes inflammation, but it can also be detrimental in that it can cause too much inflammation. Factors like this are important to consider when creating therapies for *M. tuberculosis*.

A final aspect of T cell biology relevant to tuberculosis are regulatory T cells (T_{regs}). Upon T cell activation after *M. tuberculosis* infection, T_{regs} respond quickly to help limit the damage cause by the inflammatory response[21]. T_{regs} can negatively influence the immune response in a variety of ways. One mechanism is through deactivating APCs and other myeloid cells through CTLA-4 mediated down regulation of co-stimulatory molecules CD80/CD86[22]. T_{regs} also can directly affect T cells through depletion of tryptophan in the local environment leading to T cell apoptosis[23]. Most notably, T_{regs} are known to induce apoptosis of effector $CD4^+$ T cells through depleting the local microenvironment of IL-2[24] and also contribute to immunosuppression of $CD4^+$ T cells through production of IL-10[25], TGF-β[26] and IL-35[27]. Overall T cells play a very significant part in the immune response to *M. tuberculosis*, but there are still major knowledge gaps in their function preventing the creation of an effective vaccine for tuberculosis.

B cells

The role of B cells and humoral immunity to *M. tuberculosis* remains one of the most understudied aspects in tuberculosis disease. In general, vaccine development has been heavily influenced by the fact *M. tuberculosis* is an intracellular pathogen, and therefore must require a cell mediated immune response. It was noted 20 years ago that there is no evidence that antibodies and B cells do not play a role in the immune response to tuberculosis[28] yet it still a widely accepted belief. Interestingly, it has been documented that there are antibodies to *M. tuberculosis* in infected humans, and these antibodies can have antimycobacterial effects[29]. In mice it has been noted that B-cell deficiency leads to increases in mycobacterial burden after pulmonary infection. It is also interesting to note that BCG vaccination resulted in decreased mycobacterial burden even in B cell deficient mice[30]. There is also evidence to support that when *M. tuberculosis* is phagocytosed through antibody-Fcγ receptor interactions there is increased phagolysosomal fusion and killing of the mycobacterium[31]. Independent of

antibodies, B cells also seem to contribute to the immune response to tuberculosis through interactions with other cell types. Of particular relevance is the effect B cells can have on neutrophil migration towards the site of BCG vaccination. B cells have been observed to decrease neutrophil motility, slowing down their recruitment to the injection site. With less neutrophils, macrophages and dendritic cells are able to phagocytose more bacteria allowing for enhanced antigen presentation leading to more IFN-γ producing, antigen specific, T cells and therefore a better Th1 response. A lack of B cells results in recruitment of excess neutrophils and impairs the adaptive immune response generated by BCG[32]. B cells can also influence antigen presentation at the draining lymph nodes through this same mechanism of neutrophil suppression[33]. Overall, the role of B cells in *M. tuberculosis* infection is greatly underappreciated, and more research is needed in this area.

Innate Immunity

Neutrophils

Neutrophils make up the first responders in the immune response. Upon infection with *M. tuberculosis*, neutrophils are the first immune cell recruited to the site of infection[34,35]. Because of this, neutrophils are known to accumulate in the airways of mice after aerosol challenge with either *M. tuberculosis* or BCG[36]. This process is mediated through a variety of pathogen recognition receptors (PRRs) such as the toll like receptors (TLR), G protein coupled receptors, nucleotide binding oligomerization domain-like receptors (NOD) and cytokine receptors. There are also a variety of signaling molecules that are host derived such as chemokines and pro-inflammatory cytokines responsible for this process[37,38]. Because they make such an early appearance, neutrophils play the important role of initiating the immune response and beginning to phagocytose mycobacteria and foreign particles through Fcγ receptor mediated phagocytosis or complement receptors[39,40]. Following phagocytosis, neutrophils use the production of

reactive oxygen intermediates (ROIs), nitric oxide (NO) and a variety of other bactericidal molecules such as elastases, proteinases, lactoferrin, and lysozyme to kill or inhibit the growth of mycobacteria[41]. In addition to phagocytic killing, neutrophils can also use extracellular killing methods referred to as neutrophil extracellular traps (NETs). NETs consist of chemicals such as NO that form in the granules of neutrophils, and instead of releasing them into phagosomes, they are released extracellularly to kill pathogens[42,43]. While it is known that neutrophils perform all of these functions, their efficacy in mediated killing of *M. tuberculosis* and BCG is not fully understood. Whereas the many studies sited here have indicated that neutrophils are activated and produce bactericidal components, further studies have concluded that these bactericidal components are functionally poor at actually killing of mycobacteria. In mice, *M. tuberculosis* has been shown to survive inside neutrophils even after they have been activated[44], and *M. tuberculosis* has even been observed to survive after the casting of NETs by neutrophils[43]. In humans, neutrophils have been identified as harboring live *M. tuberculosis* suggesting that they are more of a "Trojan horse" allowing for the survival of the organism rather than playing a role in killing it[45]. Given all of this information, it is clear that neutrophils play some role during infection with *M. tuberculosis* and BCG but it warrants further clarification.

Natural Killer Cells and Other Innate Lymphoid Cells

Natural killer (NK) cells are a subset of lymphocytes that cannot be classified as T or B cells based on surface marker expression[46]. NK cells are known to play a role in antiviral and anti-tumor immunity, and exhibit the function of cytotoxic T cells, without specific receptor recognition[47]. It seems logical that they would play a role in immunity to *M. tuberculosis*, but essential contributions in clearance of mycobacteria has yet to be identified[48]. NK cells express many PRRs that are also expressed by other cells of the innate immune system. It is hypothesized that the role of NK cells in mycobacterial infection may stem from their ability to sense the pathogen early on and instigate the immune response through cytokine production.

Specifically, it has been identified that NK cells can recognize mycobacterial pathogens through TLR2 and the NKp44 receptor. After recognition, NK cells become activated and produce pro-inflammatory cytokines such as IFN-γ[49]. There is also evidence that NK cells can exhibit a memory like response after recognition and activation from mycobacterial pathogens[50]. Regardless, more work is needed with these cells to determine their role, if any, in the immune response to *M. tuberculosis*.

Other innate lymphoid cells (ILCs) have also been evaluated for a role in protection against *M. tuberculosis* infection. Most notably, a recent study has identified BCG vaccination as driving recruitment and activation of ILCs to the lungs. Four weeks after intranasal BCG vaccination, type 1 ILCs were found to be recruited to the lung. They were also found to be activated, capable of producing IFN-γ[51]. Whereas this suggests that they may contribute to host defense of *M. tuberculosis*, there has been no evidence to show they play a direct role in mycobacterial killing. It is also important to note that any ILC recruitment and effector function was only noticed four weeks following vaccination, and not sooner.

A final population of ILCs are mucosal associated invariant T (MAIT) cells. MAIT cells are classified as cells from the innate immune system that are display a T cell receptor α chain with a CD3R paired with restricted Vβ segments[52,53]. MAIT cells are thought to play a role in the immune response to intracellular pathogens, but can be difficult to study as they can be difficult to isolate in mice. Any role that they have in defense to *M. tuberculosis* is thought to be through cytokine production[54]. In rhesus macaques MAIT cells were found to have limited activity during tuberculosis[55]. Studies in humans have also identified that MAIT cells show an impaired immune response to *M. tuberculosis* suggesting that they may not play a major role in directly killing mycobacterium[56]. The role of MAIT cells is currently still under review and requires further investigation.

Monocytes/Macrophages

Monocytes and Macrophages are the second responders against infection from *M. tuberculosis* and other mycobacteria after neutrophils. In the lungs, alveolar macrophages are the primary cell infected by *M. tuberculosis*[57]. The role of monocytes/macrophages in containment and clearance of *M. tuberculosis* is not exactly clear. In general, monocytes/macrophages are responsible for phagocytosing and killing pathogens. But on the other hand, monocytes/macrophages are the natural host reservoir for pathogens like *M. tuberculosis* and BCG in which they proliferate[58]. Initial uptake of mycobacterium can occur through a variety of pathways. Phagocytosis of *M. tuberculosis* has been observed through Fcγ receptors, complement receptors, and lectin receptors[59]. There is also evidence that *M. tuberculosis* gains entry into alveolar macrophages through pinocytosis[60]. Once inside the cell, *M. tuberculosis* is contained within a phagolysosome. Generally T cells are stimulated by their T cell receptor and produce IFN-γ which stimulates macrophages/monocytes to activate and fuse phagosomes, containing the mycobacteria, to a lysosome in order to kill the mycobacteria. However, *M. tuberculosis* has developed ways in which to inhibit phagolysosome maturation and escape into the cytosol where it can live and proliferate[61–64]. It is also possible that *M. tuberculosis* can reside long term inside intracellular vacuoles derived from the phagosome[65]. Either extracellularly or intracellularly, monocytes/macrophages are well characterized as being reliant on many PRRs to sense pathogen and stimulate an inflammatory response. *M. tuberculosis* and BCG are potent activators of TLRs 2, 4[66], and 9[67]. C-type lectin receptors are also important in recognizing *M. tuberculosis* through the binding of lipoarabinomannan (LAM) on the surface of the mycobacteria[68,69]. Once *M. tuberculosis* is recognized, monocytes/macrophages produce pro-inflammatory cytokines which can lead to the formation of granulomas as described in Chapter 1. Other PRRs such as nucleotide oligomerization domain 2 (NOD2) have been identified as playing a major role in the immune response generated by mycobacterial pathogens.

Research published on the interaction of BCG with NOD2 has highlighted its importance in instigating an immune response to mycobacteria through the recognition of muramyl dipeptide (MDP). MDP is found in the peptidoglycan of gram positive and gram negative bacteria[70]. The main goal of this research has centered on understanding the ability of BCG to induce immunity to non-mycobacterial infection. Upon binding of MDP by NOD2, NFκB is activated and the transcription of pro-inflammatory cytokine genes is initiated. During this process the histones surrounding these genes are rearranged leading to an epigenetic reprogramming of these genes. Any future times that NOD2 is activated these genes are accessed more easily by transcription machinery and the inflammatory response can be initiated quicker and is more robust. This has been deemed trained innate immunity[71–75]. When this phenomenon was first characterized in 2012 as having epigenetic components, a surge of studies in this field began to identify trained innate immunity as the mechanism by which BCG conferred nonspecific immunity and protection against non-mycobacterial diseases[71,73,75]. However trained innate immunity has not been characterized in terms of protection offered against *M. tuberculosis* infection. This thesis was designed to study the innate immune response to the BCG vaccine. BCG is able to induce anti-mycobacterial immunity, but the mechanisms responsible are poorly understood. The work performed in this thesis aims to increase understanding of how BCG interacts with the innate immune system, primarily macrophages, and is able to stimulate the clearance of *M. tuberculosis* through innate immune mechanisms.

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Chapter IV

Characterizing the BCG induced T cell independent mechanisms for defense against *Mycobacterium tuberculosis*

Introduction

Bacille Calmette Guérin, (BCG), is a potent immune response modifier used as a vaccine against *Mycobacterium tuberculosis* infection, a treatment for bladder cancer[1], as well as an adjuvanted delivery platform for vaccines against other pathogens[2]. BCG also causes cross resistance resulting from myeloid cell activation[3]. Since these findings, the concept of "trained-immunity" as a means of explaining the phenomenon has been proffered and shown that BCG induces epigenetic changes in human monocytes[3–5] as well as reside in bone-marrow cells[6]. These data provide strong evidence to suggest that BCG is a potent biological response modifier, but leave many questions unanswered. It has been our experience that in the C57BL/6 model of experimental tuberculosis, BCG vaccination results in a significant reduction in mycobacterial growth when mice were infected with virulent *M. tuberculosis,* 30 days after vaccination. Induction of adaptive immunity has been demonstrated by us and others and is independent of sub-strain of BCG[7], although the relative number of CD4 and CD8 T cells remained limited and did not correlate to the reduction in mycobacterial growth[8]. We hypothesized that a significant contribution to *M. tuberculosis* killing after BCG vaccination was provided by innate immune mechanisms and that this established an environment to induce adaptive immunity that was responsible for prevention of disease.

BCG induces potent T cell responses as a result of its diverse array of pathogen associated molecular patterns (PAMPs) that stimulate innate and subsequently adaptive immunity. BCG is known to stimulate multiple pathogen recognition receptors (PRRs) such as Toll-Like Receptors (TLR)-2/4/9 and nucleotide-binding oligomerization domain-like receptors (NOD)[3,9] suggesting that BCG provides multiple potent signaling mechanisms to innate myeloid

and lymphoid cells. Cells such as dendritic cells (DCs), monocytes, macrophages, neutrophils and innate lymphoid cells (ILCs) may be triggered by BCG to be activated and subsequently induce T cell mediated immunity that is required to kill *M. tuberculosis*. We asked, what if these cells had the capacity to kill *M. tuberculosis* prior to the induction of adaptive immunity, which of these cells was responsible, and how were they performing their function? Recent studies demonstrated that BCG caused epigenetic changes in macrophages, and BCG infected bone marrow-derived macrophages, when adoptively transferred, reduced the mycobacterial burden in infected recipients[6]. However these studies did not address which cells were directly responsible for killing *M. tuberculosis* and what mechanisms were used for killing. In the current studies, the mouse model was redesigned from the standard 30 day post vaccination model[10] to examine the immune response within seven days of vaccination, prior to induction of adaptive immunity, to determine how innate immune mechanisms affect the growth of *M. tuberculosis*. Using a series of depletion and knockout (KO) mouse studies, we demonstrate that after subcutaneous BCG inoculation, neutrophils, circulating monocytes, and alveolar macrophages together are sufficient to reduce mycobacterial burden, and that live BCG was required to stimulate this immune response.

The PRR NOD2, is of significant importance in BCG and *M. tuberculosis* as recent evidence has suggested that cells of the innate immune system can exhibit a memory like response through recognition of pathogens with the NOD2 receptor[3–5]. Upon stimulation with muramyl dipeptide, the NOD2 receptor induces epigenetic reprogramming around the genes responsible for initiating inflammation so that a second stimulation can produce a more robust and quicker response leading to better clearing of the pathogen in what has been called trained innate immunity[3–5,11]. Studies in humans deficient for NOD2 have shown that macrophages were unable to properly activate the inflammasome for IL-1β signaling[12,13] and showed reduced capacity to produce TNF-α[3] indicating a potential for this phenomenon to translate to humans. If so, the BCG vaccine may have certain benefits for human use and requires a better

understanding of the mechanisms by which it can induce mycobacterial killing in the mouse model. Our work demonstrated that BCG was able to induce mycobacterial killing in the absence of the NOD2 receptor, on which the training of monocytes is dependent[3]. In addition BCG induces protection in the absence of T cells, NK cells, and in LysMcre KO mice, previously characterized as lacking complete monocyte populations[14,15]. As BCG is still able to protect mice lacking immune components previously thought to play a role in immunity to *M. tuberculosis*, this study investigated the contributions of the innate immune system to BCG mediated protection.

Studies have demonstrated that BCG can induce strong T cell responses[16,17], but our work and others have suggested that it is unclear whether or not this directly correlates to protection against *M tuberculosis*[8]. Nonetheless, as this study along with others have demonstrated, BCG does adequately induce immunity to *M. tuberculosis* infection in a mouse model. Many new vaccines for *M. tuberculosis* are currently in clinical trials, and a number of them still use BCG in some form. The BCG vaccine has a future in tuberculosis prevention, but without a full understanding of the mechanism of action behind its induction of protective immunity it may not be utilized properly. Recent publications have highlighted the importance of the innate immune system during BCG vaccination and have indicated that non-specific effects of BCG vaccination may benefit young children even if protection against *M. tuberculosis* is not attained[3,18–20]. The focus of this study is on the effects of BCG on the innate immune system to better understand its mechanism of action to highlight new ways to induce mycobacterial killing for better future vaccines.

Materials and Methods

Mice: Female C57BL/6, B6.129S2-*Cd4*^{tm1Mak}/J (CD4KO), B6.129S2-*Cd8a*^{tm1Mak}/J (CD8KO), B6.129S-*Tnf*^{tm1Gkl}/J (TNF-α KO) and B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J mice (LyzMcre KO) (6-8 week-old) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at CSU for two weeks prior to experimentation. Nod1$^{-/-}$/Nod2$^{-/-}$ (NOD1/NOD2- deficient) mice were a kind gift from Dr. Andreas Baumler (University of California, Davis), and were described previously[21]. The Institutional Animal Care and Use Committee at Colorado State University reviewed and approved all experiments (Protocol ID: 16-6369A).

