THESIS

TLR9 AGONIST PRODUCES EFFECTIVE MUCOSAL IMMUNITY AS A VACCINE AGAINST MYCOBACTERIUM TUBERCULOSIS

Submitted by

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ABSTRACT

TLR9 AGONIST PRODUCES EFFECTIVE MUCOSAL IMMUNITY AS A VACCINE AGAINST MYCOBACTERIUM TUBERCULOSIS

The *Mycobacterium tuberculosis* bacillus has plagued the world for thousands of years. Researchers have found evidence of tuberculosis infection as far back as the Ice-Ages with the presence of bone lesions in mastodons[2]. The existence of tuberculosis in humans has also been found in ancient Egyptian mummies dating as far back as 4000 years ago[3]. Pulmonary tuberculosis is an airborne bacterial disease and the causative agent, *M. tuberculosis*, currently infects about 2-3 billion people globally[1]. This deadly disease is responsible for killing an average of 5,000 people every single day, and is most prevalent in developing countries, particularly in sub-Saharan Africa where the AIDS epidemic grows yearly[4]. TB is one of the first diseases to arise in these immune-suppressed patients, which furthers the need for a new effective disease control strategy. The incidence of drug resistance has been increasing steadily, also exemplifying the need for a prophylactic approach to combating the disease[1]. The only currently available vaccine for *M. tuberculosis* in use today is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), a live attenuated vaccine that has been in use since the 1930's, and with a variable efficacy rate of 0-80% the need for a new more effective vaccine is dire[5]. BCG is the mostly widely used vaccine in the world with some success in young children, but immunity wanes over time[6] due to a number of reasons. In order to replace BCG or boost BCG, a better understanding of the immune response is required, allowing for better vaccine development. Animal models provide a good basis for determining immune mechanisms that are related to protective immunity, both innate and adaptive.

Toll-like receptors (TLRs) are a key component of the innate immune response. TLRs are pattern recognition receptors (PRR), which recognize pathogen associated molecular patterns (PAMPs). These PAMPs are not produced by mammalian cells but are presented by invading microorganisms. Once PAMPs are detected the innate immune response is activated and begins the clearance of bacterial infection. PRRs are also required to activate antigen-presenting cells (APCs) that process bacterial antigens for presentation, in association with major histocompatibility complexes (MHC) to T-cells. One particularly stimulatory PAMP is CpG Oligodeoxynucleotide (ODN), which is unmethylated bacterial DNA. Once CpG ODN is introduced, the PRR TLR9 is activated thus inducing a potent T-helper 1 (Th1) response which plays a critical role in tuberculosis infection[7, 8].

Mucosally administered vaccines have been of particular interest to vaccine researches in the past twenty years[9]. Data show the nasal mucosa is an effective route of vaccine administration because it is the first point of contact to an inhaled pathogen. Since pulmonary tuberculosis is spread via aerosolized particles, vaccines targeting the mucosa may effectively prime local antigen presenting cells like alveolar macrophages and dendritic cells[10].

One method for vaccine delivery is the use of a carrier vehicle such as cationic liposomes. Cationic liposomes consist of a positively charged lipid bi-layer. The positive charge on these liposomes allows the addition of a negatively charged immune-stimulant such as CpG ODN. Liposomes carrying antigen are capable of inducing a robust cell mediated immune response or Th1 type response, which is crucial for mitigating *M. tuberculosis* infection[11]. Since protein antigen degradation occurs quickly in mucosal sites liposomes provide increased stability of these vaccine components and thus allowing prolonged antigen presentation.[10]

Given that tuberculosis is a pulmonary infection, we hypothesize that targeting the site of infection with a TLR9 agonist; a robust mucosal immune response would be induced providing protection against infection and reducing bacterial burden. Using the mouse model of tuberculosis, we determined that intranasal inoculation of a cationic liposome carrying CpG

ODN and the *M. tuberculosis* antigen ESAT-6 provides protection by inducing a robust Th1-type immune response capable of significantly reducing mycobacterial burden in the lungs after pulmonary infection, and also creating long-lasting immunity by stimulating the activation of memory T-cells.

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DEDICATION

This thesis is dedicated to my father, Donald C. Troy, who never let me give up and who reminded me who I was every single day. And to my future husband, Klifford Westcott, I would have never made it this far without your support.

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1 Literature review

1.1 M. tuberculosis Discovery and Epidemiology

The discovery of *Mycobacterium tuberculosis* involved the work of many brilliant researchers and scientists that, over hundreds of years, each played a role in its discovery. Around 460-370 BC Hippocrates identified phthisis, or consumption as the most widespread and fatal disease of its day[12]. Sylvius identified tubercles to be the consistent cause of physical changes in the lungs of consumptive patients in 1679[13]. He also described the tendency of the lesions in the lung to develop into cavities over time by performing anatomic-pathologic studies in pulmonary nodules from *M. tuberculosis* infected patients[14]. A physician named Benjamin Martin first hypothesized in 1720 that *M. tuberculosis* was caused by "wonderfully minute living creatures" that create the lesions in the lung and other symptoms of disease[15]. He also had some early epidemiological insight by stating that it might be possible to acquire the disease from prolonged close contact with an infected individual and by breathing in the expulsions from their lungs a healthy person might be affected[15].

In the 1800s doctors began recommending to patients that they seek out healthier climates and breathe in fresh mountain air as a treatment[16-18]. This actually was the first step in controlling the disease as sanatoriums were later developed and this helped separate infected individuals from healthy ones. In 1865 Jean-Antoine Villemin demonstrated that the disease could be passed from infected individuals to cattle and rabbits thus disproving the previous belief that the disease arose spontaneously[13, 15, 19]. Progress in the field of tuberculosis was further advanced in 1882 when Paul Ehrlich developed the acid-fast stain[13, 15, 20]. Prior to this, the bacteria were extremely difficult to visualize due to the mycolic acid content that prevented conventional stains and dyes from being effective.

Wade Hampton Frost was a pioneer in bacterial epidemiology and defined the subject as: "Epidemiology as comprising the whole of the unremitting effort being made to clarify the relation between the disease and disabilities which men suffer and their way of life"[21]. Frost's

work gives us some of the understanding of the epidemiology of tuberculosis that we hold today[22]. Some of Frost's work included nomenclature that helped define what is called the 'index case'[21, 22]. Due to the chronic nature of tuberculosis, multiple cases could be found in a single family, he named the first case to be found in a group the index case and this statistic was excluded from calculations to determine the risk involved with cohabitating with an infecting individual. In order to evaluate familial risk Frost needed the rates of morbidity and mortality in a large group of people over a number of years. The best records to accomplish this that were available to him were from a survey done by the Tennessee State Department of Health evaluating the African American population of Kingsport, Tennessee from 1930 to 1931 including data from 132 families[23]. By using historical analysis from the Kingsport survey he discovered that the risk of death when living with a *M. tuberculosis* infected individual in the family doubled the risk of infection compared to those who did not[23]. The highest case rates were found to be predominately in children and the elderly[23]. His papers focused on the factors that might help control the spread of disease as well as the probability for its eradication[21, 24, 25]. It was under his advice that in order to reduce the rate at which healthy individuals were exposed and infected, that infected individuals were isolated in sanatoria.

Robert Koch, born in 1843 was the son of a mining engineer in Clausthal Germany. After earning his degree as an MD and serving in the army, Koch became a physician and was given a microscope by his wife on his birthday. He set up a primitive laboratory and by 1876 Koch demonstrated the life-cycle of the anthrax bacillus and for the first time revealed a micro-organism as the cause of a disease[13, 26]. For the next few years Koch developed modern bacteriological techniques including the use of glass slides, bacterial culture on media, and disinfection by steam. In August of 1881 Koch attended a medical conference in London in which the subject of tuberculosis was of great concern[27]. Following that conference Koch set out to identify the causative agent of tuberculosis, the *M. tuberculosis* bacilli, and less than a year later he succeeded [13]. Robert Koch announced his discovery of the etiologic agent of

tuberculosis at a physiology conference on March 24th in 1882[13, 15, 19, 28]. He was the first researcher to develop a staining technique to visualize the bacterium as well as isolate and identify the *M. tuberculosis* bacteria in an infected patient[19].

Paul Ehrlich, then 28 years old was in attendance at Koch's lecture that day. Immediately following Koch's presentation Ehrlich obtained a pure culture of tubercle bacilli and that same evening took the sample to his laboratory to experiment with various stains[13]. He modified Koch's previous staining protocol in conjunction with a staining technique he developed for mast cells, while adding additional steps with nitric acid and alcohol for decolorization of the surrounding tissues. That night, Ehrlich placed the slides on what he thought was a cold stove to dry, but when he returned the next day, to his dismay he realized the stove was still lit. This turned out to be a stroke of luck when upon observing the slides he realized the accidental heating step clumped the bacilli together allowing the bacteria to be seen much more clearly because it concentrated them in a small area[13]. In May of 1882 Ehrlich published his new stain that included heat fixing and decolorization. Later, Ziel and Neelsen altered the staining protocol with a few changes and developed the Ziel-Neelsen stain that we know today[13].

Two years later Koch published a paper entitled "Koch's Postulates" which has become a staple in microbiologic studies to this day[13, 19, 26, 29, 30]. To be considered the cause of infectious disease these four criteria must be met: "1) the organism must be isolated from the diseased tissues in every case of the disease; 2) the organism must be grown in pure culture; 3) inoculation of a susceptible animal with the organism must produce the same disease; 4) the organism must be recovered from the infected animal and be grown again in pure culture"[13]. By 1890 Koch believed he held the key to the cessation of tuberculosis infection with a filtrate of the tubercle bacilli, which he referred to as tuberculin[31, 32]. He announced this as a cure for tuberculosis and a possible treatment for phthisis[33]. Although tuberculin did not prove to be an effective treatment for tuberculosis, it has been found to be a valuable diagnostic tool when

Seibert developed a purified protein derivative (PPD) of tuberculin in 1931[34]. When Koch was awarded the Nobel Prize in 1905 he spoke of the continued need for an effective treatment against the disease[35].

1.2 M. tuberculosis History

Mycobacterium tuberculosis is one of the oldest recorded human afflictions. Evidence of *M. tuberculosis* infection, the etiologic agent of tuberculosis in humans, dates as far back as 4,000 years ago when evidence of infection was found in spinal column remnants in ancient Egyptian mummies from 2400BCE[18, 36-38]. Because of the frequency in which this evidence has been found, it can be assumed that *M. tuberculosis* was relatively widespread among the population. *M. tuberculosis* in some cases can infect skeletal tissue, which creates deformities in the bone that can remain preserved for thousands of years[36]. Remnants of infected human bones have also been found in Italy, Denmark, and the Middle East at multiple Neolithic sites providing evidence that *M. tuberculosis* was relatively widespread thousands of years ago[36]. Another related organism, *Mycobacterium bovis* has evidence of older existence when what appeared to be tuberculous lesions were found in the fossilized remains of mastodons during the ice age[2, 39]. It has been hypothesized that *M. bovis* may be the predecessor for *M. tuberculosis* and the domestication of cattle tens of thousands of years ago may have lead to the evolution of the organism as we know it today, however, with modern genetic analysis technology, evidence suggests they may have evolved simultaneously[2]. The disease spread even further presumably due to the migration of Indo-European cattle herders exposing their cattle infected with the tubercle bacillus to large regions of Europe and Asia[2, 36]. There is genetic evidence of phenotypic traits such as lactose tolerance that coincide with the genetic evidence of *M. tuberculosis* infection[36].

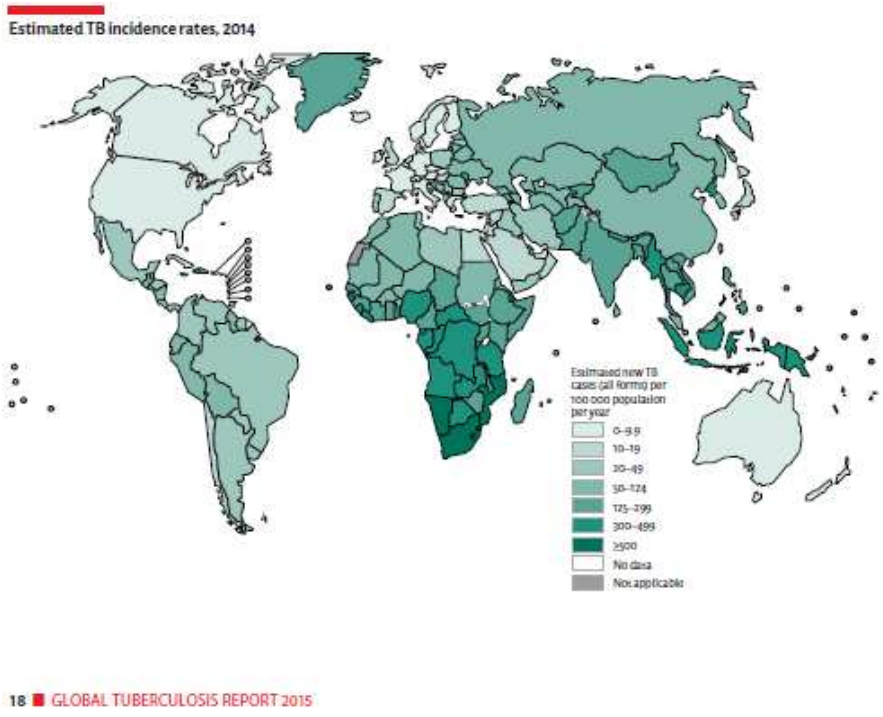
Around 460BCE the term “phthisis”, which is the Greek word for consumption was found in Greek literature and Hippocrates described the disease as being vastly widespread with symptoms that included chest pain, coughing, and bloody sputum[12, 18, 36, 37, 40, 41]. Although

Hippocrates believed that the disease was inherited, Aristotle in the 4th century BC believed it was contagious[18]. Galen, the great Roman physician of 2nd century BC also expressed his belief in its contagious natures[18]. Assyrian clay tablets from the seventh century have been found which describe consumptive individuals coughing up blood[36]. Like other actinomycetes bacteria, *Mycobacteria* can be found in the environment and in soil. Over thousands of years, some of these species eventually adapted to infecting mammalian species. The controversy remained throughout the years but in 1865 that changed when the French military physician, Jean-Antoine Villemen demonstrated that rabbits could be infected with the disease from tissue derived from a cadaver[17, 18, 37, 40]. Robert Koch supported this claim years later in 1882 when he isolated the organism and infected guinea pigs with it[15, 31].

Edward Trudeau made tremendous advances in determining the etiology of *M. tuberculosis* in the late 19th and early 20th centuries[18]. Trudeau demonstrated that rabbits could be infected with a purified culture of *M. tuberculosis* but the environment in which the animals were housed was a significant factor[18]. Because of the crowded conditions in some cages of rabbits, these animals succumbed to the disease much more quickly. This finding supported the claim that individuals would benefit greatly from breathing in fresh mountain air in the care of a sanatorium like those in Saranac Lake[18].

Due to the dramatic increase in the population of Europe, urban areas became the epicenters for the spread of disease[38]. The *M. tuberculosis* epidemic in Europe began around the 16th and 17th centuries and by the first half of the 19th century the disease coined the "White Plague" peaked, claiming the lives of one quarter of the European population[38]. This was evidenced by a post-mortem study of cadavers in Paris, which showed that one quarter of all individuals autopsied had succumbed to the disease[36]. In the years following, improved hygiene practices, health of individuals, sanitation, and housing increased thus quelling the rapid spread of the disease[18]. Later, the use of sanatoriums helped restrain growth of the disease by isolating the sick from the rest of the population[17, 18, 38, 40]. Other experimental treatments were

employed to treat the disease involving seemingly damaging practices like collapsing the lung of infected individuals to reduce lung volume, or implanting Ping-Pong balls into the pulmonary cavity[18]. *M. tuberculosis* incidence began a steady decline over time until the 1980s when a high rate of immigration from *M. tuberculosis* endemic countries into the US increased[18, 38]. To this day *M. tuberculosis* remains a significant public health concern with a fatality rate that trumps any other bacterial infection in history[1]. The WHO declared tuberculosis to be a global public health emergency in 1993[42]. Currently approximately 2 billion people are affected by tuberculosis to this day, despite the worldwide use of the live-attenuated *M. bovis* BCG vaccine, with 95% of these individuals in developing countries **(Figure 1-1)**. One out of ten individuals will become infected with the disease over the course of their lifetime[1]. Currently, there seems to be a rising trend of incidence of tuberculosis with the prevalence of antibiotic resistant bacteria on the rise, and the number of people infected with HIV increasing[1, 18, 38].