Mycobacteria: *M. bovis* BCG Pasteur (TMC#1011) was grown in Proskaur and Beck (P&B) medium containing 0.01% Tween® 80 until mid-log growth phase. It was then aliquoted and stored at -80˚C for future use. *M. tuberculosis* H37Rv (TMC# 102) was grown in P&B medium as a pellicle and then transferred to liquid cultures of P&B medium containing 0.5% Tween® 80. The cultures were passaged three times through the same media grown in a roller bottle incubator and finally aliquoted and stored at -80˚C. The number of colony forming units (CFU) and viability of all cultures was assessed after freezing and was >95% viable. The genome of the BCG Pasteur used in these studies was sequenced (Dr. M. Strong, Center for Genes, Environment and Health, National Jewish Health and University of Colorado, Denver, CO) and was >99.99% similar to the published reference genome for BCG Pasteur[22].

BCG vaccination: BCG was diluted to a concentration of 5x10^5 CFU/mL with sonicating to disrupt clumps. The vaccine was loaded into 1 mL syringe and 100 µL was inoculated to deliver 5x10^4 CFU subcutaneously, 1x10^6 CFU intravenously, 1x10^7 intranasal, 5x10^4 intramuscular per mouse. Low dose aerosol BCG vaccine given at 50-100 CFU per mouse. The inoculum was plated on 7H11 agar (Difco, BD Biosciences, San Jose, CA) to assess the accuracy of vaccine

dose. Mice were rested for 7 days post-vaccination and then infected with *M. tuberculosis*. In some studies mice received BCG that was exposed to 2.4mRads of γ-irradiation from Cs^{137} source (Irradiation Services Laboratory, CSU) that allows it to retain its metabolic activity, but not to replicate as determined by plating after irradiation procedure (data not shown).

***M. tuberculosis* infection:** *M. tuberculosis* H37Rv was diluted to a concentration of $2x10^6$ CFU/ml with prior sonication to separate aggregates. 5 mL was then loaded into a nebulizer in order to deliver 50-100 CFU to each mouse using the Inhalation Exposure System (Glas-Col, Terre Haute, IN) according to the established laboratory protocols. The inoculum was plated on 7H11 agar in addition to 5 mice being sacrificed on day of infection to assess the number of CFU implanted into the lung.

Assessment of bacterial burden: CFU in the lungs and spleens of mice were determined at 30 days post infection. Necropsy was performed to remove organs which were then homogenized in sterile saline before being diluted and plated on 7H11 agar. The plates were incubated at 37˚C for 14-18 days. Colony counts were converted into Log_{10} CFU for analysis. All CFU were performed with five mice per group unless specified otherwise.

Antibody depletion: Mice were depleted of neutrophils through intraperitoneal injection of 200 µg anti-Ly6G antibody (clone 1A8; Bio X Cell, Lebanon, NH) in PBS every 4 days. Mice were depleted of Natural Killer (NK) cells through intraperitoneal injection of 250 µg anti-asialo GM1 antibody (Accurate Chemical, Westbury, NY) in PBS every 4 days. Control mouse groups received the appropriate isotype antibody at the same concentration. Cell depletion efficiency was assessed by flow cytometry using anti-NK1.1 for natural killer cells and anti-Ly6G for neutrophils (Supplemental Table 4.1).

Flow cytometry: Single cell suspensions were prepared from lungs that were minced with a razor blade before being incubated at 37˚C in 0.5% Liberase (Sigma Aldrich, St. Louis, MO) incomplete RPMI solution (Life Technologies, Carlsbad, CA). After 45 minutes lung pieces were passed through a 70-μm nylon cell strainer (Falcon, Corning, Durham, NC) and the single cell suspension collected by centrifugation. The pelleted cells were resuspended in 2 mL ACK red blood cell lysis buffer (Life Technologies, Carlsbad, CA) and incubated at room temperature for 5 minutes before 10 mL of complete RPMI (RPMI-1640 with essential and nonessential amino acid, penicillin, streptomycin, HEPES (Sigma Aldrich, St. Louis, MO), sodium pyruvate (Sigma Aldrich, St. Louis, MO), L-glutamate (Sigma Aldrich, St. Louis, MO), and 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO) was added to stop the reaction. Cells were centrifuged and stored in complete-RPMI on ice while cells were counted and the concentration adjusted to $2x10^6$/mL in complete-RPMI. Cells were pelleted and incubated for 20 minutes at 4˚C in 2.4G2 hybridoma supernatant (Fcγ blocking antibody; ATCC® HB-197) diluted in PBS containing 5% FBS 0.01% NaN3 (FACS buffer). Cells were washed by centrifugation and resuspended in clean FACS buffer before being pelleted and stained with the fluorochrome-conjugated antibodies (Supplemental Table S1). Cells were washed again with FACS buffer before being analyzed on a BD FACS Canto II flow cytometer and data analyzed using FlowJo software (FlowJo, LLC, Ashland OR).

Macrophage stimulation assay: RAW Blue™ macrophage reporter cells (InvivoGen, San Diego, CA) engineered with a chromosomal integration of a secreted embryonic phosphatase reported construct inducible by NF-κB and AP-1 were used to assess the activity of BCG on macrophages. Cells were cultured in complete RPMI (Life Technologies, Carlsbad, CA) in 24 well tissue culture plates (Corning Incorporated, Corning, NY) and stimulated with either live BCG Pasteur or γ-irradiated BCG Pasteur. Supernatants were taken and the amount of alkaline

phosphatase was measured by addition of QUANTI-blue™ (InvivoGen). Supernatant was then transferred to a 96 well plate and optical density was measured at 72 hours at 630 nm.

Preparation of bone marrow derived macrophages (BMDM) and Real-time PCR analysis: Bone marrow cells were harvested from C57BL/6 mice and added to complete medium containing 20 ng/mL M-CSF to drive macrophage differentiation (RPMI-1640 with essential and nonessential amino acid, penicillin, streptomycin, and 10% fetal bovine serum (FBS)) (Invitrogen). Media was changed every 72 hours until the eighth day in which media was changed to exclude M-CSF and antibiotics. On day seven, cells were harvested and brought to a concentration of $1.0x10^6$ macrophages per well in a 24 well plate for stimulation for 24 and 48 hours with varying MOI of BCG or irrBCG. Supernatants and total RNA was harvested at each time point. RNA was isolated using TRIZol® (Invitrogen), and quantified using a Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific). RNA was then converted into cDNA using an iScript cDNA synthesis kit (BioRad, Hercules, CA), and RT-PCR was performed using the Qiagen RT^2 Profiler™ PCR Array for Mouse Cytokines and Chemokines on a CFX Connect™ Real-Time PCR Detection System (BioRad). Data were analyzed using the Qiagen online Data Analysis Center.

Cytokine analysis: Cytokine quantification in cell culture supernatants following stimulation was performed by enzyme-linked immunosorbent assay (ELISA). ELISA kits (Affymetrix/eBioscience INC San Diego, CA) for the following cytokines: TNF-α, IL-1, IL-6, and IL-10 were used following the manufacturer's protocol. The color intensity in wells of 96 well plates was then read using the Ultramark™ Microplate Reader (BioRad, Hercules, CA). A standard curve was also used with each assay to determine cytokine concentration in pg/mL. Lung homogenates from infected mice were pelleted and the cell free supernatant was taken to quantify cytokine concentrations, and a Cytometric Bead Array (CBA) assay was performed

using CBA mouse inflammation kit (BD Biosciences, San Jose, CA) on lung supernatants. Data from the bead assay were collected on a FACSCanto II cytometer (BD Biosciences, San Jose, CA) according to protocol with kit and analyzed using FCAP Array™ software (BD Biosciences).

Statistical Analysis: Data were analyzed using the statistical tests as described using R software (R foundation). Data from some experiments were Log10-transformed prior to analysis. Graphs were prepared using Graph Pad Prism 7 (GraphPad software, La Jolla, CA).

Results

CD4/CD8/TNF-α independent M. tuberculosis killing mechanisms induced by BCG:

In the mouse model, BCG is well established as conferring protective immunity against experimental pulmonary *M. tuberculosis* infection. To determine if BCG induced immunity was not solely dependent on adaptive immunity, CD4KO and CD8KO mice were vaccinated with $5x10^4$ BCG Pasteur thirty days prior to infection, the time at which adaptive immunity is active. Mice vaccinated with BCG thirty days prior to infection also showed a 1 Log_{10} reduction in CFU in lungs (Figure 4.1A) and spleens (data not shown) representing a 90% reduction in mycobacterial burden in the absence of either $CD4^+$ or $CD8^+$ T cell mediated immunity. We then wanted to determine if TNF–α, a key cytokine required for protective immunity was essential for killing *M. tuberculosis* after BCG vaccination. No significant difference was observed between BCG-vaccinated C57BL/6 and TNF-α KO mice at day 30 post-vaccination (Figure 4.1A). Our data showed that three major adaptive immune elements were not an absolute requirement for BCG mediated killing of *M. tuberculosis*.

BCG-induced innate immune mechanisms are sufficient to kill M. tuberculosis

We then altered the paradigm to ask if a similar reduction in CFU was observed at an earlier time (prior to induction of T cell mediated immunity) in immunocompetent mice. To this end, when mice were vaccinated 7 days before pulmonary infection, there was a significant reduction in CFU in the lungs and spleens of mice (Figure 4.1B). As 7 days was not a sufficient time to mount an adaptive immune response, this led us to hypothesize that adaptive immunity was not an absolute requirement for BCG induced reduction of the mycobacterial burden. As such, we hypothesized that protection induced by BCG vaccination was due to innate immunity which worked independently of adaptive immunity. T cell activation at day 7 post-vaccination, examined by ELISpot assay revealed no difference in antigen-specific T cells in the lungs, spleens and, lymph nodes between vaccinated and non-vaccinated, naïve mice (data not

Figure 4.1

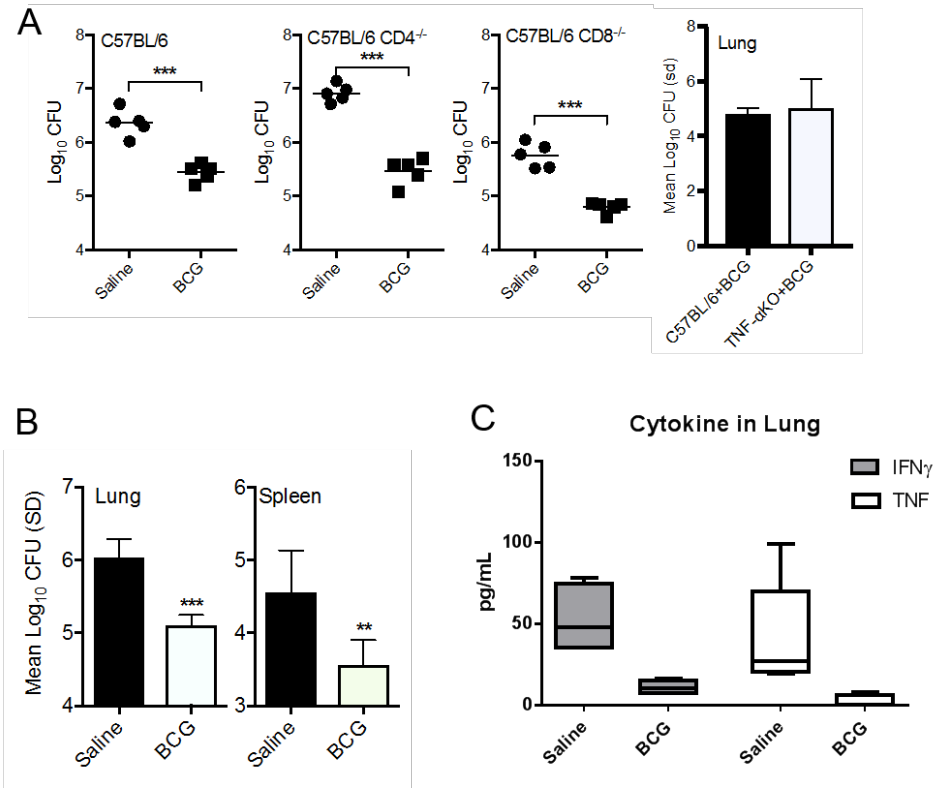

Figure 4.1
C57BL/6 CD4, CD8, and TNF-α knockout mice were vaccinated subcutaneously with $5x10^4$ CFU BCG, rested for 30 days and then infected with a low dose aerosol of *M. tuberculosis* H37Rv. CFU were determined at day 30 post infection (**A**). C57BL/6 mice vaccinated with $5x10^4$CFU BCG Pasteur seven days before infection and the CFU determined 30 days after infection (**B**). The concentration of IFN-γ and TNF-α at 30 days post infection in lung tissue after mice were vaccinated with BCG seven days before infection (**C**). Experiments were performed with N=4-5 mice per group and are representative of multiple iterations. **p≤0.01, ***p≤0.001.

shown). To better understand the nature of the early immune response in the lungs, cytokine analysis was performed on lung tissue after infection (Figure 4.1C). IFN-γ and TNF–α were significantly reduced in the lungs of infected mice that had been vaccinated 7 days prior, compared to non-vaccinated mice that were subjected to the same infection suggesting a nonessential role for these cytokines in BCG induced immunity.

BCG-induced macrophage changes in the lung after subcutaneous vaccination:

As BCG induced protective immunity within 7 days we wanted to better understand the mechanism by which a subcutaneous vaccination induced protection in the lungs in that time. Others have reported that subcutaneous BCG vaccination induced systemic responses, such as increased $CD14^+$ monocytes[3], but the response in the lungs has not been defined. We assessed changes in lung cell populations seven days after subcutaneous BCG vaccination. Lungs from vaccinated and non-vaccinated mice were collected to identify differences in monocyte/macrophage populations using CD11b, Ly6C, F4/80 and CD14 markers (Figure 4.2A). BCG vaccinated mice had increased percentages of $CD11b^+$ cells in their lungs (Data not shown). Further analysis of the $CD11b^+$ population showed that vaccinated mice had higher percentage of $CD11b^+$ $F4/80^+$ cells but a lower percentage of $CD11b^+$ $F4/80^+$ $Ly6C^+$ cells (Figure 4.2B & C) indicating that particular population of monocytes were losing Ly6C expression. The Ly6C marker has been used to distinguish circulating monocytes versus cells migrating into tissues[23] suggesting that upon BCG vaccination, the lungs experience an infection like state in which monocytes are recruited from the blood to help control the infection, although in this case in the absence of a lung infection. There is evidence that circulating monocytes can differentiate into interstitial macrophage populations once they reach tissue such as the lungs that have the capability to self-renew,[23,24] suggesting a possible mechanism by which BCG induces innate immunity in the lungs. There was also a significant increase in

Figure 4.2

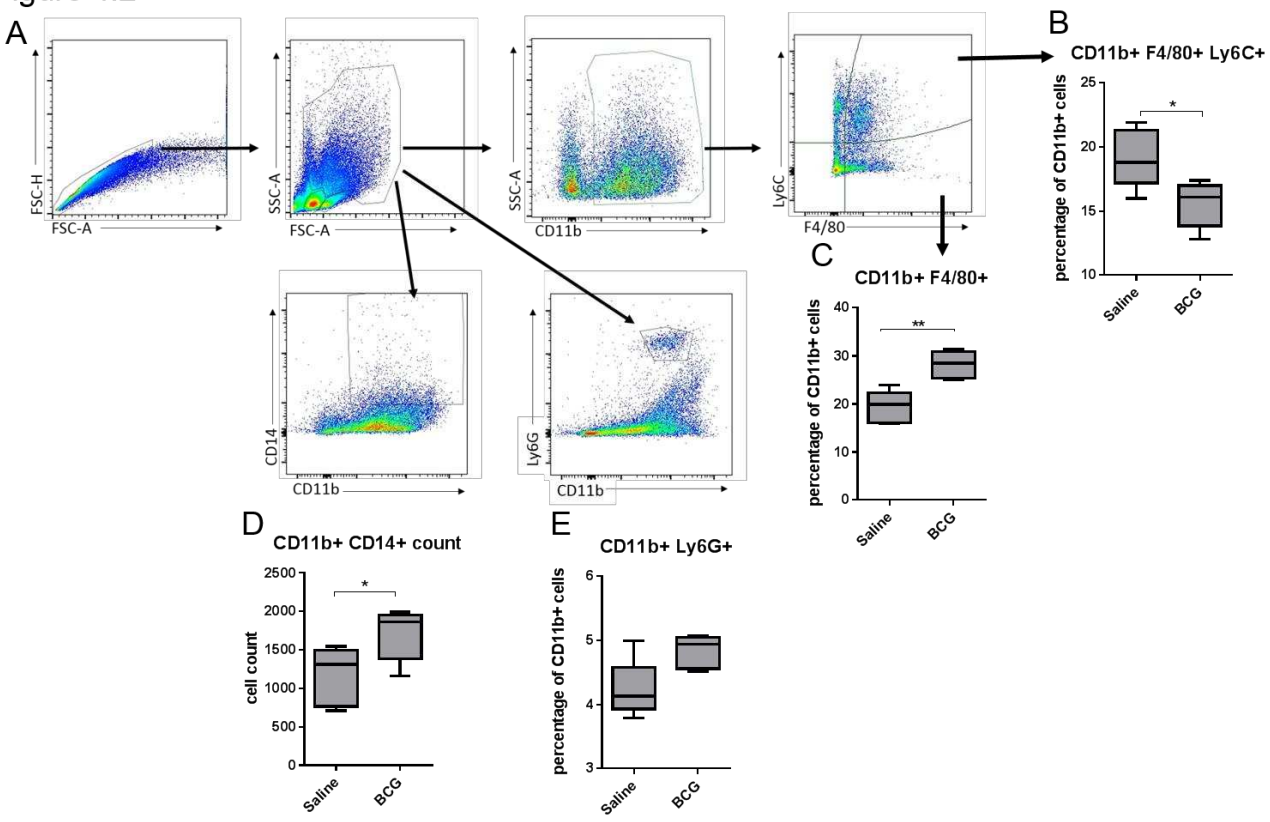

Figure 4.2
Flow cytometry gating strategy; singlets were identified using FSC-A and FSC-H, and granulocytes were gated based on size and granularity. The markers CD11b, Ly6C, and F4/80 were used to identify subpopulations of macrophages and Ly6G was used to identify neutrophils (**A**). C57BL/6 mice, vaccinated subcutaneously with $5x10^4$ CFU BCG Pasteur, showed an increase of $CD11b^+$ cells in the lungs and a lower percentage of those cells are positive for the marker F4/80 and Ly6C at day seven post-vaccination (**B**). The lungs from BCG vaccinated mice also showed an increase in the percentage of $CD11b^+$ cells that were also $F4/80^+$ (**C**). BCG vaccinated mice also showed an increase in the number of double positive cells in the lungs for CD11b and CD14 expression (**D**). Lungs from vaccinated mice also had a small increase in $CD11b^+$ $Ly6G^+$ neutrophils (**E**). N=5 mice per group, *p≤0.05 **p≤0.01 (Wilcoxon rank sum test).

$CD11b^+$ $CD14^+$ cells in the lungs of vaccinated mice (Figure 4.2D), that has been observed in the spleen by others[3]. There was also a very minimal increase of neutrophils ($CD11b^+$ $Ly6G^+$) in the lungs (Figure 4.2E). Our data suggested that BCG stimulation increased phagocytic cells in the lungs, but their phagocytic potential was not shown.

Innate immunity induced by BCG requires neutrophils:

Prior to induction of adaptive immunity, BCG induced a significant reduction in the mycobacterial burden (Figure 4.1B), thus we focused on the contribution of innate immunity to killing of *M. tuberculosis*. It is generally accepted that IFN-γ produced by T cells is essential for controlling *M. tuberculosis* infection, through activation of infected cells such as macrophages[25,26], although a prior study identified an IFN-γ independent mechanism for killing mycobacterium, that was however CD4 T cell-dependent[27]. As a T cell mediated IFN-γ response was not detected at this time to induce killing of mycobacterium (Figure 4.1C) our first approach was to target a potential source of IFN-γ in the innate immune system. In addition to their ability to produce IFN-γ, we were also interested in the role of Natural Killer (NK) cells during BCG vaccination for their contributions in controlling intracellular pathogens, especially *M. tuberculosis*[28]. To better understand their role, we used anti-asialo-GM1 antibody to deplete NK cells in mice during infection with *M. tuberculosis* in both vaccinated and unvaccinated mice. Our data suggested that NK cells did not play a role in killing *M. tuberculosis* in either group (Figure 4.3A). Depletion of IFN-γ after aerosol infection, either immediately after or from day 15 post-infection, resulted in a significant reduction in the mycobacterial burden, suggesting the presence of alternative mechanisms for killing the intracellular mycobacteria (Figure 4.3B).

Given their importance in the development of immunity, we next wanted to examine the role of neutrophils during BCG vaccination[29]. Neutrophils are the first cells at the site of infection and are responsible for initiating immune responses by activating other cells including

Figure 4.3

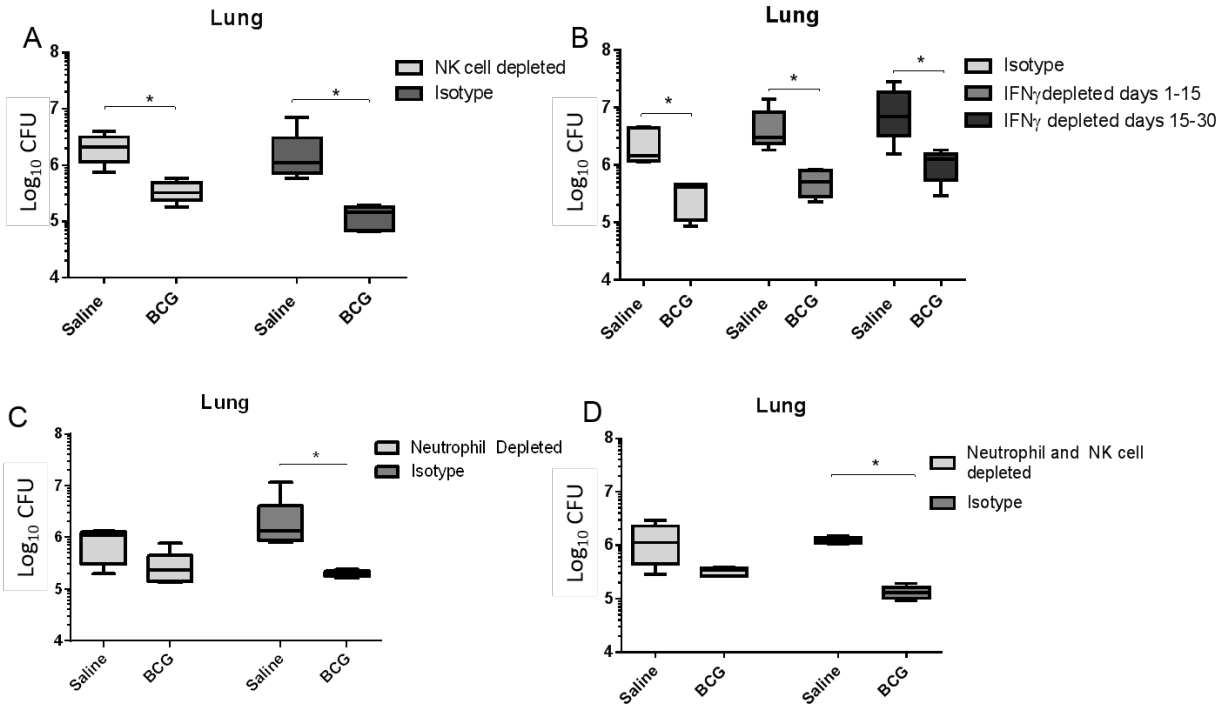

Figure 4.3
BCG vaccinated C57BL/6 mice, depleted of NK cells using anti-asialo GM1 antibody during infection were able to significantly reduce the mycobacterial lung burden similar to isotype antibody-treated control mice (**A**). NK cell depletion was confirmed through flow cytometry three days after depletion (**Supplementary figure 4.1.1B**). BCG vaccinated C57BL/6 mice depleted of IFN-γ during infection at either days 1 to 15, or 15 to 30 post-infection had significantly reduced mycobacterial lung burdens, similar to isotype antibody-treated infected control mice (**B**). BCG vaccinated C57BL/6 mice, depleted of neutrophils during vaccination and up to 4 days post-vaccination, reduced their lung mycobacterial burden by approximately 0.5 Log_{10} CFU compared to isotype antibody-treated infected control mice (**C**). Neutrophil depletion was confirmed through flow cytometry three days after depletion (**Supplementary Figure 4.1.1A**). BCG vaccinated C57BL/6 mice, depleted of neutrophils during vaccination, and up to 4 days post-vaccination, and NK cells from the time of infection, had reduced their mycobacterial lung burden by 0.5 Log_{10} CFU compared to isotype antibody-treated infected control mice, which are able to reduce burden by 1 Log_{10} (**D**). N=5 mice per group for all experiments, *p≤0.05 (T-test).

macrophages and dendritic cells[30]. To examine the role of neutrophils during BCG induced immunity, Mice were treated with anti-Ly6G antibody to deplete these cells prior to and during vaccination, and the neutrophil population was allowed to return before infection on day seven, as antibody treatment was stopped on day 4 post vaccination. Administering anti-Ly6G prior to and during BCG vaccination resulted in a 0.5 Log_{10} CFU reduction in mycobacteria compared to the 1 Log_{10} CFU reduction observed in the isotype control group (Figure 4.3C) and the same result was observed when neutrophils were depleted during vaccination and NK cells depleted during infection (Figure 4.3D). These data suggest that neutrophils played a role in establishing innate immunity, possibly through an early inflammatory response that initiates the reduction in mycobacterial burden. However it does not imply any role for neutrophils in the direct killing of mycobacteria as the neutrophil populations were only depleted during vaccination and allowed to return during infection.

Trained innate immunity is not a factor in BCG induced killing of M. tuberculosis at seven days post vaccination

In order to assess the importance of trained innate immunity to BCG induced protection against *M. tuberculosis* infection, we utilized a mouse model deficient in the NOD1 and NOD2 receptors. Even in the absence of the NOD receptors, BCG induced immunity resulted in a significant reduction in the mycobacterial burden in the lung similar to wild type mice (Figure 4.4A) indicating that NOD2-dependent trained innate immunity was not required for BCG induced protection against *M. tuberculosis* infection at day 7 post-vaccination. This suggested an alternative mechanism for induction of innate immunity and subsequent killing of mycobacteria. To determine if the route of vaccination affected BCG induced mycobacterial reduction within 7 days, mice were vaccinated through five different routes; subcutaneous, intravenous, intranasal, low dose aerosol, and intramuscular. BCG vaccination induced a

Figure 4.4

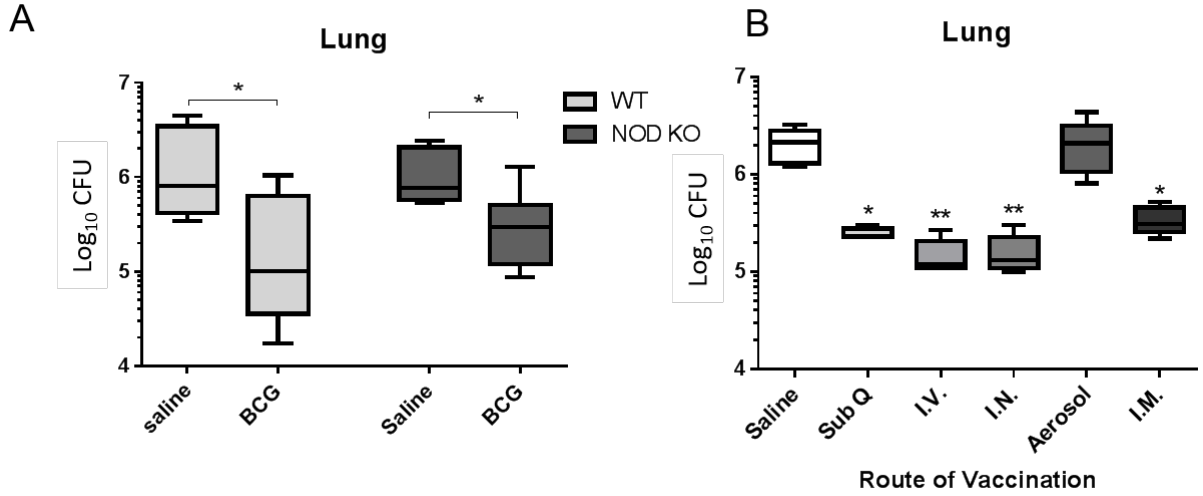

Figure 4.4
C57BL/6 mice deficient for both NOD1 and NOD2 (NOD KO) were vaccinated subcutaneously with either $5x10^4$ CFU BCG Pasteur or sterile pyrogen-free saline as controls. When vaccinated seven days before infection, C57BL/6 NOD KO mice show a capacity to significantly reduce the mycobacterial burden in lung (**A**). C57BL/6 mice vaccinated with BCG seven days before infection by route specified; subcutaneous (sub Q), intravenous (I.V.), intranasal (I.N.), low dose aerosol (Aerosol), intramuscular (I.M.). Mice are able to reduce the mycobacterial burden regardless of route of vaccination (**B**). N=5-6 mice per group, *p≤0.05. **p≤0.01 (Kruskal Wallis test with Dunn post hoc test).

significant reduction in mycobacterial lung burden regardless of route of vaccination with the exception of aerosol vaccination (Figure 4.4B). This was expected as aerosol vaccination deposited approximately 100 CFU BCG into the lungs of mice which may have been insufficient to generate a protective immune response.

BCG replication is required for the induction of protective immunity

Although trained innate immunity did not seem to be the principal component for BCG induced killing of *M. tuberculosis*, it did not preclude innate immune system involvement. BCG is an intracellular pathogen, and a known potent stimulator of the innate immunity[31] that has the ability to protect against non-mycobacterial diseases as well as certain cancers[11]. Past studies have demonstrated that when given as a subcutaneous vaccine, live BCG can be found in various organs of mice including lungs[7] and bone marrow[6]. There is also some suggestion that the numbers of BCG found are misrepresented as some live BCG may not replicate when taken out of the host[32]. All of this suggests that BCG is able to survive in the endosomes of macrophages for extended periods of time, and some groups have reported this *in vitro*[33,34] with evidence that BCG can inhibit phagosome maturation *in vivo*[35]. However, it is unknown if induction of protective innate immunity depends on BCG remaining viable after inoculation and whether it needs to replicate. To address this question mice were vaccinated subcutaneously with either viable $5x10^4$ CFU BCG Pasteur or an equivalent dose of γ-irradiated BCG Pasteur. In addition, another cohort of mice were inoculated with either viable BCG or γ-irradiated BCG intravenously to determine if alternate routes worked as effectively as subcutaneous vaccination. Our findings indicated that the ability of BCG to actively replicate played a role in reducing the mycobacterial burden as γ-irradiated BCG groups did not produce the same level of mycobacterial killing, regardless of the route (Figure 4.5A). If BCG replication was important for the induction of strong innate immunity, it will be important to identify the

Figure 4.5

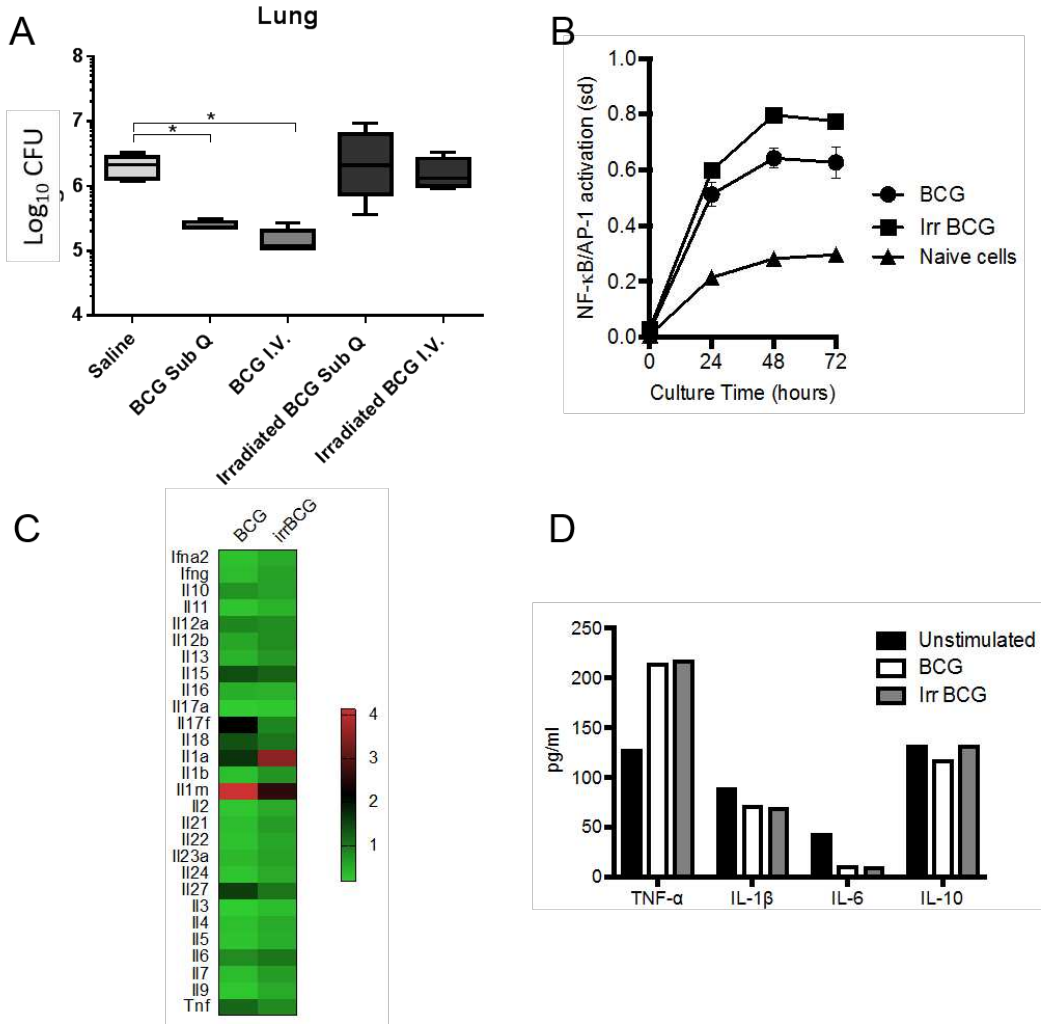

Figure 4.5
C57BL/6 mice were vaccinated with either live BCG or γ-irradiated BCG (irradiated BCG) subcutaneously or intravenously. γ-irradiated BCG was unable to stimulate a reduction in mycobacterial burden compared to live BCG which was able to reduce mycobacterial burden by 1 Log_{10} (**A**). N=5-6 mice per group, *p≤0.05. The reporter RAW-Blue® cell line to assess NF-kB and AP-1 activation was used to assess the ability of either live BCG or γ-irradiated BCG to stimulate NF-kB and AP-1 signaling pathways. The activation of NF-kB and AP-1 signaling pathways was similar, regardless of whether the BCG was viable or non-replicating (**B**). Cytokine mRNA expression was examined in C57BL/6-derived BMDM, stimulated with BCG for 24 hours, using the RT2 Profiler™ PCR Array for Mouse Cytokines and Chemokines (**C**). Supernatants were also collected from these cultures and assessed for the production of protein cytokines TNF-*α*, IL-1β, IL-6 and IL-10 (**D**). Data are representative of two in vitro studies.

mechanisms and how this translates to humans as BCG vaccines used in humans are lyophilized and coupled with improper storage can lead to only around 30% viability[36].