[1] WHO. 2015. Global Tuberculosis Report 2015. In © *World Health Organization 2015*, 20 ed. World Health Organization. 193.

Figure 1-1: "Estimated TB incidence rates, 2014"

1.3 M. tuberculosis Bacilli Structure and Characteristics

Mycobacterium tuberculosis is a non-motile bacillus ranging in length from 1-4μm and is related to Actinomycetes bacteria[33, 43]. It is traditionally thought of as an obligate aerobe (with some exception)[44] and in pulmonary tuberculosis, the bacteria thrive in the oxygen rich environment in the upper lobes of the lung[45]. The bacteria are facultative intracellular parasites and generally infect resident alveolar macrophages in pulmonary alveolar sacs[46]. The generation time for the bacteria is 15-20 hours which translates to an incubation time of 4-6 weeks on Middlebrook's or Lowenstein-Jensen agar to develop colonies[47-51]. Although the bacterial cell wall does contain peptidoglycan, *M. tuberculosis* is not considered Gram-negative or Gram-positive, but acid-fast due to its impermeability to dyes and stains caused by the mycolic acids in the membrane[43, 52]. The stain used to visualize the bacteria microscopically is called the Ziehl-Neelsen stain which contains acidified organic compounds[15]. This contains the dyes carbol-fuchsin, methylene blue, and acid-alcohol to perform decolorization, staining the bacteria pink[33, 53].

M. tuberculosis possesses a cell wall unique to prokaryotes, and although it does contain peptidoglycan, 60% of the cell wall is lipids[43, 54]. These consist mainly of mycolic acids, cord factor and wax-D[43, 52, 55-58]. Mycolic acids make up about 40-50% of the dry weight of the cell envelope and are strongly hydrophobic which affects the permeability of the surface[43, 59]. This also is thought to be a significant virulence factor of the bacteria by protecting it from complement deposition, lysozyme, cationic proteins, and oxygen radicals produced by phagocytes[60]. Due to its lipid based cellular envelope, *M. tuberculosis* is resistant to desiccation and disinfection, which makes it a robust organism capable of surviving harsh conditions making it viable for up to 10 weeks outside of a host[60].

In 1884 Robert Koch discovered the structural virulence factor of *M. tuberculosis* that was described as a rope-like structure in bacterial cultures, which he named cord factor[55, 56]. This chain of mycolic acids was found to play no role in the growth of the bacteria, however,

when the cord factor was removed, the virulence of the organism was reduced by inhibiting phagosome-lysosome fusion in phagocytic cells[55]. Cord factor causes the bacteria to grow in a serpentine formation and is toxic to cells and inhibits neutrophil movement[57]. Another component of the cellular envelope called Wax-D, is also used to create the potent Th2 type immune activator, Freund's complete adjuvant (CFA)[61].

Cord factor, which contains mycolic acid residues, is another key virulence factor of mycobacteria[56, 62]. This is the most abundant glycolipid produced by *M. tuberculosis* and it is this component that allows the bacteria to survive and replicate within infected macrophages by preventing phago-lysosomal fusion[56, 62]. Despite the ability of these bacteria to disable components of the immune response, *M. tuberculosis* is highly immunostimulatory and induces pro-inflammatory cytokine production, particularly in macrophages, which include interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α[56, 63]. Cord factor has also displayed highly antigenic and toxic effects within granulomas, causing caseation necrosis and cavitary tuberculosis resulting in severe histopathology[56, 64].

The mycobacterial cell wall has an upper and a lower segment, and beyond the membrane is peptidoglycan covalently attached to arabinogalactan, all of which are attached to mycolic acids[58]. Interspersed throughout cell wall proteins, are free lipids including phosphatidylinositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM)[52, 58]. Each of these glycolipids plays a role in either virulence and pathogenesis, or modulating and inducing an immune response upon infection to *M. tuberculosis*[52, 58]. For instance, LAM is able to bind to toll-like receptors (TLRs), and even insert itself into cellular membranes resulting in manipulation of host immunity[58]. Innate recognition of these glycolipids involves binding by various TLRs, and they are also recognized by CD1-restricted T-cells[54]. LAM has an array of other immunomodulatory functions that include T-cell activation[65], inhibition of phagosome maturation[57], inhibition of IFN-γ and macrophage function[66], scavenging cytotoxic free radicals[67], and stimulating a wide array of cytokine production[54, 68-73].

Other mycobacterial glycolipids also play an immunomodulatory role in the infected host in a number of ways. For example, Cambier *et al*. has shown that phthiocerol dimycocerosate (PDIM) in the bacterial cell wall inhibits signaling TLRs via inhibition of the MyD88 adaptor protein[74, 75]. This prevents recruitment by evading detection by the immune system and preventing recruitment of microbicidal macrophages to the site of infection[74, 75]. Cambier has also shown that this glycolipid is also responsible for inhibiting inducible nitric oxide synthase (iNOS) and reactive nitrogen intermediates (RNIs) in the macrophages of infected mice, thus reducing their microbicidal abilities[74, 75].

1.4 M. tuberculosis Pathogenesis

Mycobacterium tuberculosis is the etiologic agent of tuberculous and is responsible for more deaths than any other single bacterial pathogen[14, 76]. The spread of *M. tuberculosis* is predominantly via aerosolized particles and results initially in pulmonary disease. Droplet nuclei less than 5μm in size, and containing as few as 1 – 3 organisms are capable of transmitting infection[77-80]. Once particles gain access to the pulmonary alveoli, resident alveolar macrophages engulf the bacteria via mannose, compliment, and Fc receptors; and attempt to clear the invading organisms through phagosome-lysosomal fusion and respiratory burst[59, 77, 81, 82]. In some cases, Type II alveolar epithelial cells may engulf bacteria instead of alveolar macrophages[36, 83]. Another key cell in detecting bacterial invasion is the dendritic cell (DC), which comes soon after detection of infection by macrophages[36, 83]. The DC is essential for T-cell activation and thus plays a critical role in bridging the gap between the innate and adaptive immune response to *M. tuberculosis*[36, 83]. DCs are migratory however and may contribute to the dissemination of disease, and may play a role in lymphatic dissemination[36].

Phagocytes recognize and take up bacteria after detection of pathogen associated molecular patterns (PAMPs)[59]. These are detected by pattern recognition receptors (PRRs) like complement or mannose receptors[36, 77]. Surfactant proteins in alveolar surfaces can also increase uptake of *M. tuberculosis* by upregulating the presence of mannose receptor activity on

the surface of macrophages[36]. Another PAMP on *M. tuberculosis* is the 19-kDa lipoprotein contained in the bacterial cell wall that is recognized by a critical PRR, toll-like receptor (TLR)2[36, 59, 84, 85]. Macrophages have a number of defense mechanisms to combat invading organisms following phagocytosis and subsequent phagosome-lysosomal fusion[59]. The lysosome contains a highly acidic pH, reactive oxygen intermediates (ROIs), reactive nitrogen intermediates (RNIs), enzymes, and antimicrobial peptides[36, 77, 82]. It has been shown that mice deficient in the gene responsible for producing RNIs are more susceptible to intracellular pathogens[36, 86-89]. After detection and infection of alveolar macrophages by *M. tuberculosis*, the phagocyte begins chemokine production in order to recruit neutrophils, monocytes, and lymphocytes[82, 90]. These recruited cells are not able to effectively clear infection[82], and the immune response attempts to 'wall off' the bacterial infection by creating the beginnings of a granuloma[79, 81, 83, 91, 92]. These focal lesions consist mostly of macrophage-like giant cells and lymphocytes[79, 83, 91]. As more lymphocytes are recruited, infected macrophages are killed off causing caseous necrosis at the center of the granuloma, effectively containing the infection within this lesion[79, 83, 91, 92]. The outer perimeter of the granuloma contains lymphocytes, monocytes, and fibroblasts[79, 83, 93]. Within this granuloma, the bacteria can reside in a dormant state for decades though they generally do not replicate at this stage due to the hostile environment of the granuloma that contains low levels of oxygen, low pH, and toxic fatty acids[83, 93]. If the cellular immune response of the host is not robust enough to control infection, disease may progress further[83, 92]. If the immune response effectively contains the infection, it could remain in this latent stage indefinitely[83]. After the healing of granulomas a fibrous, calcified lesion remains[83]. However, if the individuals immune response is weakened due to immunosuppression from HIV infection, age, or other stressors, the center of the granuloma could become liquefied and provide and environment that permits unchecked bacterial replication which can spread to other parts of the lung or become widely disseminated[76, 83, 92].