Given that γ-irradiated BCG did not induce protective immunity at day 7 post-vaccination, we wanted to determine if this was due to the inability of γ-irradiated BCG to activate antigen presenting cells such as macrophages. Using the reporter RAW-Blue® cell line to assess NF-κB and AP-1 activation, cells were cultured for 24, 48 and 72 hours in the presence of either BCG or γ-irradiated BCG. NF-κB and AP-1 activation was similar between the two cultures, suggesting that γ-irradiated BCG was a good as viable, replicating BCG at activating macrophages (Figure 4.5B). To confirm our finding, we analyzed cytokine mRNA expression in C57BL/6-derived BMDM cultured with either BCG or γ-irradiated BCG, using the Qiagen RT^2 Profiler™ PCR Array for Mouse Cytokines and Chemokines, and found no significant difference between the two stimuli in their ability to modulate mRNA expression of key pro- and anti-inflammatory cytokines (Figure 4.5C). Furthermore, at the protein level, production of TNF-α, IL-1β, IL-6 and IL-10 were similar between the two culture conditions (Figure 4.5D), reinforcing the fact that metabolically active, non-replicating BCG was a good as replicating BCG in producing key cytokines. Interestingly, the array data highlighted differences in mRNA expression for IL-17F, IL-27, IL-1a and IL-1rn. Analysis of the ability of BCG and γ-irradiated BCG to induce phosphorylation of IRF3 and IRF7 signaling pathways by Western Blotting showed no significant difference in the activation of either pathway between viable and γ-irradiated BCG (data not shown).

BCG provides protection in a LysMcre monocyte/macrophage knockout mouse

To further understand the role of monocytes/macrophages in BCG vaccination we utilized a LysMcre knockout mouse in which the M-Lysozyme gene has been deleted[14]. The M lysozyme gene is responsible for the production of M lysozyme by macrophage cells which

Figure 4.6

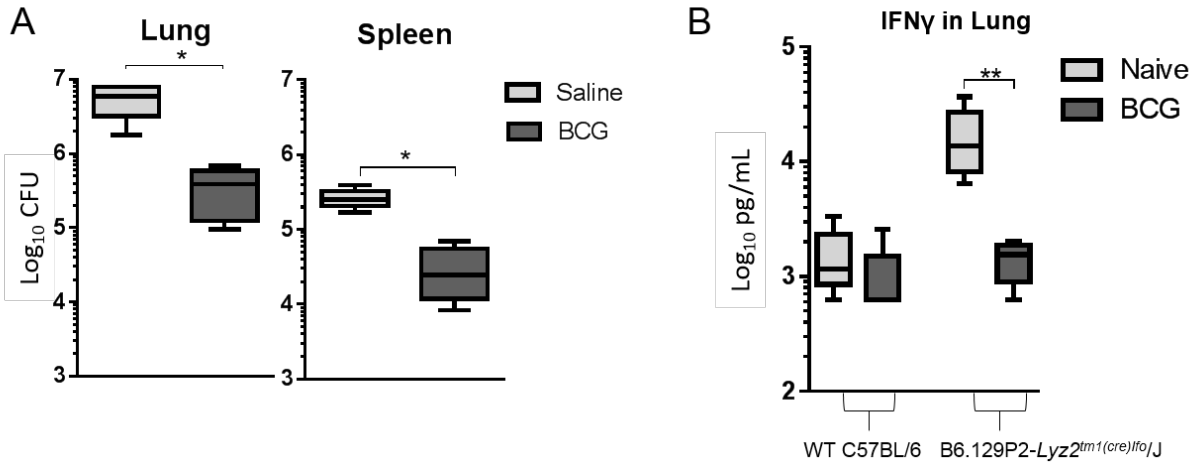

Figure 4.6
C57BL/6 LysMcre-KO mice, unable to differentiate myeloid cells into mature granulocytes and monocytes, retained the ability to reduce mycobacterial burden when vaccinated with BCG, seven days before infection (A). Concentration of IFN-γ in lung supernatants from 30 days after infection in WT C57BL/6 and LyzMCre-KO imce as determined by ELISA (B). N=5 mice per group *p≤0.05 **p≤0.05 (T-test).

can be used to identify macrophages in addition to its highly antimicrobial properties[37]. It is highly expressed during monocyte and macrophage development suggesting it plays a role in the process[38]. Mice deficient of the M lysozyme gene have been extensively characterized[15] and these data lend support to the notion that M lysozyme plays a role in differentiation and maturation[38,39]. These mice were vaccinated seven days prior to infection and CFU and IFN-γ determined 30 days later. Again BCG induced immunity resulted in a significant reduction in the mycobacterial burden in the lungs and spleen by 1 Log_{10} CFU (Figure 4.6A). These findings confirmed our prior observations in which Liposomal Clodronate (Encapsula NanoSciences LLC) was used to deplete macrophages (data not shown). This also validated previous findings by others that M lysozyme was not required to kill *M. tuberculosis*, despite lysozyme being a highly abundant antimicrobial peptide in pulmonary airways[40] as mycobacterial lipoproteins act as a lysozyme inhibitor[41]. IFN-γ concentrations were elevated in the lungs of knockout mice 30 days after infection when compared to wild type mice. However we observed that when vaccinated with BCG and mycobacterial killing was induced, the concentration of IFN-γ decreased to levels of those found in wild type mice (Figure 4.6B).

Discussion

In 2012 Aeras and the TuBerculosis Vaccine Initiative published a strategic blueprint for the next decade of tuberculosis vaccines, which called for research into alternative mechanisms for dealing with infection of *M. tuberculosis*[42]. The current studies demonstrate that there is still much to be learned about BCG induced mechanisms of action. It is interesting that BCG has been in use for so long without an adequate understanding of the types of immune responses it generates. Given that BCG is the most widely used vaccine in the world, administered to millions of infants annually, it is imperative to understand the effects that BCG has on the innate immune system while remaining viable and possibly proliferating. Although others have explored how BCG interacts with other innate immune cells, the majority have focused on adaptive immunity[43], although there is now a push to better understand the innate immunity. The finding that BCG induced protective immunity was independent of $CD4^+$ and $CD8^+$ T cells provided evidence to suggest that alternative mechanisms could play a role in killing *M. tuberculosis*. Furthermore, the finding that TNF-α was not an absolute requirement for BCG to induce protective immunity lead us to analyze these alternative mechanisms. Our studies subsequently demonstrated that BCG induced mycobacterial killing within 7 days of vaccination, in the absence of T cells, NK cells, and in mice lacking normal monocyte development, so the question must be asked; what was responsible for the early mycobacterial killing induce by BCG vaccination?

With the goal of long-term protection in mind, we looked to the immediate innate immune response to better understand ways of initiating strong immunity against *M. tuberculosis*. In measuring the immediate response we first examined the role of neutrophils. Neutrophils played a significant role, as their depletion during and after BCG vaccination significantly abrogated the initial clearance of mycobacteria. This was not surprising as neutrophils are the second highest cell in abundance only to lymphocytes in broncho-alveolar lavage of human TB

patients, suggesting that there are strong chemo-attractants that cause them to extravasate into the lung during infection[44]. There have also been many proposed roles for neutrophils in killing *M. tuberculosis*, and if subcutaneous BCG vaccination was able to increase the recruitment of neutrophils to the lungs it seems this may be a potential mechanism for the induction of mycobacterial killing so early after BCG vaccination[45,46]. Some other potential innate immune cells with a role are mucosal associated invariant T (MAIT) cells that behave very similarly to T cells, and play a role in mycobacterial infection and other bacterial pathogens[47]. In addition, *M. tuberculosis* infection induces migration of MAIT cells into the lungs suggesting a role for them in either a bactericidal role or in cell recruitment[48]. However, the full function of MAIT cells during tuberculosis infection is currently unknown. There are some data to suggest that MAIT cells contribute to an IFN-γ mediated clearance of pulmonary tuberculosis and that blockade of PD-1 may enhance this ability suggesting the potential for a future PD-1 based therapy[49], but it is unknown how this can influence innate immunity. Some studies have indicated that presence of MAIT cells are variable at best during infection with *M. tuberculosis* suggesting that their role in early clearance would be negligible[50].

Other innate immune cells that may play a role in mediating clearance of *M. tuberculosis* are innate lymphoid cells (ILCs). There are 3 types of ILCs and each have been reported to participate in various aspects immunity from allergy and asthma[51] to phagocytosis and antigen presentation[52]. Others have shown that subcutaneous BCG vaccination induced the recruitment of ILC's to the lungs of mice,[53] which supports our findings that indicated the presence of an immune response in the lungs seven days after BCG vaccination. There has also been some suggestion that type 3 ILCs mirror the function and cytokine production of $CD8^+$ T cells and may therefore be an early responder to BCG in the lungs[54]. Identifying the role and function of ILCs in relation to BCG vaccination and induced mycobacterial killing will be important if new mechanisms are to be utilized for vaccine development. However, all

indications are that ILCs are not recruited to the lungs until around 30 days post subcutaneous BCG vaccination suggesting they do not play a role during the early response studied here[53].

Another interesting aspect of the current studies is the ability of BCG to affect immunological change in the lungs so soon after vaccination. Our preliminary data suggest that this change may actually occur within a much shorter time window than 7 days. If live BCG is present in the lung it is no surprise that there is an immune response already mounting by the time the infection occurs in our model. It will be important to identify which parts of that immune response are responsible for mycobacterial killing, and if this immune response can be enhanced or applied to formulate an effective and long-lasting vaccine.

We focused our attention on the γ-irradiated BCG effect on macrophages, which is important for killing of *M. tuberculosis* and our data represented the first time that BCG had failed to induce a 1 Log_{10} CFU reduction. We asked if it was due to the inability of γ-irradiated BCG to trigger vital signaling pathways required to activate macrophages, but our data suggested that γ-irradiated BCG was as good as replicating BCG to activate key cytokine activation pathways. Targeted mRNA data showed differences in the IL-17F, IL-27, IL-1a and IL-1rn gene expression between BCG and γ-irradiated BCG stimulated BMDM, some of which have been shown by others to be up-regulated in BCG stimulated human peripheral blood mononuclear cell cultures[55]. IL-17F, produced by Th17 helper T cells, type 3 innate immune cells (ILCs), γδT cells, NK T cells and $CD8^+$ T cells, and by activated monocytes is a pro-inflammatory cytokine involved in host defense against bacterial and fungal infection[56,57]. The role of IL-17A in pulmonary tuberculosis has been shown by others to be required for protection and is associated with Th17 immunity[58]. The current set of studies identified IL-17F as a possible requirement for *M. tuberculosis* killing in the absence of a T cell response. Very few reports have focused on IL-17F or other members of the IL-17 family in relation to tuberculosis

or vaccination. IL-1β and IL-1RN were identified as major pro-inflammatory cytokines induced by BCG[59] and belong to the IL-1 cytokine family[60].

Of particular note, our finding that BCG vaccination in LyzMcre KO mice caused a significant reduction in CFU. These mice have almost all of the innate immune system depleted, with the only fully intact population being alveolar macrophages and partially intact peripheral blood monocytes and neutrophils[15]. As small amounts of peripheral myeloid cells remain in this model, it suggests that only a fraction of available myeloid cells are needed to induce this response. Future studies using these mice could identify how much of an immune response they are still capable of. This also suggests that the response may not be entirely cell mediated, as the immune cell populations are largely deficient. There is potential that the immune response observed is due to soluble factors or cytokines that are able to magnify the response signal so that large cell populations are not needed. It is also interesting that unvaccinated LyzMcre KO mice had such an increase in IFN-γ production along with an increase in CFU. This further supports that IFN-γ is not required for mycobacterial killing at this stage, as alveolar macrophages and IFN-γ would have been present together in ample supply. Altogether, our data support a model in which neutrophils are required during initial inoculation to instigate an inflammatory response. From there monocytes are recruited from the blood by neutrophils and stimulated by the BCG. Our flow cytometry data showed that there was an increase in these monocytes in the lung after BCG vaccination and must somehow work with alveolar macrophages to induce killing of mycobacterium. As IFN-γ was not required for mycobacterial killing, there could be a role for serine proteases which have been observed to induce killing of mycobacteria when produced by neutrophils[61] or monocytes[62,63]. Our data suggests a nonessential role for dendritic cells, basophils, eosinophils, and NK cells given that they are almost completely depleted in the spleens of the LyzMcre KO mice[15]. However future studies are needed to confirm this.

Our findings support the fact that when given to infants, BCG stimulates undefined changes to the immune system in different organs such as the lungs and its presence prevents tuberculosis in this cohort. If a BCG vaccine program is to be adopted in developed countries for its non-specific effects it would be sensible to better characterize these changes to further understand the mechanisms by which BCG is altering the immune system. In addition our NOD KO model along with the γ-irradiated BCG studies and *in vitro* data propose that conventional mechanisms of immune stimulation may not be critical to this immune response. Our results indicated that NOD2 dependent trained innate immunity was not a requirement for a reduction in mycobacterial lung burden within seven days. Recent reports have suggested that trained innate immunity was induced optimally through intravenous vaccination, in which BCG interacted with bone marrow cells[6]. These studies on trained innate immunity also required that BCG vaccination occur 30 days prior to infection to observe an effect[3,6]. As our study involving different routes of vaccination demonstrated, the induction of mycobacterial killing was not dependent on route, and occurred within seven days supporting the fact that our observations do not fit the paradigm of trained innate immunity and may suggest a mechanism prior to induction of trained immunity. This suggests the potential for an unconventional innate immune mechanism for killing *M. tuberculosis* that is induced by BCG early, or within seven days of vaccination. Our future studies plan to elucidate these mechanisms and hope to learn new ways to induce killing of *M. tuberculosis* through vaccination.

The development of a functional vaccine for tuberculosis is critical to reducing the spread and numbers of people who suffer from the disease. Many vaccines currently in trials focus on the stimulation of a strong T cell response[64], as that has been the method of success for other vaccines. However the research presented here opens the door for developing other types of immunity with a vaccine that may effectively reduce mycobacterial burden and potentially the mortality associated with active tuberculosis infection. The role of neutrophils in instigating an immune response to tuberculosis needs to be better characterized, and could

possibly lead to the discovery of alternative mechanisms to induce mycobacterial killing through vaccination.