Wallgren has described the pathogenesis of *M. tuberculosis* in four stages[36, 94]. The first stage begins 3 to 8 weeks following inhalation of infectious particles[36, 94]. Once the bacteria are implanted within pulmonary alveoli, they form the Ghon complex after dissemination to regional lymph nodes[36, 94]. Tuberculin reactivity begins after this stage[36, 94]. After a period of about 3 months the second stage commences which is indicated by dissemination of bacteria throughout the bloodstream, reaching other organs as well as other areas of the lung[36, 94]. If bacteria become disseminated, miliary tuberculosis may establish infection throughout the body, or meningitis may occur[36, 80, 94]. This is the stage in which acute infection or fatalities due to symptoms may occur[36, 94]. The third stage symptoms can range from 3 months to three years following stage 2 in which inflammation of the plueral cavity may become established causing chest pain[36, 94]. Following dissemination, bacterial fragments presented by APCs are detected by sensitized CD4+ T-cells which begin induction of pro-inflammatory cytokines[36, 94]. The final stage of disease may take up to three years but concludes with resolution of the Ghon complex, or primary complex, in which the disease progresses no further[36, 94]. It is at this stage that extrapulmonary lesions might progress, however this is not common and disease usually halts at this point[36, 94]. A small percentage of infected individuals become reinfected with the disease at some point later in their lives, this however is thought to be due to reactivation of the existing infection as opposed to additional exposure[36, 94]. However, 50% of HIV positive individuals who have been infected are at risk of recurrence of infection[36]. *M. tuberculosis* infection in adults almost always manifests as pulmonary disease and results in lung damage including necrosis, bleeding, and cavity formation[36, 94] .

Due to a number of virulence and evasion mechanisms that will be discussed, the bacteria ultimately escape macrophage phagosomes upon death of the cell and eventually escape into the bloodstream and the lymphatics[77]. Mechanisms of virulence of other intracellular bacteria have been hypothesized to be potential mechanisms that *M. tuberculosis* employ include: survival of phago-lysosomal fusion by inhibition of the fusion process, alternative

mechanisms of uptake that circumvent phago-lysosomal fusion, resistance to the contents of the lysosome, inactivation of anti-microbial enzymes, and escape into the cytoplasm[77, 82]. Studies by McDonough *et al*. have shown that degradation of mycobacterial membrane within the phagolysosome is comparatively slow which may be due to mycolic acids in the mycobacterial cell wall[77, 82]. This delay may be a virulence mechanism in which *M. tuberculosis* is able to 'hide' in the endocytic vesicle evading or delaying detection by CD4+ T-cells[77]. This group also displayed the ability of *M. tuberculosis* to bud out from fused phagolysosomes into vacuoles that created a tight membrane around the bacterium protecting it from secondary lysosomes[77]. This later resulted in an increased multiplication in the bacteria, which was not observed in attenuated strains such as BCG and H37Ra[77]. Another virulence mechanism of *M. tuberculosis* however is a resistance to clearance by RNIs, which is thought to be associated with mycolic acids in the bacteria cell wall[36, 58]. It has also been shown that *M. tuberculosis* can inhibit $Ca2^{+}$ signaling in human macrophages, which may be a mechanism of preventing phagosome-lysosomal fusion[36, 95]. There are a number of tools that can be used to determine virulence mechanisms; one example would be various *M. tuberculosis* mutants that are similar to clinical strains yet display impaired virulence. For example, one of these strains, *M. tuberculosis* SigH exhibits normal growth and survival but has reduced histopathology compared to the wild type (WT) strain[36, 96, 97]. To this day, virulence mechanisms remain largely uncharacterized and their theories remain controversial.

1.5 Immune Response to M. tuberculosis

1.5.1 Toll-Like Receptors

Innate immunity is the first line of defense specializing in non-specific recognition of invading pathogens. The innate immune system acts through highly conserved germ-line-encoded pattern recognition receptors (PRRs) which bind to pathogen-associated molecular patterns (PAMPs) i.e. foreign antigens on bacteria, viruses, and fungi[98]. In contrast, adaptive immunity recognizes very specific components of pathogens in association with major

histocompatability complexes (MHC) and is dependent on upon the innate immune response for initial exposure of these components[99].

The Toll protein (Toll) was first discovered in 1996 in the fruit fly *Drosophila melanogaster*. Toll in the fruit fly is a PRR, which recognizes microorganisms and fungi as well as having other functions. In vertebrates, Toll is referred to as toll-like receptors (TLRs) because of their homology and similarity to that of the receptor molecule in *Drosophila*. The first TLRs in humans were described by Janeway (1997) in which he explored the dependence of the adaptive immune response on the expression of various signals from antigen presenting cells (APCs) such as co-stimulatory signals and cytokine induction[100]. Janeway cloned and characterized the human homologue of Toll and determined that it is a type I transmembrane protein, which indicates a conserved defense pathway from *Drosophila* to humans that can induce signals for stimulating both innate and adaptive immunity in vertebrates[101].

Toll and TLRs also have a leucine-rich repeat (LRR) domain, and both have a cytoplasmic domain homologous to human interleukin (IL)-1 receptor[101-103]. The LRR motif provides the structural framework for protein-protein interaction that facilitate cell adhesion, cellular trafficking, enzyme inhibition, and hormone-receptor interactions[104]. Both the IL-1 receptor and *Drosophila* Toll signal through activation of the nuclear factor (NF)-κB pathway[101, 103, 105]. NF-κB induces the transcription of genes such as the co-stimulatory molecule B7.1, required for T-cell activation, and the pro-inflammatory cytokines interleukin (IL)-1, IL-6, and IL-8[101]. Since Janeway's discovery, nine other TLRs have been identified in humans and 13 in mice[106, 107]. Some TLRs such as TLR3,7,8, and 9 are expressed intracellularly in endosomal compartments[108]. Although some other cell types contain TLRs, the majority of TLRs are found on the surface of innate and adaptive cells[109]. Exogenous lipoproteins, lipids, and proteins from microbes are recognized by TLRs located on the cell surface[108-110]. Bacterial and viral nucleic acids are recognized by intracellular TLRs in endocytic vesicles[109, 111]. The expression of some TLRs differs between humans and mice[106]. For example, TLR2 expression in mice is low, but

constitutive in humans with the exception of T-cells. TLR3 is expressed in mice on dendritic cells (DCs) and macrophages and is induced by LPS, but in humans TLR3 is expressed only by DCs and is not induced by LPS. TLR9 is expressed on all myeloid cells, plasmacytoid DCs (pDCs), and B-cells in mice, but in humans TLR-9 is expressed only on B-cells, pDCs, and neutrophils[109]. TLR10 in mice is a pseudogene, whereas in humans it is widely expressed[106]. It has been shown that alveolar epithelial cells and alveolar macrophages express TLR2 which recognizes gram-positive PAMPs as well as a number of mycobacterial products including lipoteichoic acid, lipoproteins, and lipoarabinomannan[112, 113].

Recognition of PAMPs by TLRs results in increased production of anti-microbial peptides and pro-inflammatory cytokines demonstrating a critical role for TLRs in detecting and eliminating pathogens. Activation of TLRs in alveolar macrophages following lung infection results in greater phagocytic activity of the cell, increased oxidative burst, and the secretion of pro-inflammatory cytokines[112]. The connection between innate and adaptive immunity results from the interaction of PRRs like TLRs that activate and induce APC maturation such as macrophages and dendritic cells upon exposure to pathogens and PAMPs[109]. TLRs enable the activation of DCs which then migrate to lymph nodes where they prime of naïve T-cells, making TLRs and integral component of activation of adaptive immunity[109].

Upon macrophage activation, a signaling pathway is initiated by TLRs in which the adaptor molecule, myeloid differentiation primary response protein 88 (MyD88) plays a key role (except for TLRs 3 and 4)[114]. MyD88 links the TLRs that recognize *Mycobacterium tuberculosis* components and links them to the interleukin-1 receptor associated kinase (IRAK) which recruits TNF receptor-associated factor 6 (TRAF6), then TGF-β-activated protein kinase (TAK1), and mitogen-activated protein kinase (MAPK). NF-κB promotes transcription of the genes responsible for pro-inflammatory cytokine production such as tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8[101, 114].

Studies performed by Bafica *et al* have shown that TLR9 and TLR2 play an integral role in controlling *M. tuberculosis* infection, especially in cooperation with each other[115-117]. The mechanism by which this protection takes place was shown to involve TLR9 stimulating the production of key cytokines such as IL-12p40 and interferon (IFN)-γ[105]. Further, the critical role of TLRs in *M. tuberculosis* immunity was evidenced by studies in which MyD88$^{-/-}$ mice were challenged with *M. tuberculosis*[112, 118]. MyD88 is an adaptor molecule required for signaling by most TLRs[59, 108]. MyD88 $^{-/-}$ mice show increased susceptibility to aerosol infection with the pathogen[112, 118], similar to IFN-γ$^{-/-}$ mice[119]. MyD88$^{-/-}$ mice also showed decreased pro-inflammatory cytokine production, which resulted in decreased IFN-γ production and decreased nitric oxide synthase-2 (NOS2) expression[118]. When MyD88$^{-/-}$ APCs are co-incubated with *M. tuberculosis in-vitro*, a marked reduction in the synthesis of IL-12, TNF-α, and nitric oxide (NO) was observed[118]. The role of MyD88 in *M. tuberculosis* infection may be due to the fact that *M. tuberculosis* has been shown to contain some highly stimulatory CpG motifs that are recognized by TLR9[59, 120]. TLR9 is localized in endosomes and phagolysosomes and could be triggered following endocytosis of *M. tuberculosis* by APCs. It has been shown that TLR9 also plays a key role in the induction of Th1 immunity to *M. tuberculosis in-vivo*[115, 118].

The TLRs that play a role in *M. tuberculosis* recognition include TLR2, TLR4, TLR9, and possibly TLR8[113, 115, 120, 121]. *M. tuberculosis* glycolipids are recognized by TLR2 heterodimers combined with TLR1 or 6. TLR2 recognizes the glycolipids lipoarabinomannan (LAM), lipomannan (LM), and phosphatidylinositol mannoside (PIM)[59 120, 122, 123]. It also recognizes the glycoproteins 38-kDa, and 19-kDa, and triacylated (TLR2/ TLR1), or diacylated (TLR2/ TLR6) lipoproteins[59, 113, 120, 122]. TLR2 is involved with granuloma formulation, also initiates an innate immune response by inducing TNF-α and IL-12 production by macrophages[115, 120]. TLR2 and TLR6 were also implicated in stimulation of IL-1β production[59]. TLR4 plays a role in TNF-α production by macrophages and is also activated by heat shock protein 60/65 which is secreted by multiple *M. tuberculosis* species[124, 125]. It has been shown that TLR9, which recognizes

unmethylated bacterial DNA, also is a key component for induction of IL-12 production in DCs[115]. TLR9 has also been shown to stimulate IL-12p40 production beyond primary infection and extend production throughout secondary and chronic infection[126, 127]. Although TLR8 recognizes single-stranded RNA, protein expression of the receptor in macrophages has been shown to increase after BCG infection, and following *M. tuberculosis* infection in humans[128]. Redundant roles have been shown with TLRs, for example knocking out TLR2 or TLR9 alone did not significantly decrease protection compared to wild type animals, but when both were knocked out simultaneously defects in IL-12 and IFN-γ were apparent[115].

The role of each particular TLR in relation to *M. tuberculosis* infection is sometimes conflicting and unclear. For example, some studies have shown that although TLR2 may not play an essential role in pathogenesis of tuberculosis, it is important for defense against mycobacterial infection[117]. In this experiment, mice were infected with the Kurono strain of *M. tuberculosis* administering approximately 100 bacteria per animal[117]. The absence of TLR2 was not lethal to the mice, however, pro-inflammatory cytokines and their transcription factors were significantly reduced[117]. In other studies, it has been shown that TLR2 is solely responsible for pro-inflammatory cytokine production in response to H37Rv *M. tuberculosis* infection[84], and another found that TLR2 is required for macrophage killing of mycobacteria in both humans and mice[129].

Many vaccine strategies involve the targeting of various TLRs by including peptide antigens in subunit vaccines that strongly induce a robust immune response upon vaccination[108, 122]. CpG oligodeoxynucelotides (ODN) for example is recognized by TLR9 and is a strong inducer of immunity; in particular, the subtype CpG-C stimulates the proliferation of pDCs which produce large quantities of IFN-α[99, 105]. This adjuvant in conjunction with highly immunogenic *M. tuberculosis* antigens induces a protective effect in mice and guinea pigs upon challenge with H37Rv and is the basis of the current set of experiments.

1.5.2 Macrophages

Pulmonary infection with *Mycobacterium tuberculosis* is initiated with the inhalation of respiratory droplets from an individual with active tuberculosis infection[130-132]. Upon inhalation of infectious particles, the bacteria encounter the first line of cellular defense in the lung, the alveolar macrophage[59, 77, 85, 131, 133-136]. Bacteria reach these macrophages if other innate components of the immune system and mechanical barriers fail. Some of these mechanisms include the mucocilliary escalator to mechanically remove particles from the airway, antimicrobial peptides such as α and β-defensins produced by airway epithelia, and the collectin surfactant protein-A has been shown to stimulate TLR-4 signaling[137-140]. Other innate cells that play a role early during infection by destroying invading bacteria non-specifically include natural killer cells (NK), natural killer T-cells (NKT), and mucosal innate lymphoid cells (ILC)[139, 141]. These cells however, do not bridge the gap between innate and adaptive immunity as the alveolar macrophages do[142].

Bacteria that reach the depths of the alveolar sacs are then phagocytosed by the macrophage through a variety of cell surface receptors, including complement receptors, mannose receptors, CD14, scavenger receptors, and Fc receptors[85, 133, 143]. Upon infection, alveolar macrophages phagocytose *M. tuberculosis* bacteria and antigens are processed in the endosomal compartment of the macrophage and presented to recruited T-lymphocytes by major histocompatibility complex II (MHCII) molecules. Although CD8+ T-cells and even γ/δ T-cells play a role in immunity to *M. tuberculosis*[8], CD4+ T-cells are a dominant force in protection against infection, and they are activated by this antigen presentation and then in turn, activate the macrophage through stimulation with interferon (IFN)-γ[144, 145]. Upon activation, the macrophage is then able to inhibit the growth of the bacteria and kill the bacteria sequestered in phagolysosomes by releasing oxygen radicals and hydrolytic enzymes like lysozyme to degrade the bacterial cell wall. Activated macrophages also produce tumor necrosis factor (TNF)-α which inhibits bacterial growth[146] and works synergistically with IFN-γ to increase the ability of

macrophages to kill bacteria[147]. Eventually, granulomatous focal lesions begin to form in order to sequester remaining bacteria by macrophages forming epithelioid giant cells and foamy macrophages in conjunction with lymphocytes[82].

Activation of the host macrophage then leads to generation of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) thus allowing for direct bacterial killing[82]. Although many of the bacteria are effectively killed at this stage, bacteria remain and continue to multiply logarithmically within macrophages unable to promote bacterial killing[59, 114, 148]. T-cell mediated immunity at the site of infection is initiated by recruitment of primed antigen specific T-cells to the site of infection. T-cells undergo clonal expansion and multiply within early lesions or tubercles while releasing pro-inflammatory cytokines such as IFN-γ, which in turn activates more macrophages thus enabling more proficient killing of bacteria[82].