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Chapter V

Characterizing the Monocyte Derived immune response to BCG vaccination

Introduction

BCG vaccination against *Mycobacterium tuberculosis* infection has been used for almost a century and is still used for millions of people worldwide[1,2]. An efficacy of 0 to 80% suggests that it is not able to provide long lasting protection against pulmonary tuberculosis infection[3]. Studies have indicated that it does induce T cell responses, but these do not correlate to protection against *M. tuberculosis*[4,5]. It is known that BCG can have a variety of effects on the innate immune system and currently, these non-specific effects have been classified as trained innate immunity[6–8], but many of these effects have gone uncharacterized. Trained innate immunity can take up to 30 days to become effective[9], and other studies show they are dependent on epigenetic reprogramming instigated through the NOD2 receptor signaling pathway[7]. Our previous studies have indicated that BCG can induce protective immunity against *M. tuberculosis* infection within 7 days, and in the absence of the NOD2 receptor suggesting the role of additional innate immune mechanisms stimulated by BCG. This study aims to better characterize the innate immune response within 7 days of BCG vaccination to better understand the mechanisms behind the ability of BCG to induce a reduction in mycobacterial growth. *In vitro* studies indicated that macrophages infected with BCG exhibit an increased capacity to kill BCG when infected again 2 days later. Past data have suggested that BCG is able to remain viable after vaccination and can be found in the lungs and spleen[10]. As BCG needs to be alive to induce protection against *M. tuberculosis* this suggests a potential mechanism by which BCG can stimulate innate immunity in the lungs of mice. Using a recombinant BCG expressing TdTomato, we found live BCG in the lungs of mice 2 days after subcutaneous vaccination. Past flow cytometric analysis has revealed the increased presence of cells in the lungs of BCG vaccinated mice expressing macrophage phenotypic markers in mice. Using a 20 color flow cytometry panel and automated clustering analysis, we were able to

better characterize this population of cells and found that macrophages from the lungs of vaccinated mice have a predisposition for the M1 phenotype. Through this study we aim to better understand the role that these macrophage cells could play in reducing mycobacterial growth and demonstrate their potential for a new vaccine or therapeutic.

Materials/Methods

Mycobacteria: *M. bovis* BCG Pasteur (TMC#1011) was grown in Proskaur and Beck (P&B) medium containing 0.01% Tween® 80 until mid-log growth phase. It was then stored at -80˚C f. *M. tuberculosis* H37Rv (TMC# 102) was grown in P&B medium as a pellicle and then transferred to liquid cultures of P&B medium containing 0.5% Tween® 80 in roller bottles. The cultures were passaged three times through the same media grown and finally stored at -80˚C. Colony forming units (CFU) and viability of all cultures was assessed after freezing at 95% viability. The genome of the BCG Pasteur used was sequenced (Dr. M. Strong, Center for Genes, Environment and Health, National Jewish Health and University of Colorado, Denver, CO) and was >99.99% similar to the published reference genome[11].

BCG vaccination: BCG was diluted to a concentration of $5x10^5$ CFU/mL in endotoxin free PBS and sonicated to disrupt clumps. BCG was loaded into 1 mL syringe and 100 µL was inoculated to deliver $5x10^4$ CFU subcutaneously per mouse. Inoculum was plated on 7H11 agar (Difco, BD Biosciences, San Jose, CA) to check the accuracy of dose. In some studies mice received BCG that was exposed to 2.4mRads of γ-irradiation from Cs^{137} source (Irradiation Services Laboratory, CSU) that allows it to remain metabolically active, but not to replicate as determined by plating (data not shown).

MPL/DDA/MDP vaccination

Lipid A, monophosphoryl (MPL) (Sigma Aldrich, St. Louis, MO) was used at a concentration of 25 µg/animal. MPL was prepared by diluting to a concentration of 1 mg/mL in sterile 0.5% triethanolamine (Sigma Aldrich, St. Louis, MO). Solution was heated to 65C and sonicated for dispersal. The pH was then adjusted to 7.4 with 1.0M HCL. Dimethyldioctadecylammonium bromide (DDA) (Sigma Aldrich, St. Louis, MO) was used at a concentration of 250 µg/animal. DDA was prepared as a 5mg/mL solution in endotoxin free water. N glycolyl Muramyl di peptide

(MDP) (InvivoGen, San Diego, CA) was used at a dose of 10 µg/animal and was prepared in vaccigrade water (InvivoGen, San Diego, CA). Before vaccination, all components were mixed at appropriate concentration and delivered subcutaneously in the scruff of the neck at a volume of 100 µL/mouse.

In vitro stimulation

RAW Blue™ macrophage reporter cells (InvivoGen, San Diego, CA) were cultured in complete RPMI (RPMI-1640 with essential and nonessential amino acid, penicillin, streptomycin, HEPES (Sigma Aldrich, St. Louis, MO), sodium pyruvate (Sigma Aldrich, St. Louis, MO), L-glutamate (Sigma Aldrich, St. Louis, MO), and 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO)RPMI (Life Technologies, Carlsbad, CA) in 24 well tissue culture plates (Corning Incorporated, Corning, NY) and stimulated with either live BCG Pasteur or γ-irradiated BCG Pasteur at a multiplicity of infection (MOI) of 5:1. Cells were treated with 100 ng/mL Hygromycin for 6 hours to kill all BCG and then cells were rested overnight. Cells were then stimulated with Hygromycin resistant BCG and incubated for 24 hours. Cells and supernatnant were plated on 7H11 agar plates + 50 µg/mL hygromycin to determine CFU.

Creation of TdTomato BCG

BCG Pasteur was grown according to methods previously listed. Frozen stocks were thawed and grown in 7H9+OADC + Tween80 until an OD600 of 0.8 to 1 was reached. Methods for transformation have been previously described[12]. Briefly, BCG was pelleted by centrifugation and then incubated in 5 mL 10% glycerol solution for 1 hour to make competent for electroporation. 5 µL ptdtomato vector (Clontech/Takara, Kusatsu Japan) was combined with 400 µL BCG and put into a 0.2cm electrode gap cuvette and incubated on ice for 10 minutes. The cuvette was pulsed at 2.5 kV, 25 µF, with the resistance set to 1000 Ω and the sample was recovered from the cuvette and diluted up to 1 mL in 7H9+OADC+Tween. Samples were then

plated on 7H11 plates + 50 µg/mL hygromycin (Sigma Aldrich, St. Louis MO) at 37C for 21 days to select for positive colonies. A single colony was then chosen nd grown in 7H9+OADC supplemented with hygromycin.

Flow Cytometry

Single cell suspensions were prepared from lungs that were minced with a razor blade before being incubated at 37˚C in 0.5% Liberase (Sigma Aldrich, St. Louis, MO) incomplete RPMI solution (Life Technologies, Carlsbad, CA). After 45 minutes lung pieces were passed through a 70-µm nylon cell strainer (Falcon, Corning, Durham, NC) and the single cell suspension collected by centrifugation. The pelleted cells were resuspended in 2 mL ACK red blood cell lysis buffer (Life Technologies, Carlsbad, CA) and incubated at room temperature for 5 minutes before 10 mL of complete RPMI (RPMI-1640 with essential and nonessential amino acid, penicillin, streptomycin, HEPES (Sigma Aldrich, St. Louis, MO), sodium pyruvate (Sigma Aldrich, St. Louis, MO), L-glutamate (Sigma Aldrich, St. Louis, MO), and 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO) was added to stop the reaction. Cells were centrifuged and stored in complete-RPMI on ice while cells were counted and the concentration adjusted to $2x10^6$/mL in complete-RPMI. Cells were pelleted and incubated for 20 minutes at 4˚C in 2.4G2 hybridoma supernatant (Fcγ blocking antibody; ATCC® HB-197) diluted in PBS containing 5% FBS 0.01% NaN3 (FACS buffer). Cells were washed by centrifugation and resuspended in clean FACS buffer before being pelleted and stained with the fluorochrome-conjugated antibodies (Supplemental Table 5.1). Cells were washed again with FACS buffer before being analyzed on a BD FACS Canto II flow cytometer or a Cytek Aurora flow cytometer. Data was analyzed using FlowJo software (FlowJo, LLC, Ashland OR).

Cytokine analysis

Lung homogenates from infected mice were pelleted and the cell free supernatant was taken to quantify cytokine concentrations.Cytokine quantification in cell culture supernatants following stimulation was performed by enzyme-linked immunosorbent assay (ELISA). ELISA kits (Affymetrix/eBioscience INC San Diego, CA) for the following cytokines: TNF-α, IL-1, and IL-6 were used following the manufacturer's protocol. The color intensity in wells of 96 well plates was then read using the Ultramark™ Microplate Reader (BioRad, Hercules, CA). A standard curve was also used with each assay to determine cytokine concentration in pg/mL.

Tissue culture and Real-time PCR analysis:

RAW Blue macrophage cells and MHS Macrophage cells were grown in tissue culture and brought to a concentration of $1.0x10^6$ macrophages per well in a 12 well plate for stimulation for 72 hours with 5:1 MOI of BCG or irrBCG either directly or indirectly using costar transwell 12 mm inserts with 0.4μm pore size (Corning, Corning NY). Supernatants and total RNA was harvested at each time point. RNA was isolated with TRIZol® (Invitrogen), and quantity and purity was assessed using a Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific). RNA was then converted into cDNA using an iScript cDNA synthesis kit (BioRad, Hercules, CA), and RT-PCR was performed using the Qiagen RT^2 Profiler™ PCR Array for Mouse Cytokines and Chemokines on a CFX Connect™ Real-Time PCR Detection System (BioRad). Primers for iNOS have been previously published[13]. Upon reinfection, cells in tissue culture were infected at an MOI of 5:1, incubated for 24 hours and then 0-3 dilutions were plated on 7H11 plates. The plates were incubated at 37C for 12-18 days until colonies formed and were counted to calculate CFU from original sample.

tSNE and FlowSOM analysis

t-Distributed Stochastic Neighbor Embedding (tSNE) and FlowSOM (self-organized map) analysis were conducted using FlowJo and FlowSOM protocol was performed using the

previously published protocol[14]. Live cells from singlets gate were identified using viability dye 510 (BD Biosciences, San Diego CA). The $CD45^+$ cell population was then gated and the resulting populations were run through the downsample plugin in FlowJo to reduce population sizes to 10,000 events. All samples were concatenated into a single file and then analyzed through forward scatter (FSC-A) and side scatter (SSC-A) to identify granulocyte populations (Figure 5.3A). These populations were then run through the tSNE and FlowSOM® plugins available in FlowJo using all compensated parameters excluding channels for live/dead and CD45. tSNE was run at 1000 iterations with a perplexity of 20 and Eta of 200. FlowSOM set up 8 meta clusters and was first performed on whole concatenated file. The same analysis was subsequently performed on the vaccinated and naïve samples and results were mapped to the first tree, ensuring comparability between results. Eight meta clusters were designated after a few trials determined this provided optimal population resolution.

Heatmap and statistical analysis

Heatmaps were created and data were analyzed using the statistical tests as described using R software (R foundation). Data from designated experiments were Log_{10}-transformed prior to analysis. Graphs were prepared using Graph Pad Prism 7 (GraphPad software, La Jolla, CA).

Results

BCG infected macrophages are present in mouse lung after BCG vaccination

As BCG can induce killing of mycobacteria in the lung within seven days, we wanted to determine if BCG could be found in the lungs of subcutaneously vaccinated mice. Our past work has demonstrated that BCG induced a change in innate immune cell populations in the lung, and that BCG needed to be alive to induce protective immunity. From here we hypothesized that the monocyte recruitment to the lungs after BCG vaccination was due to the presence of BCG in the lungs. To this end, we utilized a strain of recombinant BCG producing TdTomato to look for the presence of live BCG in the lungs after subcutaneous vaccination. It has been previously shown that viable BCG can be found in the lungs 30 and 42 days post BCG vaccination[10] but it has not been determined how early after vaccination it is present. Through this method we found that two days after vaccination, BCG was already present in the lungs and was still capable of producing TdTomato protein (Figure 5.1A). However, the concentration of $CD11b^+$ cells carrying BCG were too low to be isolated by FACS and used for downstream application. We were unable to detect live BCG in the lymph nodes or spleen through our flow analysis (data not shown). Cells from whole lung were plated, stimulated with BCG, and analyzed for TNF-α, IL-1β, and IL-6 production via ELISA which determined that the cells in the lungs of vaccinated mice do not produce more cytokine than naïve mice after BCG vaccination (Figure 5.1B).

Monocytes are stimulated by BCG vaccination

We have previously demonstrated that subcutaneous BCG vaccination induced monocyte recruitment to the lungs. To further understand the immune response to BCG we characterized how this immune response was generated. We first looked into ways to artificially stimulate an influx of monocytes into the lungs. Previous studies have shown that BCG induced trained innate immunity can lead to protection against non-mycobacterial diseases. We sought to

Figure 5.1

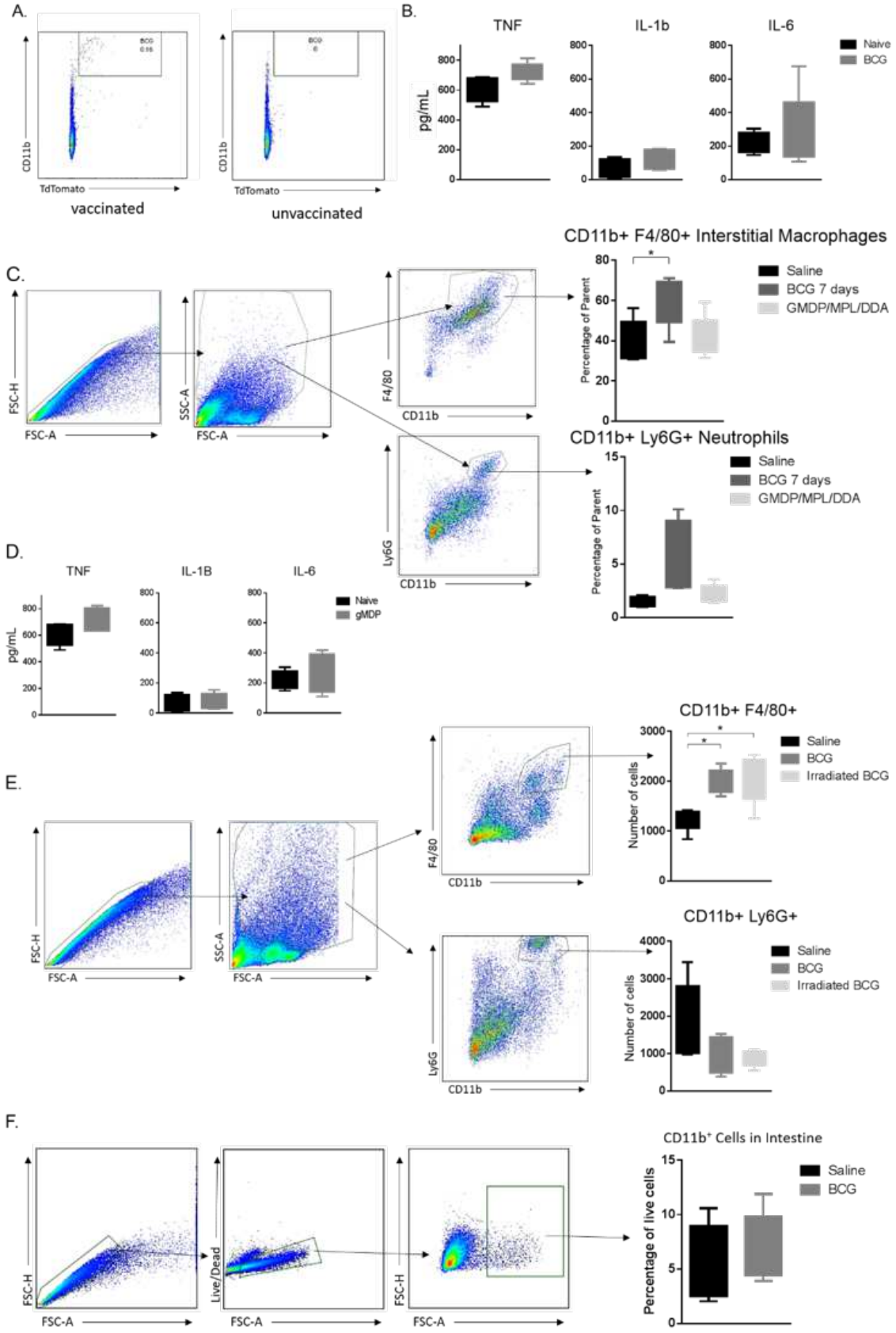

A.
CD11b
TdTomato
vaccinated
unvaccinated
B.
TNF
IL-1b
IL-6
pg/mL
Naive
BCG
C.
FSC-H
FSC-A
SSC-A
F4/80
CD11b
Ly6G
CD11b+ F4/80+ Interstitial Macrophages
Percentage of Parent
Saline
BCG 7 days
GMDP/MPL/DDA
CD11b+ Ly6G+ Neutrophils
D.
TNF
IL-1B
IL-6
pg/mL
Naive
gMDP
E.
CD11b+ F4/80+
Number of cells
Saline
BCG
Irradiated BCG
CD11b+ Ly6G+
F.
Live/Dead
CD11b⁺ Cells in Intestine
Percentage of live cells
Saline
BCG

Figure 5.1
Mice vaccinated with $1x10^6$ CFU BCG expressing TdTomato are observed to have TdTomato positive cells in their lungs as soon as 2 days after vaccination compared to naïve mice (A). Lungs of BCG vaccinated mice were forced into single cell suspension and cultured overnight. Cells were left unstimulated or infected with BCG (5:1 MOI) and cytokine production determined by ELISA (B). No statistical significance was determined between vaccinated and unvaccinated mice (N=5). Mice were vaccinated with either $5x10^4$ CFU BCG Pasteur or a mixture of glycolyl muramyl dipeptide (GMDP), monophosphoryl lipid-A (MPL), and dimethyldioctadecylammonium liposomes (DDA). Only vaccination with BCG allowed for recruitment of macrophages into the lungs of mice. Neutrophil numbers were also elevated though not statistically different (C) (N=5). Lungs from GMDP/MPL/DDA vaccinated mice were forced into single cell suspension and stimulated with GMDP/MPL/DDA and TNF-α, IL-1β, and IL-6 production was measured through ELISA (D). No statistical significance was determined between vaccinated and unvaccinated animals (N=5). Mice vaccinated with $5x10^4$ CFU BCG Pasteur and $5x10^4$ γ-irradiated BCG Pasteur show the same influx of macrophage cells into the lung 7 days after vaccination. This trend was not noticed with neutrophils (E).

recreate the observed monocyte influx without live BCG, but rather through PRR activation, using trained innate immunity. Trained innate immunity is instigated primarily through activation of the nucleotide oligomerization domain 2 (NOD2) receptor[7]. To this end, we formulated the TLR-4 agonist monophosphoryl lipid-A (MPL), and dimethyldioctadecylammonium liposomes (DDA) adjuvant along with a NOD2 agonist glyoclyl muramyl dipeptide GMDP. GMDP is known to potently activate the NOD2 receptor[15] and past studies have suggested that MDP can induce monocyte recruitment to the lungs to protect against infection[16]. MPL/DDA was chosen for its ability to incite recruitment and activation of monocytes to the injection site[17]. Mice were inoculated with GMDP/MPL/DDA and 7 days later the lungs were assessed for recruitment of interstitial macrophages and neutrophils after 7 days (Figure 5.1C). The percentage of $CD11b^+$ $F4/80^+$ macrophages in the lungs of GMDP/MPL/DDA inoculated lungs was not significantly different from saline-treated mice, while they were significantly increased in BCG inoculated mice. To determine if NOD2 stimulation could lead to lung monocyte activation, lung cells from GMDP/MPL-DDA inoculated mice were isolated and plated in 24 well tissue culture plates. Cells were stimulated with BCG and assessed for TNF$-\alpha$, IL-1β, and IL-6 expression. Cytokine production in the GMDP/MPL/DDA inoculated mice was not statistically different from saline treated mice (Figure 5.1D). Additionally, we looked at the ability of $\gamma-$irradiated BCG (BCG fully intact but unable to replicate) to induce changes in the cell populations in the lungs. We found that Irradiated BCG and live BCG both were able to induce a similar change in macrophage cell population, but no change in neutrophil populations was observed (Figure 5.1E). Finally, we evaluated the immune response to BCG vaccination at a different mucosal site by analyzing the gut mucosal surfaces via flow cytometry for increased monocyte recruitment. BCG did not increase recruitment of monocytes into the gut after 7 days (Figure 5.1F).

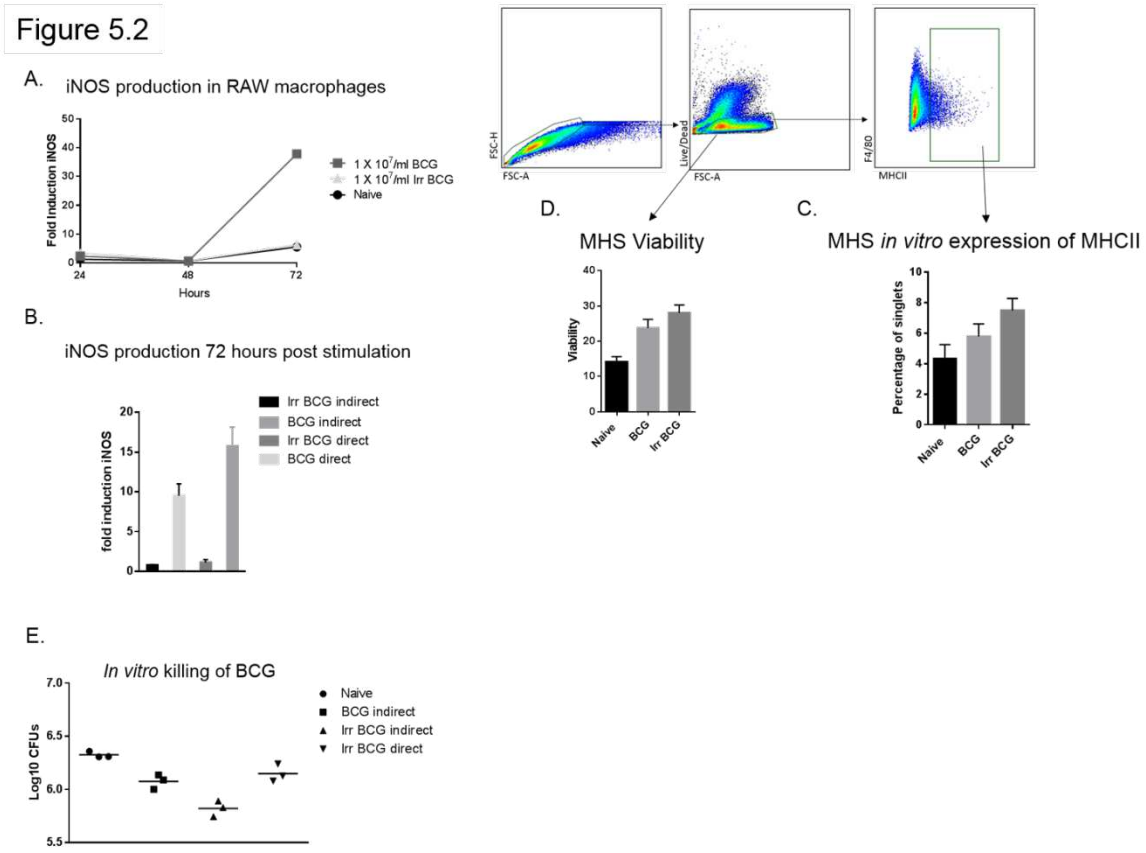
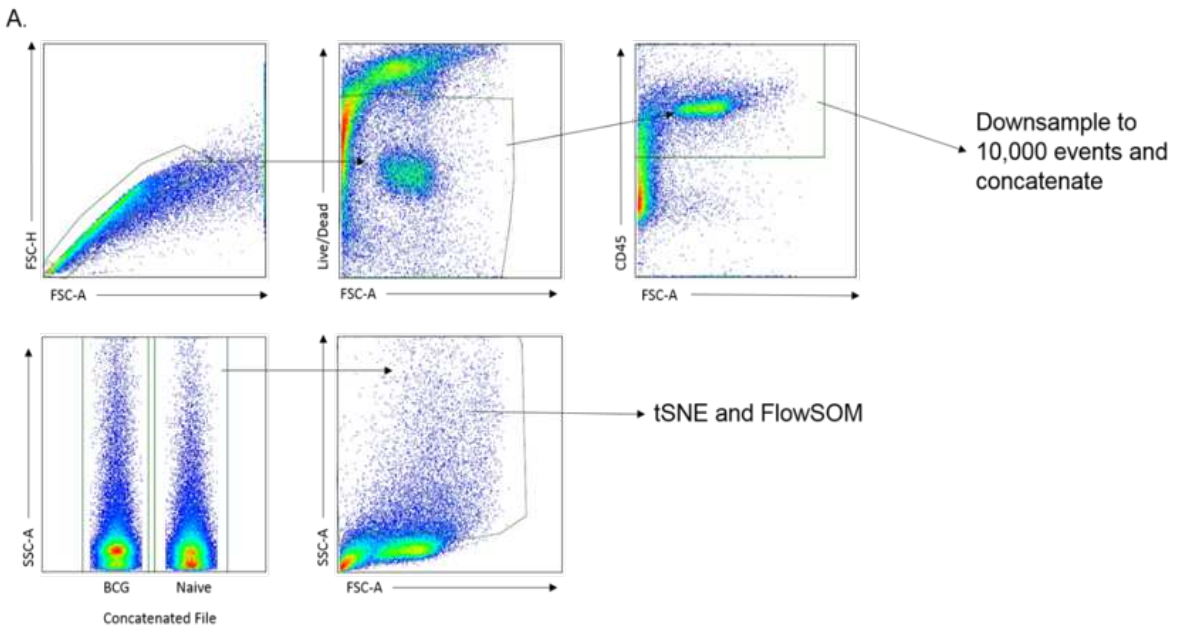


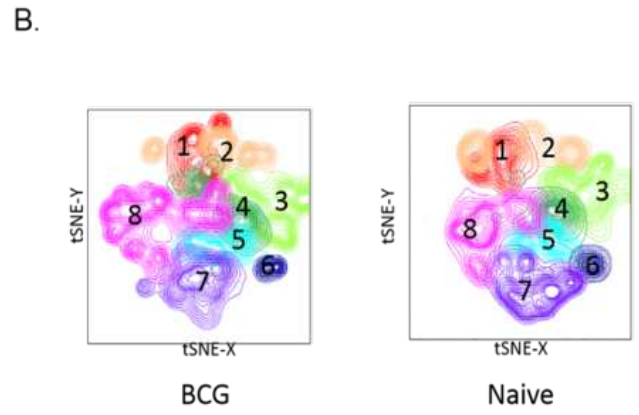
Figure 5.2
RAW macrophage cells in tissue culture plates at $1.0x10^6$ cells per well and stimulated at an MOI of 5:1 with BCG, γ-irradiated BCG, or unstimulated (Naïve) for 24, 48, and 72 hours. Cells stimulated with live BCG show a 40 fold induction of iNOS production compared to γ-irradiated BCG stimulated and Naïve cells (A) (N=3). MHS macrophage cells in tissue culture plates at $1.0x10^6$ cells per well and stimulated either directly with live BCG or indirectly with live BCG in a 12mm transwell insert with 0.4 μm pores above the cells. BCG does not need to directly stimulate the cells to induce a 10 fold increase in iNOS production (B) (N=3). MHS cells stimulated with BCG either directly or indirectly in a transwell system increase MHCII expression (C) and also increase viability after 3 days (D) (N=3). MHS cells stimulated with BCG in a transwell insert (BCG indirect), γ-irradiated BCG in a transwell insert (Irr BCG indirect) or γ-irradiated BCG directly (Irr BCG direct) show increased killing of mycobacterium when infected 3 days after stimulation (E) (N=3). Transwell inserts have pores 0.4 uM in diameter which allow transfer of soluble components, but no transfer of bacteria. *=$p<0.05$ using the kruskal wallis test.

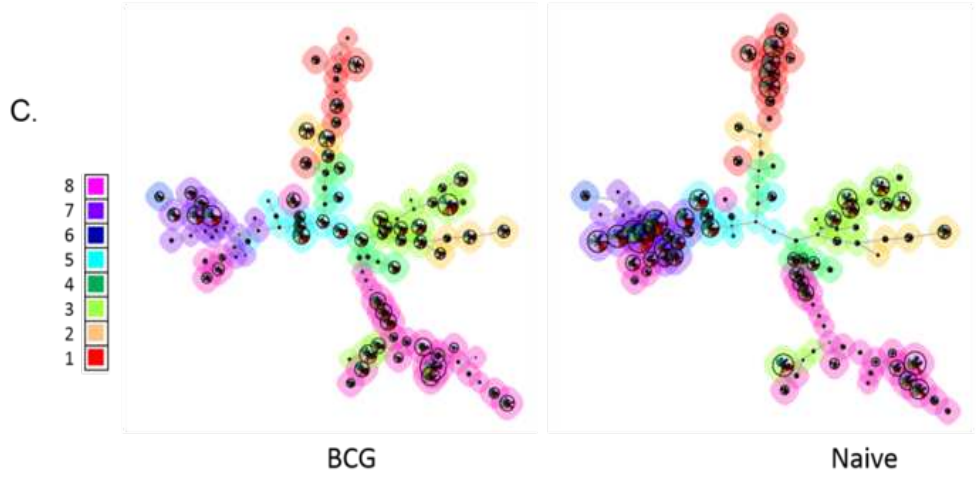
Macrophage cells stimulated by BCG show increased production of iNOS and increased *mycobacterial killing properties.*

It has been demonstrated in the past in studies characterizing trained innate immunity[7,8,18] that previous exposure to a NOD2 agonist can increase the killing properties of macrophages. However, these studies were carried out over a 7 day period using NOD2 agonists to characterize NOD2 dependent training of monocytes whereas we were interested in the more rapid response of activated macrophages. It is known that nitric oxide can play a role in controlling mycobacterial growth in the mouse model[19] and nitric oxide production can be induced through the enzyme inducible nitric oxide synthase[20] (iNOS or NOS2). To determine the potential role of iNOS in BCG induced mycobacterial killing, RAW macrophage cells were stimulated with viable BCG or γ-irradiated BCG for up to 72 hours to assess changes in gene transcription of iNOS. At 24 and 48 hours after initial stimulation, there were no significant differences in iNOS production between groups, but at 72 hours after stimulation with live BCG there was a 40 fold induction of iNOS whereas the γ–irradiated BCG and naïve groups did not show this (Figure 5.2A). Next we assessed the ability of BCG to induce iNOS in other macrophage cell lines more closely related to lung resident macrophages. MHS macrophages were evaluated for their response to soluble components produced by BCG as well as direct stimulation. MHS macrophages were cultured and stimulated either directly with BCG, or indirectly using microporous inserts with 0.4 μM size pores, where BCG was retained only in the insert, unable to reach the cells. Seventy two hours later RNA was extracted from the cells in the lower wells for analysis by real time PCR. MHS macrophage cells directly stimulated with BCG showed a 15 fold induction of iNOS. We also found that having BCG present in the insert, but not directly stimulating the cells also led to about a 10 fold induction of iNOS, suggesting the release of soluble factor(s) by BCG that activated macrophages (Figure 5.2B). After 72 hours in

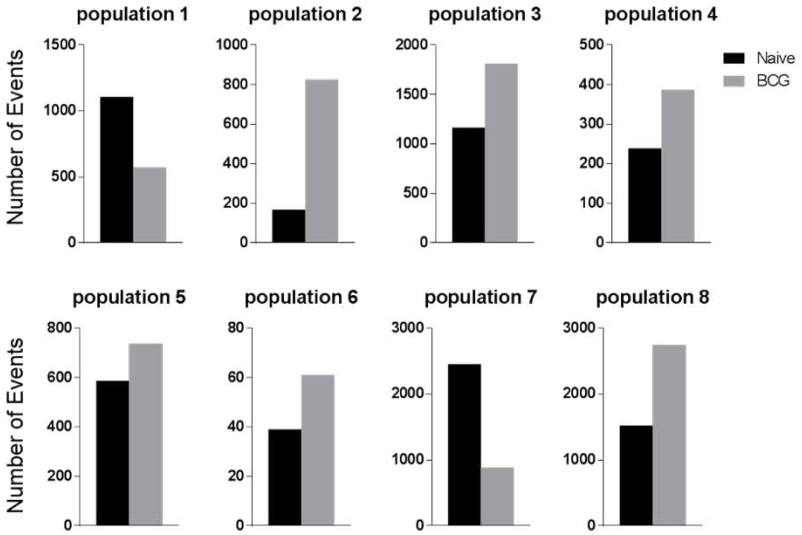
Figure 5.3


A.
FSC-H
FSC-A
Live/Dead
FSC-A
CD45
FSC-A
Downsample to
10,000 events and
concatenate
SSC-A
BCG
Naive
Concatenated File
SSC-A
FSC-A
tSNE and FlowSOM

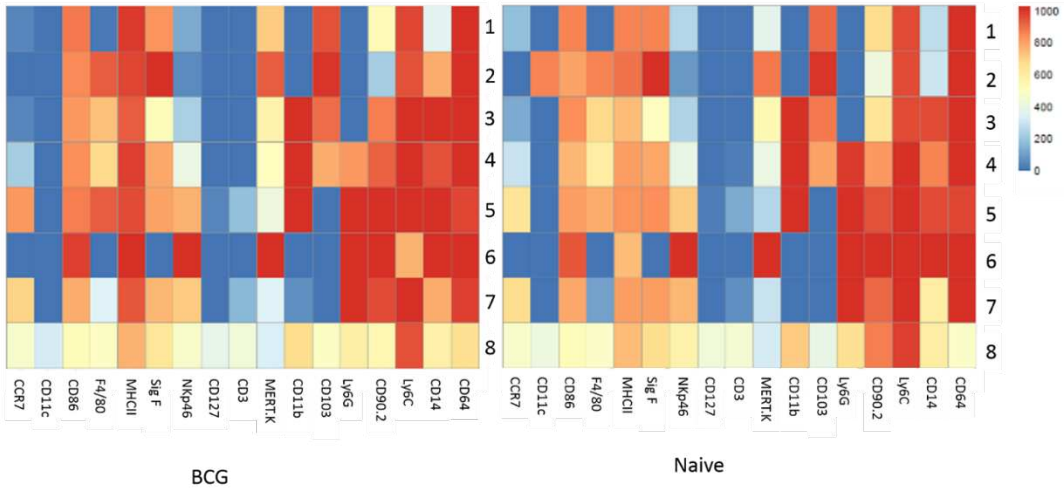

B.
tSNE-Y
tSNE-X
1
2
3
4
5
6
7
8
BCG
tSNE-Y
tSNE-X
Naive


C.
8
7
6
5
4
3
2
1
BCG
Naive

D.


population 1
population 2
population 3
population 4
population 5
population 6
population 7
population 8
Number of Events
Naive
BCG

E.