Bacteria are recognized by resident macrophages by the pattern recognition receptors (PRRs) on the host cell surface. The PRRs recognize pathogen associated molecular patterns (PAMPs) associated with the bacteria. A variety of PRRs are involved including toll-like receptors (TLRs), complement receptors (CRs), surfactant protein A (Sp-A) receptors, nucleotide-binding-oligomerization-domain (NOD) like receptors (NLRs), and mannose receptors (MRs)[114]. A major route of entry into host cells involves the uptake of bacteria via complement receptors and complement opsonization found on the alveolar macrophage cell surface[114]. Specific complement receptors involved include CR1, CR3, and CR4 on the macrophage cell surface thus promoting ingestion of the bacteria. During the secondary immune response, uptake of bacteria into host cells also involves opsonization with antigen specific antibodies via Fc receptors. This route actually promotes uptake of bacteria into the phagolysosome which may ultimately lead to death of the bacterium[149]. Although B-cells do not play a primary role in controlling infection, studies have shown that antibody may confer a protective effect[150, 151].

TLRs on macrophages are highly specialized in facilitating the uptake of bacteria into the host cell and also are responsible for initiating a signaling cascade that leads to the production of pro-inflammatory cytokines. The key TLRs involved in recognition of mycobacterial antigen includes TLR2, TLR4, and TLR9[90]. TLR2 forms heterodimers with TLR1 or TLR6 and recognizes various mycobacterial lipoproteins found on the bacterial cell surface[152]. It also interacts with lipomannan and phosphatidyl-myo-inositol mannoside (PIM)[90]. Upon macrophage activation, a signaling pathway is initiated by TLRs in which the adaptor molecule, myeloid differentiation primary response protein 88 (MyD88) ultimately results in the activation of the transcription factor nuclear factor (NF)-κB[153]. This transcription factor upregulates the genes responsible for pro-inflammatory cytokine production such as: TNF-α, interleukin (IL)-1β, IL-6, and IL-8[153, 154].

Once a TLR is activated, cell surface molecules such as Fc receptors are expressed, which can then bind to antibody, if present. When the macrophage engulfs the pathogen, it then sequesters it in a membrane-bound vesicle called the phagosome. Once the cell matures, the pH of the phagosome is lowered for acidification, which is mediated by a proton-ATPase complex associated with the membrane of the phagosome[155]. The invading pathogen is then transported to the acidified phagosome for killing. After fusion of the phagosome with the lysosome, the pH is then reduced to about 4.0 thus facilitating the degradation of phagocytosed pathogens[156]. Bacterial survival is dependent upon various virulence mechanisms that *M. tuberculosis* displays to prevent its destruction[148, 157]. One method is by preventing the acidification of the phagosome by selectively inhibiting the fusion of vesicles containing proton ATP-ase[82]. This allows a higher pH in the vesicles, reducing the bacteriostatic effect of IFN-γ as well. One study has shown the bacteriostatic effect of IFN-γ may be mediated through acidification of the phagosome through the activation of proton pumps[155]. *M. tuberculosis* prevents phago-lysosomal fusion by arresting phagosome maturation with the cell membrane component lipoarabinomannan (LAM)[156]. This glycolipid is not only a virulence factor of the

organism, but it also retains its structural integrity[158]. Glycolipids in the bacterial membrane provide protection by decreasing permeability and susceptibility to antibiotics as well as macrophage killing[158]. LAM prevents membrane trafficking and intracellular signaling by blocking the cytosolic Ca^{2+} increase[156, 159]. Other cell wall components that contribute to virulence and preservation of the organism include lipomannan (LM), phosphatidylinositol (PI), and phosphatidylinositol mannosides (PIMs)[158].

Upon engulfment of *M. tuberculosis* bacteria, macrophages produce an array of chemokines including CCL2, CCL3, CCL5, CCL7, CCL12, CXCL2, CXCL8, and CXCL10[160]. Chemokines are chemotactic cytokines produced by a number of cells including lymphocytes, monocytes, dendritic cells, and epithelial cells in secondary lymphoid organs or infected tissue. These chemical messengers play a role in the trafficking of lymphocytes to sites of infection, as well as cellular activation. Chemokines are generally named CC for the two N-terminal cysteine residues located at the N-terminus, and L or R depending on if it is the ligand or chemokine receptor[161].These proinflammatory chemokines promote microbicidal activity and trafficking of various cell types to the site of infection, and also play a key role in granuloma formation[162]. This chemokine profile recruits monocytes for further differentiate, promotes macrophage activation, attracts dendritic cells to the site of infection, and also recruits T-lymphocytes[163]. The chemokine CCL2 is a critical cytokine during infection as it plays a role in monocyte recruitment and macrophage activation. It has been shown that transcription factors like NF-κB signal through various TLRs to induce CCL2 production[164]. CCL5 is another key chemokine produced by macrophages, among other cells. This chemokine recruits DCs, T-lymphocytes, and other cells to the site of infection and has been shown to play a role in granuloma formation[165-167]. Overall, the host responds to infection by releasing an overabundance of chemokines that can cause recruitment of cells required to combat *M. tuberculosis*.

It has been shown that a Th1-type immune response is critical for control of *M. tuberculosis* infection[168]. The polarization of the immune response towards Th1 type immunity

is directed by IL-12 production predominantly by macrophages and dendritic cells[169-172]. The major cytokines involved with a Th1 response against *M. tuberculosis* include: IFN-γ, TNF-α, IL-1α/β, IL-2, IL-6, and IL-12[173-178]. Macrophages produce TNF-α; which is a key cytokine involved in granuloma formation, stimulating production of IL-1, IL-6[179], and early chemokines, as well as induction of RNIs and ROIs [89, 180-182]. Macrophages also produce IFN-γ, which is essential for antigen presentation and the activation of phagocytes[183, 184]. It is critical for the regulation of T-cells in response to *M. tuberculosis* infection and promotes cellular proliferation, apoptosis, cell adhesion, and granuloma formation[85]. IFN-γ contributes to the production of RNIs, ROIs, nitric oxide (NO), and induces a respiratory burst in macrophages, which plays a key role in killing of *M. tuberculosis*[89, 183, 185]. The cytokines TNF-α, and IFN-γ activate the enzymatic pathway controlled by inducible NO, that results in the production NO and other RNIs derived from L-arginine[89].

1.5.3 Dendritic Cells

Ralph Steinman and his colleagues first characterized dendritic cells (DCs) in 1972[186]. It was known at the time that an 'accessory cell' of some kind worked in conjunction with lymphocytes to execute an immune response but the nature of this cell was unknown[187, 188]. Steinman knew there were similarities between these cells and macrophages, but these 'accessory cells' were adherent to tissue culture surfaces, lacked Fc receptors on their surface, and exhibited unusual 'tree like' or dendritic processes[189]. In order to study these cells, which comprised only 1% of cells in the spleen, Steinman enriched these cells and cultured them for purity until enough were obtained for studies[190]. DCs were then found to originate in the bone marrow and reside in close proximity to T-cell areas of lymphoid tissue and they play a key role in T-cell activation[191]. After DCs are generated, they then enter circulating blood and later traffic to tissues where they become resident immature DCs[188]. At this stage, the immature DC expresses high levels of major histocompatability complex (MHC) II and are highly phagocytic

and endocytic, which makes them especially proficient at capturing antigen in peripheral tissues[192].

DCs are derived from hematopoietic stem cells and originate from both lymphoid and myeloid lineages; where myeloid DCs are also called 'classical' or conventional DCs and lymphoid DCs are called plasmacytoid DCs. Myeloid DCs express TLR2, 3, 4, and 7; and human myeloid DCs express the cell surface markers CD11c[193] and HLA-DR. Lymphoid DCs or plasmacytoid DCs (pDCs) are $CD11c^-$, only express TLR 7 and TLR 9, and produce large amounts of interferon (IFN)-α in response to viral infection[194]. By incubating stem cells or peripheral blood-adherent monocytes with interleukin (IL)-4 and granulocyte-macrophage colony stimulating factor (GM-CSF), DCs can also be derived *in-vitro*[195]. Another DC type is the Langerhans cell, which is found in an immature state in the epidermis and mucosal epithelia[196]. These cells are partly responsible for tissue graft rejection by sensitizing host T-cells after transplantation[197].