CCR7
CD11c
CD86
F4/80
MHCII
Sig F
NKp46
CD127
CD3
MERT.K
CD11b
CD103
Ly6G
CD90.2
Ly6C
CD14
CD64
BCG
Naive

F.

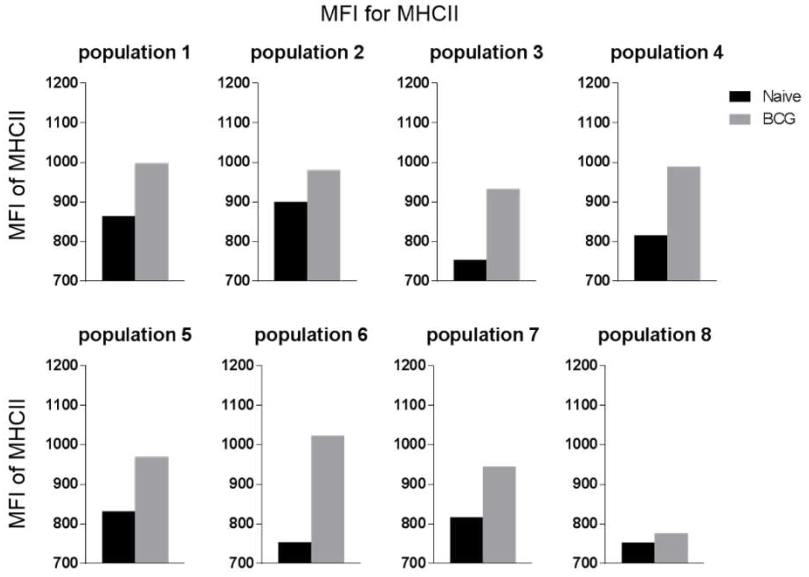

MFI for MHCII
population 1
population 2
population 3
population 4
population 5
population 6
population 7
population 8
MFI of MHCII
Naive
BCG

Figure 5.3
Lung cells taken from BCG vaccinated and naïve mice were analyzed by flow cytometry using the gating strategy depicted (A). tSNE charts of the 8 populations generated through FlowSOM analysis that display some of the differences between the lungs of BCG vaccinated and naïve mice (B). Minimal spanning tree (MST) created by FlowSOM depicting the relationship between the 8 populations generated by the FlowSOM algorithm (C). The number of events in each population created by FlowSOM (D). Heatmaps displaying the phenotypic characteristics of each population generated by FlowSOM in terms of mean fluorescence intensity (MFI) highlighting the differences between the populations in the lungs of BCG vaccinated and naïve mice (E). The difference in the mean fluorescence intensity (MFI) of MHCII between lungs of vaccinated and unvaccinated mice (F).

tissue culture, BCG infection not only increased MHCII expression (Figure 5.2C), but also boosted survival of MHS macrophages (Figure 5.2D). Interestingly, γ-irradiated showed just as much MHC II stimulation and viability suggesting activated macrophages, but not iNOS expression (Figure 5.2B and D). This supports what we have seen *in vivo*, as Irr BCG did induce an influx of MHCII macrophages into the lungs, but no killing of *M. tuberculosis* was observed. Only live BCG increased iNOS suggesting this as a possible mechanism for killing *in vivo*. An increase in cell viability also suggested increased activation (Figure 5.2D). Past studies have shown that live BCG and γ-irradiated BCG are very stimulatory for monocytes[21]. We decided to test the effect of indirect stimulation, that is culturing cells in a well below a transwell insert with either live BCG or γ-irradiated BCG in it. As was the case during *in vivo* BCG vaccination, the live BCG was anticipated to shed proteins or produce soluble factors that could stimulate cells, but there was no contact between the bacteria and the cells. MHS macrophage cells that had been stimulated with live BCG or γ-irradiated BCG in a transwell system were infected with BCG and incubated 24 hours. The cells were then lysed and BCG was plated to count remaining CFU. Cells that had been stimulated by live BCG in the transwell (indirectly) had fewer CFUs of BCG when plated compared to non-stimulated cells (Figure 5.2E). Interestingly we found that cells stimulated indirectly with γ-irradiated BCG indirectly were more efficient at killing mycobacteria than those stimulated with γ-irradiated BCG directly (Figure 5.2E). We hypothesize that this may be due to activation of PRRs on the surface of the cells vs PRRs that are intracellular. Overall the data suggested that BCG can stimulate alveolar macrophages to produce iNOS and nitric oxide and that it can also stimulate increased killing of mycobacteria. However more tests are needed to link these results together.

Flow Cytometry analysis shows an increase in innate immune cells in the lungs after BCG vaccination

To better analyze the populations of cells present in the lungs after BCG vaccination we utilized a 20 color flow cytometry panel (**supplementary table 5.1**). Mice were vaccinated with BCG Pasteur and lungs were taken for analysis 7 days later. Populations analyzed through tSNE and FlowSOM were identified using the gating strategy shown (Figure 5.3A). To perform tSNE and FlowSOM analysis, samples were first downsampled to 10,000 events then concatenated, and tSNE and FlowSOM was performed on the single resulting file. tSNE analysis represents the phenotypic shift of each population found through the flowSOM clustering algorithm (Figure 5.3B). Changes and relationships between these populations are shown through minimal spanning trees (MST) (Figure 5.3C). FlowSOM analysis showed that not only were there differences between BCG vaccinated and unvaccinated mouse lungs in terms of cell populations present (Figure 5.3D), but there was also a difference in the mean fluorescence intensity (MFI) of the markers for MHCII on the cell surface between cells in these groups (Figure 5.3E). The increase in the MFI of the MHCII marker in the lungs of BCG vaccinated mice for the populations analyzed can be represented graphically (Figure 5.3F).

Discussion

Our previous studies have concluded that although BCG does induce trained innate immunity, it is not an absolute requirement for BCG mediated protection against *Mycobacterium tuberculosis* in a mouse model. As we demonstrate here, innate immunity to tuberculosis is inducible within seven days, and it is possible that this immune response was due to changes that occurred in monocyte populations after subcutaneous BCG vaccination. Of interest in this study, was the fact that in an *in vitro* culture system, we demonstrated that BCG stimulated increased mycobacterial killing in macrophages through the release of soluble factors (Figure 5.2B). Although mice were vaccinated with $5x10^4$ CFU live BCG, only a small fraction of live BCG reach the lung and stimulate an immune response (Figure 5.1A). As BCG is an intracellular pathogen, only a small number of cells in the lung were infected with BCG, yet there was an effective immune response mounted to combat pulmonary *M. tuberculosis* infection. Because such a small amount of BCG are able to stimulate a large immune response, we have hypothesized that cell signaling factors that can amplify a signal are involved in killing *M. tuberculosis* at this early stage of infection. There is also potential that BCG can induce mycobacterial killing through multiple mechanisms, but further evidence is needed to support this.

Our attempt to increase cell recruitment to the lung by inoculation with MDP showed that a wider activation of PRRs was required for a suitable immune response. γ-irradiated BCG, which carries multiple PRRs, can deliver multiple signals, but when vaccinated before infection, γ-irradiated BCG was not able to induce a significant reduction in the mycobacterial burden (Chapter 3). Altogether we now hypothesize that while γ-irradiated BCG stimulated the necessary cell recruitment, live BCG is required for adequate immune priming for effective clearance of mycobacteria. Overall this suggests that activated cells are required for reduction in mycobacterial burden, and that the level of activation can only be achieved by live, proliferating BCG. However, the cells responsible for this killing or controlling the growth of *M.*

tuberculosis remain unknown. Recent attempts have been made to identify more fully the populations of macrophage cells present in the lungs arriving at the conclusion that there are distinct populations of interstitial macrophages conserved across a variety of tissues[22]. Our previous work has demonstrated that BCG vaccination provides protection against infection with *M. tuberculosis* even in M lysiozyme (LysM) gene deficient mice and therefore devoid of circulating macrophages. These recently identified populations of interstitial macrophages are thought to arise from monocyte populations[22] which would not have been present in a LysM knockout. However, the alveolar macrophage populations are present[23], as they are embryonically established, capable of self renewal[24,25] and fully functional without input from circulating monocytes[26]. This suggests a potential for alveolar macrophages to be critical for BCG mediated protection against *M. tuberculosis.* Others have reported that in the early stages of M. tuberculosis infection, alveolar macrophages are the primary target for infection. When infected these cells re-localize to the interstitium and from there the infection can disseminate to other immune cells[27]. It is possible that interstitial macrophages play a role in clearance of mycobacteria with multiple populations of interstitial macrophages having been identified in the lungs of mice[28] each with a diverse set of functions that are yet to be fully understood. It is known that exposure to bacteria can activate these macrophages[29] and are capable of altering the functions of other innate immune cells[30]. However, our previous results using LysM knockout mice suggested that populations of monocyte-derived macrophages were non-essential to BCG mediated protection shifting the focus to alveolar macrophages (Chapter 3 Figure 6).

Our findings that iNOS production increased in MHS macrophages after stimulation with live BCG and not γ-irradiated BCG also supported the hypothesis that alveolar macrophages were involved in the killing of *M. tuberculosis*. As previously stated, nitric oxide is required for controlling mycobacterial infection in the mouse model[19]. We have demonstrated that BCG can induce macrophages to upregulate iNOS production, and therefore nitric oxide, through

releasing soluble components with direct stimulation a non-requirement. We have also demonstrated here that BCG can be found in the lungs of mice within 2 days after vaccination to produce soluble components. This has potential to translate to human as TB patients have been found to exhibit increased iNOS production in granulomas[31], and in alveolar macrophages[32]. Interestingly, others have shown a link between NOD2 activation and nitric oxide production in human macrophages[33] further strengthening the link between BCG trained innate immunity. However, we have found in the past that BCG still induced mycobacterial killing in mice lacking the NOD2 receptor. Although this did rule out a role for NOD2 and trained innate immunity in BCG induced mycobacterial killing, there is a strong likelihood that nitric oxide is involved.

The analysis by FlowSOM suggested that there were changes in granulocyte cell populations in the lungs of BCG vaccinated mice, when compared to naïve mice. Most notable was the increase in mean fluorescence intensity (MFI) of the MHCII marker that all of the populations exhibited, suggesting an elevated activation state in readiness to stimulate adaptive immunity. BCG vaccination induced increased expression levels of the MHCII marker in these cell populations in addition to increasing recruitment of these cells. Macrophages can take on two distinct polarizations, M1 or M2[34,35]. An increase in MHCII expression is an indication that the macrophages in BCG vaccinated mice might be taking on a predisposition to M1 phenotype instead of M2. Classic M1 macrophage phenotype is characterized by increased expression of MHCII and costimulatory molecules like CD86[36,37]. It has long been known that stimulation with interferon-γ can increase MHCII expression on monocytes through Jak-STAT activation[38]. It has also been commented that LPS activated macrophages take on this predisposition suggesting alternative mechanisms for M1 polarization[39]. It is then possible that the overall predisposition of the innate immune system in the lungs is redefined upon BCG vaccination with a shift to M1 polarization. Not only would this increase cytotoxicity of macrophages upon stimulation with IFN-γ[39], but it may permit the macrophages to resist colonization by

mycobacteria resulting in the decreased growth observed in BCG vaccinated animals. There is also evidence that LPS stimulation and tolerance in macrophages leads to a hybrid polarization state after recovery during which macrophages have increased ability to be pro-inflammatory and microbicidal[40]. Pathways such as this could also contribute to the anti-mycobacterial response after BCG vaccination

Our experiments have demonstrated that BCG vaccinated mice have macrophage-like cells infiltrating into the lung. As shown in Figure 5.3 C and D, population 5 was more prevalent in vaccinated mice and also showed an increased MFI for CCR7 suggesting that such cells are being recruited to the lung. It has been suggested that surface expression of CCR7 is common of M1 macrophages[41], and this supports our hypothesis. Population 2 in BCG vaccinated mice had increased events compared to unvaccinated mouse lungs. These cells were positive for the macrophage markers CD64, MERTK, Siglec F, MHC II, F4/80, and CD86[42] with the expression of MERTK suggesting they may be alveolar macrophages[43]. The BCG vaccinated groups also showed an increase in CD14 in this population. These cells were potentially alveolar macrophages that were present in the LysM knockout mouse, and showed increased activation after BCG vaccination. Other populations of interest were population 3 and population 4 as lungs from BCG vaccinated mice showed increased presence of these cells. These populations can be characterized as monocyte-derived and macrophage like based on their surface marker profiles. Although the cells in population 3 had a similar phenotype in BCG and naïve mice, population 4 showed an increase in expression of CD14, CD90.2, MERTK and MHCII and decreased Ly6G expression after BCG vaccination. Population 8 had higher numbers in vaccinated mice, but it was difficult to determine the phenotype based on the cell expression profile. Overall, these results showed that not only did BCG vaccination induce changes in the number of macrophage-like cells in the lungs, it also induced changes in the relative expression (demonstrated by the changes in MFI) for specific markers associated with activation. This is consistent with the hypothesis that BCG vaccination induces the activation of

macrophages in the lungs and suggests that these activated macrophages are present in the lungs 7 days after subcutaneous BCG vaccination.

The results of this study are significant for the future of TB research for two main reasons. First, it suggests a mechanism by which T-cell dependent vaccines for tuberculosis could be combined with BCG to not only produce strong T cell responses to *M. tuberculosis* infection, but give the host a predisposition to switch to M1 polarized macrophage over M2. If this occurs these macrophages may be harder to infect and more capable of mycobacterial clearance after infection. It also suggests that switching the majority of macrophages to an M1 state could be utilized to some effect by a therapeutic to help clear mycobacteria after infection or prevent the spread of infection. However there is still the question of how the signal is being transmitted to the macrophages. A recent study has linked the importance of the innate immune response to clearance of *M. tuberculosis*[44], and understanding these mechanisms is crucial for future use of these mechanisms in vaccines or therapies.

We previously hypothesized that innate lymphoid cells could play a role in BCG induced immunity. Past studies have identified populations of innate lymphoid cells in the lungs 4 weeks after BCG vaccination but not sooner[45]. Our flow cytometry analysis supported this as we were not able to detect significant numbers of these cells present in the lungs within 7 days (data not shown). Our previous experiments have shown that neutrophils were a partial requirement for BCG vaccination to induce mycobacterial killing. In mapping out an overall picture of innate immunity induced by BCG, neutrophils, usually the first cell population at the site of infection[46], could be responsible for a swift, strong immune response that recruits macrophages to the injection site resulting in BCG stimulated macrophages. These now infected cells must return to the circulation, going possibly to lymph nodes, but eventually bringing the BCG to the lung where they would be producing cytokine that can stimulate a switch to M1 polarization. Upon infection with *M. tuberculosis*, the macrophage cells musts be more pro-inflammatory, cytotoxic, and potentially harder to infect resulting in a decreased mycobacterial load in BCG vaccinated

animals. It is unknown how long the activated macrophages remain in the lungs, but past experiments in T cell knockout mice have suggested that it can last up to 30 days. As macrophage activation demonstrated the ability to limit the growth of *M. tuberculosis*, this is a point of interest for future vaccine development and is a potential immunotherapy target. Future studies must be conducted to classify the mechanism.

This study identified one potential mechanism through which mycobacterial killing could be achieved. Our *in vitro* studies comparing live BCG to γ-irradiated BCG suggested that the former is more adept at inducing the production of iNOS and therefore nitric oxide. We hypothesize this difference to be due to the fact that live BCG can live and proliferate inside macrophage cells by inhibiting phagosome maturation[47]. Whereas γ-irradiated BCG would be immediately destroyed by any phagocytic cell, live BCG would be able to provide prolonged stimulation to different PRRs. BCG induced induction of iNOS has been studied in the past and has been described as a mechanism by which BCG induces protection against *F. tularensis*[48]. This study identified a mechanism by which BCG induces production of iNOS and nitric oxide through autocrine signaling. This supports our theory of the importance of BCG replication as live, replicating BCG could continually induce autocrine signaling of the required cytokines for iNOS production leading to increased macrophage activation and killing. It should be noted that this study identified TNF-α and IFN-γ as the necessary cytokines required for increased NO production. Our past studies have identified that TNF and IFN-γ are not required for BCG mediated protection. However, NO production can be stimulated by a multitude of pathways[20] and there is potential for a compensatory mechanism in the absence of these cytokines. Future studies are needed to confirm this. Our findings from our iNOS study also support our hypothesis of activated macrophages in the lung as nitric oxide production is indicative of an M1 phenotype and iNOS expression is capable of inducing further gene expression concordant with the M1 phenotype such as interferon regulatory factor 5 (IRF5)[49]. Macrophage activation is a widely studied phenomenon that is important across a variety of physiological diseases.

Perhaps the results from this study will be useful in identifying BCG not only as an important candidate for use in vaccination against tuberculosis, but also as a potential biological response modifier that is usable across the world of immunotherapy any place an M1 phenotype in macrophage populations is desired.

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Chapter VI

Concluding Remarks

Mycobacterium tuberculosis has survived as a pathogen for thousands of years. Even with current technology, there seems little hope in completely eliminating the threat that *M. tuberculosis* poses against human populations. The research described within this thesis hopes to provide new potential areas for not only vaccine research, but also immunotherapy research. With the increase of antibiotic resistance, the need for effective, alternative treatment methods is paramount. The past ten years have drastically altered effective cancer treatments with immunotherapies being added alongside traditional radiation therapy[1]. Maybe the tuberculosis field can learn from a successful combination therapy model, with antibiotics and new immunotherapies.

The Importance of Macrophages to *M. tuberculosis* Infection

Macrophages play an important role in the pathogenesis of M. tuberculosis for a variety of reasons. *M. tuberculosis* is an intracellular pathogen infecting macrophages. Upon infection, granulomas, which are a hallmark of tuberculosis, are a macrophage derived structure. Alveolar macrophages are of particular importance. Alveolar macrophages are defined as tissue resident macrophages that have taken up residence in the alveolar sacs of the lungs. These populations are capable of self-renewal making them distinct from other macrophage populations[2] as is shown by work detailing the downregulation of MafB and cMaf, transcriptional regulators that repress macrophage self-renewal[3]. These cells are especially significant when the results of our LysMCre study are taken into account. LysMCre KO mice are known to have severely depleted circulating monocyte populations with alveolar macrophages being fully intact[4]. As these mice are protected from aerosol infection by BCG vaccination, these remaining cell populations are viable candidates for the immunity induced by BCG. In addition, alveolar macrophages are known to be a major host reservoir for *M. tuberculosis* during early

infection and provide a pathway for dissemination of infection[5]. As we have also found, these populations of lung macrophages are activated, a phenotype that is known to be microbicidal, and capable of iNOS production. If BCG vaccination activates alveolar macrophages either directly or indirectly, their role in preventing dissemination of early *M. tuberculosis* infection provides a mechanism by which BCG vaccination may reduce mycobacterial growth in the lungs.

Combination Therapy for Tuberculosis

With the success of new immunotherapies in cancer and other inflammatory diseases, there arises the potential for the use of combination antibiotics and immunotherapies for increased efficacy. Cancer immunotherapies have recently shown increased success through the modulation of the tumor microenvironment. Successful treatment methods so far have relied on immune checkpoint inhibitors with targets such as programmed cell death protein 1 (PD1), programmed death ligand 1 (PDL1), and cytotoxic T-lymphocyte associated protein 4 (CTLA-4). Checkpoint inhibitors like this have become commonly used for their role in helping modulate the regulatory aspects of the immune system.

When utilized the right way, controlled downregulation of regulatory immune pathways can lead to increased efficacy of the immune systems clearance of an infection or tumor[6]. However, careful consideration of the microenvironment is required to effectively adjust the regulatory immune system. Chronic infections such as tuberculosis are similar to cancer in that there is a prolonged period of time where the immune system is exposed to antigens. Just as with cancer, this prolonged exposure to tuberculosis antigens does lead to increased expression of PD-1 on CD4 and CD8 T cells, and blocking PD-1 or PD-L1 with an antibody increases CD4 and CD8 T cell activity[7]. There is also some evidence that blocking of Tim-3 and PD-1 can increase T cell activity after tuberculosis infection in HIV patients by reversing T cell exhaustion[8], and PD-1 blockade shows some promise in saving effector T cells from apoptosis

during pulmonary infection[9]. As T cell exhaustion has been shown to restrict the functionality of ESAT-6 specific CD4 T cells during *M. tuberculosis* infection[10], there is potential for anti-exhaustion therapies to improve the immune response to tuberculosis. However, a better understanding of the role that T cell exhaustion plays in immunity to tuberculosis is required for these therapies to be utilized effectively.

Current work also suggests that T cell recruitment in the lung results in two primary localizations, parenchymal T cells and vascular T cells. Although these populations of cells can both be pathogen specific effector T cells, the cells that localize to the parenchyma have shown enhanced protective capacity against *M. tuberculosis* infection. Interestingly, although they were not as effective at fighting infection, the vascular T cells produced higher amounts of IFN-γ[11]. From this it has been hypothesized that although IFN-γ plays an important role in mycobacterial killing in the spleen, it may not be as important for protection from tuberculosis in the lung[12]. Another molecule of the tumor necrosis factor (TNF) subfamily called CD153 has been found to be necessary to control *M. tuberculosis* infection in the lung, and may be more important than IFN-γ in this role[13,14]. There is suggestion that CD153 deficiency upregulates PD-1 in CD8 T cells in certain tumors and this pathway could lead to the development of an effective immunotherapeutic approach[15]. Others have reported that Anti-PD-1/PD-L1 therapies may be beneficial for use in chronic infections like tuberculosis[16] and a future study characterizing the involvement of both CD153 and PD-1 during *Mycobacterium tuberculosis* infection would provide a good basis for a development of an immunotherapeutic based on these mechanisms. A more complete understanding of the microenvironment of the lung during early infection and late stage infection is needed to fully utilize any types of immune therapy. The granuloma microenvironment of immune cells generated by the response to tuberculosis bears many similarities to the tumor microenvironment; at the center of it are macrophages.

Innate Immune Training and BCG

Much of this thesis is concerned with innate immune training and its role in clearance of *M. tuberculosis*. Up until this point, trained innate immunity has demonstrated its ability to protect against non-mycobacterial infection such as *Candida albicans and Staph aureus*[17]. Protection from trained innate immunity against *M. tuberculosis* infection has been identified, but innate immune training and epigenetic remodeling was observed 30 days post vaccination, and only upon vaccination with the intravenous route[18]. As our results show, BCG can still induce immunity in the C57BL/6 model through multiple routes of vaccination, and protection is established as early as 7 days post vaccination. While this does not discount the importance of trained innate immunity, it does suggest that the mechanisms behind trained innate immunity are not an absolute requirement for BCG mediated protection. Our results show that BCG induces physiological changes in the innate immune populations in the lungs. There is potential that these changes are induced through a similar epigenetic reprogramming event, as we did not rule it out as a potential mechanism. However there is little evidence to support this. There is also potential that both trained innate immunity, and the activation of macrophages we observed are induced by the same thing. BCG is a known inducer of autophagy, and some evidence suggests that autophagy pathways are also responsible for activating trained innate immunity[19]. There is also some evidence that autophagy can induce the killing of *M. tuberculosis*[20]. Perhaps further investigation into BCG induced autophagy will allow full characterization of the immune response stimulated by BCG against *M. tuberculosis*.

The Future of BCG in *M. tuberculosis*

There is no doubt that the BCG vaccine has a role in the future of tuberculosis prevention and elimination. Although its limited efficacy has been outlined in this thesis, BCG still has much to offer. The sheer volume of people who have been vaccinated with BCG make it a good platform to build upon for a booster vaccine. It also has a wide variety of immunostimulatory effects that help it provide protection to non-mycobacterial diseases[17]. In developing nations

and areas of high rates of endemic diseases, this protection can be life saving for the children that BCG is given to. Finally, BCG has a strong biological response modifier ability. This has been widely used as a successful treatment for bladder cancer[21,22] and as shown in this thesis, has potential to modulate the immune system systemically. Although the mechanisms though which BCG interact with the innate immune system are not fully understood, it does provide every indication that it has earned its spot as the most widely used vaccine in the world and will continue to be used to fight disease.

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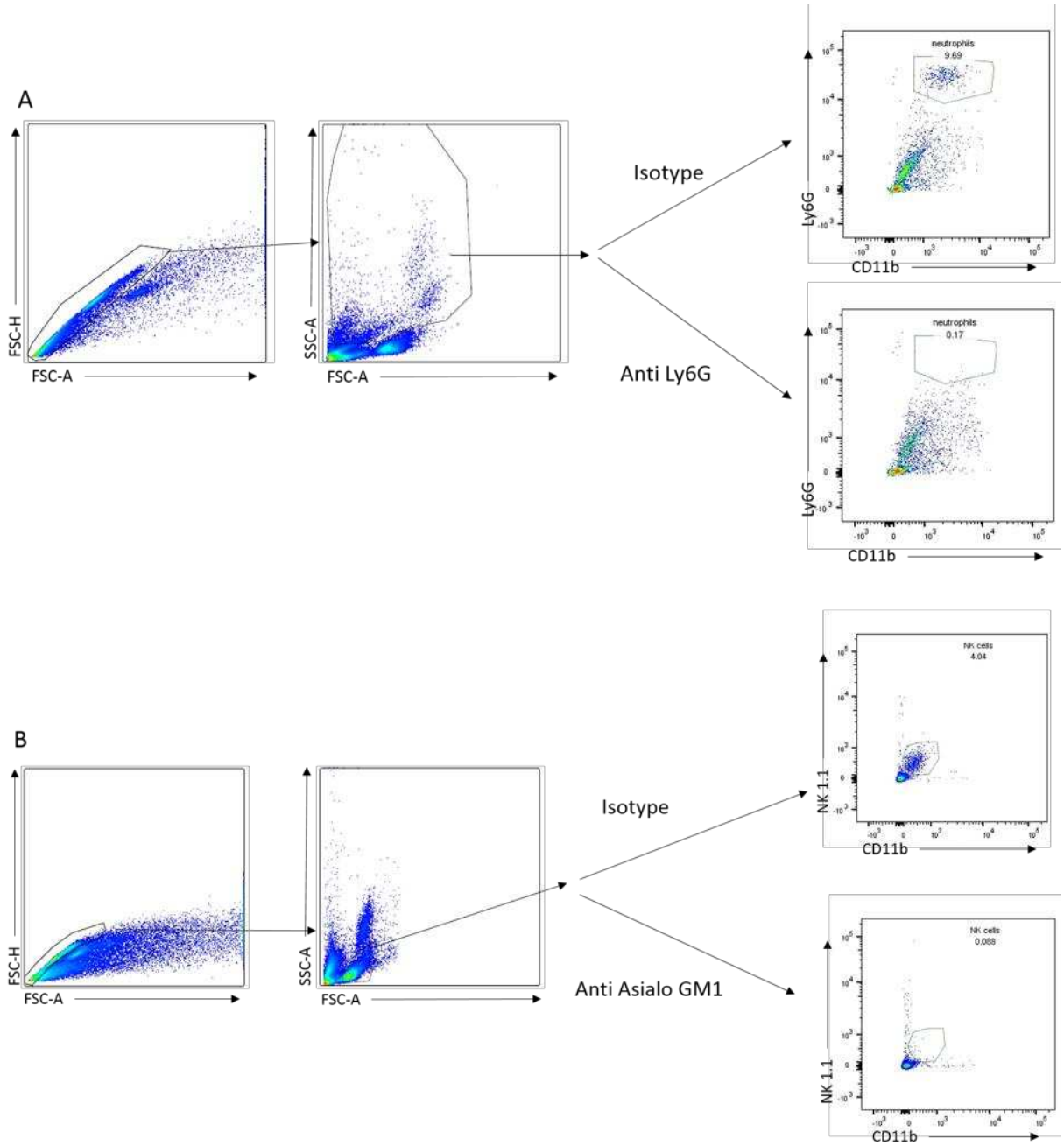
APPENDIX I

Supplementary Figures

Supplementary Figure 4.1.1



Supplementary Figure 4.1.1
Flow cytometric confirmation of neutrophil depletion using a Ly6G antibody (**A**) and of natural killer cell depletion using an NK1.1 antibody (**B**). Peripheral blood mononuclear cells were used to examine the efficacy of depletion.

Supplementary Table 4.1
Details of antibodies used for flow cytometry in the current studies.

Supplementary Table 4.1

Marker	Fluorophore	Isotype	Clone	Vendor
CD11b	Alexa Fluor 700	Rat IgG2b	M1/70	BD Biosciences
CD11c	BV510	Arm Ham IgG1	HL3	BD Biosciences
Siglec F	PercP cy5.5	Rat IgG2a	E50-2440	BD Biosciences
Ly6G	PEcy7	Rat IgG2b	RB6-8C5	Tonbo Biosciences
Ly6C	APC	Rat IgG2a	1G7.G10	Miltenyi Biotec
F4/80	PE	Rat IgG2b kappa	BM8.1	Tonbo Biosciences
I-A[b] MHCII	BV421	Mouse IgG2a	AF6-120.1	BD Biosciences
CD14	FITC	Mouse IgG1 kappa	X54-5/7.1	Biolegend
NK 1.1	PE	Mouse IgG2a kappa	PK136	Tonbo Biosciences

Supplementary Table 5.1
A table showing the flow cytometry antibodies used in the 21 color panel used to generate the FlowSOM and tSNE data.

Supplementary Table 5.1

Marker	Fluorophore	Isotype	Clone	Vendor
Live Dead	BV510	NA	NA	BD
CD 127	FITC	Rat IgG2ak	A7R34	Biolegend
MERT K	PE	Rat IgG2ak	2B10C42	Biolegend
CD24	PE cy5	Rat IgG2bk	M1/69	Biolegend
CD103	PercP Cy5.5	Rat IgG2ak	M290	BD
CD90.2	PEcy7	Rat IgG2ak	53-2.1	BD
CD43	APC	Rat IgG2b	S11	Biolegend
Ly6C	AF700	Rat IgM	AL-21	BD
CD14	APC-Cy7	Rat IgG2ak	sa14-2	Biolegend
CCR7 (CD197)	BV421	Rat IgG2a	4B12	BD
CD86	BV480	Rat IgG2ak	GL1	BD
F4/80	BV605	Rat IgG2ak	T45-2342	BD
Siglec F	BV711	Rat IgG2ak	E50-2440	BD
CD335 NKp46	BV786	Rat IgG2ak	29A1.4	BD
CD11c	Pacific Blue	Arm ham IgG	N418	Thermofisher
Ly6G	PercP e fluor 710	Rat IgG2ak	1A8	Thermofisher
I-Ab MHCII	BV650	Mouse IgG2ak	25-9-17	BD
CD11b	Pe Texas Red	Rat IgG2b	M1/70.15	Thermofisher
CD62L	AF 647	Rat IgG2ak	MEL-14	Biolegend
CD45	PercP	Rat IgG2bk	30-F11	BD
CD3	AF532	Rat IgG2bk	17A2	Thermofisher

LIST OF ABBREVIATIONS

C57BL/6	Genetically modified mice used as animal model of disease
CCR7	C-C chemokine receptor type 7
CD11b	Cluster of Differentiation 11b; integrin alpha M
CD11c	Cluster of Differentiation 11c; integrin alpha X
CD127	Cluster of Differentiation 127; Interleukin 7 Receptor
CD14	Cluster of Differentiation 14; Co-receptor with TLR4
CD3	Cluster of Differentiation 3; T cell coreceptor
CD4	Cluster of Differentiation 4; Marker for T cells
CD43	Cluster of Differentiation 43; Leukosialin (Sialophorin)
CD45	Cluster of Differentiation 45; Protein tyrosine phosphatase receptor type, C
CD8	Cluster of Differentiation 8; Marker for T cells
CD86	Cluster of Differentiation 86 (B7-2); Co-stimulatory molecule to activate T cells
CD90.2	Cluster of Differentiation 90.2; Thymosite antigen 2
CFU	Colony Forming Units
DIM	Phthiocerol dimycocerosate
DNA	Deoxy Ribonucleic Acid
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1
IFNγ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
ILCs	Innate Lymphoid Cells
INH	Isoniazid
iNOS	Nitric Oxide Synthase
LAM	lipoaribinoannan
LM	lipomannan
Ly6C	Lymphocyte antigen 6 complex locus C1
Ly6G	Lymphocyte antigen 6 complex locus G
MAIT Cells	Mucosally Associated Invariant T cells
MDR	Multi Drug Resistant
MERT-K	Proto-oncogene tyrosine protein kinase mer
MHCII	Major Histocompatibility complex II
MTB	*Mycobacterium tuberculosis*
NK Cell	Natural Killer Cells
NKp46	Natural cytotoxicity triggering receptor 1
NO	Nitric Oxide
NOD2	Nucleotide Oligomerization Domain 2
PCR	Polymerase Chain Reaction
PIM	phosphatidylinositol mannosides
qPCR	Quantitative Polymerase Chain Reaction
RIF	Rifampin
RNA	Ribo nucleic Acid
Sig F	Siglec F; Sialic Acid-binding immunoglobulin-type lectins
TB	Tuberculosis
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor alpha
XDR	Extensively Drug Resistant