DC progenitors are derived in the bone marrow and travel through the blood to the skin, gut, airway epithelium, the lung, and interstitial spaces of multiple organs[198]. At this point the DC is immature and expresses cell surface molecules enabling optimal antigen uptake and antigen presentation; however, they are not yet able to initiate a T-cell response[192]. Upon uptake of antigen and exposure to inflammatory signals, DCs then migrate to secondary lymphoid organs via blood or afferent lymphatics where they encounter circulating T-cells[199]. After trafficking the DCs are 'activated' and have undergone a process of maturation that resulted in the partial loss of ability for antigen uptake and processing, and increased ability for antigen presentation and T-cell priming[197, 200]. This T-cell priming is enabled by secretion of pro-inflammatory cytokines such as IL-12[201], expression of elevated levels of MHC class I and MHC class II on the surface of the DC, as well as the expression of the co-stimulatory molecules B7.1 (CD80), B7.2 (CD86), and CD40[200]. The expression of IL-12 by DCs skews the immune response towards a Th1 type response characterized by IFN-γ producing T-cells[201, 202]. DCs are

one of the most potent antigen presenting cells (APCs), and are important for initiating primary T-cell mediated immune responses[203]. DCs provide the critical link between innate and adaptive immunity by acting as APCs that take up antigen and present peptides to circulating T-cells[204]. In the absence of DCs, T-cell activation was significantly impaired[205].

The major function of DCs sample antigens in the periphery through endocytic pathways associated with receptors such as the mannose receptor, DEC-205, DC-SIGN, Fc receptors, and complement receptors[206-209]. Other important receptors are TLRs, which recognize pathogen associated molecular patterns (PAMPs). After a DC takes up antigens, the cell migrates to the draining lymphoid tissue, where it matures possessing lower phagocytic capabilities, and expressing high levels of MHC class II, CCR7, and co-stimulatory molecules like CD80/86[210, 211]. This migration of DCs and T-cells to local lymph nodes is facilitated by the binding of the CC-chemokine receptor 7 (CCR7) to its ligands CC-chemokine ligand 19 (CCL19) and CCL21[211].

Immature DCs present large amounts of MHC-II molecules and adhesion molecules including CD11a (LFA-1), CD11c, CD50 (ICAM-2), CD54 (ICAM-1), CD58 (LFA-3), and CD102 (ICAM-3)[210, 212]. DCs also express the co-stimulatory molecules CD80 (B7.1), and CD86 (B7.2), which are only upregulated during activation[210, 213]. DCs become activated after encountering antigen and then mature into APCs[214]. Once DCs are activated they then secrete cytokines like IL-12, critical for Th1 polarization, and present cognate antigens to naïve T-cells thus priming them[214, 215].

Mycobacterium tuberculosis infects humans primarily in the lung, initially replicating within resident alveolar macrophages. DCs are also present in the airway epithelium and lung parenchyma where they are exposed to the *M. tuberculosis* bacteria[216, 217]. After acquiring antigen, the DC migrates to the local draining lymph nodes where they encounter naïve T-cells to prime and activate them by presenting peptides on MHC molecules[192, 204, 218]. Infection of *M. tuberculosis* is controlled by cell-mediated immunity, making antigen uptake by DCs and

subsequent T-cell activation a key event upon infection[192, 219]. It has been shown that *in-vivo* depletion of $CD11c^+$ DCs delays the CD4+ T cell response to *M. tuberculosis* and exacerbates the outcome of infection[205]. In addition to elevated cell surface molecules upon ingestion of *M. tuberculosis*, key Th1 type cytokines are also produced by the DC including tumor necrosis factor (TNF)-α, IL-1α and IL-1β, and IL-12[192]. One important event produced by DCs in *M. tuberculosis* follows the priming of antigen specific T-cells is production of the key cytokine, IL-12[201, 210, 214, 215, 218, 220]. In one study in which IL-12p40 deficient mice were infected with *M. tuberculosis*, lymph node (LN) migration was decreased when compared to wild type mice suggesting that IL-12 facilitated migration of DCs plays a critical role in controlling *M. tuberculosis* infection in part through affecting the expression of the homing receptor CCR7[218]. Treating DCs with IL-12p40 *in-vivo* restored migration to the LN[218]. DC migration is a crucial component of CD4 T-cell activation and is required for protection against *M. tuberculosis* infection[85, 215].

In order to combat clearance through cell-mediated immunity, *M. tuberculosis* employs various virulence factors derived from cell wall components such as, mannose-capped lipoarabinomannan, (ManLAM), and phosphatidylinositol mannosides (PIMs). Macromolecules like glycolipids from the mycobacterial cell wall induce strong divergent effects on DCs and PIM in particular achieves this effect by inhibiting DC cytokine responses[221].

In addition to T-cell activation and providing the link between innate and adaptive immunity, DCs are also responsible for creating immune tolerance and B-cell stimulation[194, 222, 223]. Another role for DCs may involve cross-talk between *M. tuberculosis* infected neutrophils which leads to DC maturation facilitated by the binding of DC-SIGN on DCs to Mac-1 on the neutrophil. The signaling of early response neutrophils facilitates the maturation of DCs and increases endocytosis of antigen that eventually leads to activation of T-cells in local draining lymph nodes[224]. pDC will be investigated in the experiments reported here, through activation of TLR9 to determine the importance of these cells in vaccine mediated immunity.

1.5.4 Lymphocytes

Thymocytes, T-cell precursors, originate in the bone marrow and migrate to the thymus for further differentiation and selection[225, 226]. B lineage cells, or B-cells also originate in the bone marrow, but unlike T-cells, B-cells remain in the bone marrow for differentiation as well[227]. Although the role of B-cells in *Mycobacterium tuberculosis* immunity remains controversial, the importance of B-cells is more apparent with every study that focuses on them[150, 228, 229]. Both B-cells and T-cells are distinguished by the receptors on their surface as well as their function[230-233]. T-cells undergo differentiation in the thymus as indicated by the cellular surface markers they express[234]. Initially T-cells are double negative (CD4-, CD8-), during further development they all become double positive (CD4+, CD8+), and finally they mature into single positive CD4+ or CD8+ T-cells[234]. After exiting the thymus cell CD4+ and CD8+ subpopulations each carry diverse functions. Different T-cell subsets include double-negative (DN/CD4-CD8-)[235], CD4+, CD8+, regulatory T-cells (Treg), natural killer T-cells (NKT), and γ/δ T-cells[230, 231, 236-238]. These cells are capable of recognizing a diverse range of microbial peptides, with a high degree of specificity[239]. Developing thymocytes undergo a process in which an antigen receptor (the T-cell receptor, TCR) is generated from the assembly of a composite set of genes with extensive diversity created by somatic recombination of many gene segments contained on one chromosome[240, 241]. Pathogen recognition by T-cells is dependent upon these diverse TCRs which are composed of heterodimers that form structures on the surface of the cell similar to antibody Fab-like structures, referred to as immunoglobulin like structures, and create different classes named either α/β or γ/δ T-cells[225, 238, 242-245].

There are other cellular phenotypes involved during infection as well, although not to the degree that CD4+ and CD8+ T-cells play a role. Many of these cells play a role in the innate immune response early in infection [246]. The role of these cells is to clear invading organisms, non-specifically, generally by binding via innate pathogen-associated molecular patters (PAMPs). Some of these cell types include neutrophils, NK cells[247], NKT cells, mucosal-

associated invariant T-cells (MAIT) and innate lymphoid cells (ILCs)[246, 248-250]. These cells play a role in early clearance of *M. tuberculosis* before an adaptive immune response is initiated [246]. Usually innate immunity is not sufficient to eliminate all of the pathogen and this is why adaptive immunity is generated. If early clearance is not achieved, an adaptive immune response then begins upon activation of T-lymphocytes and B-cells by antigen presenting cells (APCs). These cells act by killing of bacteria or infected cells, or by production of pro-inflammatory cytokines like interferon (IFN)-γ in response to binding of PAMPs [246].

After initial development in the thymus, a primary lymphoid organ (which also includes the bone marrow); naïve T-cells traffic through the blood to secondary lymphoid organs such as the lymph nodes, spleen, or mucosal lymphoid organ[161]. Once reaching secondary lymphoid tissue, they can then begin interacting with macrophages and dendritic cells (DCs) expressing major histo-compatibility complex (MHC) class II molecules presenting peptides, which can then be distinguished between 'self' and 'non-self'[231]. This circulation makes it possible for these highly specific cells to encounter their cognate antigens present on DCs. When the CD4+ T-cell encounters its cognate antigen presented by the DC, the T-cell becomes activated and undergoes a phenotypic transformation[242]. This activated cell, now an effector T-cell, can produce growth factors and undergo clonal expansion thus allowing the dissemination of antigen specific progeny to the site of infection.

TCRs recognize antigen by binding to their ligand, MHC molecules on the surface of phagosomal cells, which come in two forms: MHC class I, and MHC class II[244]. MHC class I molecules are present on most cells in the host and are recognized by CD8+ T-cells[244]. Professional antigen presenting cells including B-cells, dendritic cells (DCs), and macrophages present MHC class II molecules on their surface, which are recognized by CD4+ T-cells[225]. Professional phagocytic cells present peptides generated in their endosome and display these antigens on their surface bound to MHC molecules for recognition by CD4+ T-cells[244]. CD4+ T-cells (and CD8+ T-cells) are highly specific adaptive immune cells and play a key role in antigen

recognition[225]. The T-cell undergoes clonal expansion with signals from the cytokine interleukin (IL)-2 that is responsible for directing the growth and differentiation of T-cells[251]. Antigen presenting cells (APCs) present peptide antigen to T-cells, thus providing the link between innate and adaptive immunity, and activate the cell specific to the antigen being presented. The second signal required for activation of naïve T-cells involves the binding of the co-stimulatory molecules CD28 on T-cells to CD80 (B7.1) and CD86 (B7.2) on APCs[252, 253]. A third signal is necessary for T-cell maturity, and for CD8+ T-cells this is the presence of the cytokine IL-12 and for CD4+ T-cells IL-1β is thought to be necessary for activation[252, 254, 255].

Although the immune response to tuberculosis is a complex process involving many cell types, CD4+ T-cells play a dominant role[245, 256]. CD4+ T-cells, or T-helper cells (Th) circulate throughout the body searching for its cognate antigen presented on APCs and distinguish between 'self' and 'non-self' (antigen) peptides[225, 254]. Th cells direct antibody class switching in B-cells, direct the activation and growth of cytotoxic T-lymphocytes (CD8+ T-cells), and activate phagocytes such as macrophages to enable bactericidal effects of the cells[225].

Upon activation, CD4+ T-cells can differentiate into a number of subsets defined by their cytokine production profile. The main subsets include: Th1, Th2, and Th17 cells[244, 257]. In general Th1 cells produce IFN-γ, and support cell-mediated immunity[258, 259]. Th2 cells produce IL-4 and support humoral immunity[258, 259]. Th17 cells play a role in inflammatory and autoimmune diseases and are involved during early infection[260, 261].

The Th1 type immune response is thought to play the predominant role in controlling *M. tuberculosis* infection[8, 262-265]. CD4+ T-cells provide a protective immune response by activation of macrophages through antigen specific cytokine production that then enables intra-cellular killing of bacteria[262]. Although Th1 cells produce a number of inflammatory cytokines including IL-2, IL-6[266], IFN-γ, tumor necrosis factor (TNF)-α, and TNF-β[267, 268]; IFN-γ appears to be a key cytokine during *M. tuberculosis* infection[258, 262, 269]. IFN-γ mediates protection in mice by the induction of reactive nitrogen intermediates (RNIs) in the macrophage, which are essential for

the killing of intracellular mycobacteria[262]. It is generally accepted that cellular immunity, meaning T-cells and mononuclear phagocytes, provides protection against *M. tuberculosis* infection, as opposed to humoral immunity[144, 270]. The role of CD4+ T-cells has been shown by studies in which CD4+ T-cell depleted mice were infected with *M. tuberculosis*. Although it is widely accepted that CD4+ T-cells and the Th1 response plays a critical role in containing *M. tuberculosis* infection[8, 266], there is still more to be elucidated about the mechanism by which protection is conferred.

Numerous studies have demonstrated the critical need for CD4+ T-cells to control *M. tuberculosis* infection, showing that disabling CD4+ T-cells, mice and humans quickly succumb to infection[256, 271-275]. CD4+ T-cells are also crucial in maintaining control over time, and the antibody (Ab) depletion of CD4+ T-cells 6-months post-infection also reactivated disease and resulted in a fatal increase in bacterial burden[276]. Through Ab depletion[277] adoptive transfers[270, 278], or gene disrupted mice[279], it has been shown in multiple models that disabling CD4+ T-cell function is devastating to the *M. tuberculosis* infected mouse. CD4+ T-cells contribute to *M. tuberculosis* immunity in a number of ways. One important route is through activation of *M. tuberculosis* APCs and production of pro-inflammatory cytokines, thus recruiting more immune cells to the site of infection as well as enabling APCs to produce reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) to kill the *M. tuberculosis* bacterium[262, 277]. CD4+ T-cells are MHC class II restricted and are activated through signals from APCs, particularly macrophages and dendritic cells[149, 212]. Once activated, the T-cell then in turn activates the macrophage or dendritic cell, enabling *M. tuberculosis* killing, by secreting IFN-γ[280] and TNF-α[149, 212 281]. T-cells have also been shown to play a key role in containing infection independent of IFN-γ and NOS2 production[276]. Other possible mechanisms of protection involve binding of co-stimulatory molecules such as CD40L to CD40 on macrophages and dendritic cells although this is not a critical event[282]. Another possible mechanism of action for

CD4+ T-cells on *M. tuberculosis* infection could be apoptosis or lysis of infected cells with perforin and granulysin in humans as well as TNF-α production[283, 284].

CD4+ T-cells are also needed for B-cell function, however, the precise role of B-cells in *M. tuberculosis* infection is as of yet unclear[229, 285]. One study demonstrated the efficacy of conferring some protection using a monoclonal antibody against arabinomannan by possibly enhancing the cellular immune response against *M. tuberculosis*; with 30-60% survival after <75 days whereas untreated mice succumbed to infection within only 30 days[151]. Other monoclonal antibody treatments have also been shown to have an effect, which may be useful for prophylactic and therapeutic treatment of *M. tuberculosis*[228]. B-cells have also been shown to modulate responses of innate-like T-cell populations as well[286], and other studies have implicated B-cells as important facilitators of granuloma formation[151, 287]. One other important role of humoral immunity is the opsonization of *M. tuberculosis* bacilli allowing more efficient uptake of bacteria into alveolar macrophages via phagocytosis[288]. When bacteria are first opsonized, various virulence factors can then more effectively be overcome like the hindrance of phago-lysosomal fusion within the macrophage as well as preventing the acidification of the lysosomal compartment[288].

In studies performed by Sullivan *et al.*, it has been shown that knocking out the transcription factor, T-bet, results in loss of protection against *M. tuberculosis* infection as evidenced by increased bacterial burden following infection[289]. Since IFN-γ production is dependent upon the presence of T-bet, adequate immune response is not generated without this cytokine[289]. Recent studies by Sakai *et al.* have shown that as an alternative to the pivotal role that IFN-γ and T-bet signaling play in controlling infection, the ability of CD4+ T-cells to effectively enter the lung parenchyma is what is responsible to control infection[290]. This study showed that two subpopulations of CD4+ T-cells can be found in the lung parenchyma and vasculature and lung-homing ability is a key feature of protective CD4+ T-cells[290]. Another type of CD4+ T-cell, the T-regulatory (T-reg) cell, expresses the transcription factor FoxP3 and plays

a key role in regulating the immune response through the production of anti-inflammatory cytokines like TGF-β that prevent immuno-pathology by dampening the immune response after infection is under control[291].

Data regarding the importance of CD8+ T-cells[236, 275, 292], as well as a possible role of γ/δ T-cells in *M. tuberculosis* infection have been conflicting, however, it is clear that these cells indeed play an important role[238, 245, 272, 277, 293]. One reason why the role of CD8+ T-cells has been dismissed in the past is the location of *M. tuberculosis* in infected macrophages. It is generally thought CD8+ T-cells recognize cytoplasmic antigens more readily, and that the usually vacuole-bound bacteria would not be recognized by CD8+ T-cells[149]. In one study, the role of class-I restricted T-cells in resistance to *M. tuberculosis* infection was assessed[294]. By creating a targeted disruption in the β-2 microglobulin gene, MHC-I molecules are not formed, and mice were unable to generate functional CD8+ T-cells. Upon infection with *M. tuberculosis*, 70% of β-2m$^{-/-}$ mice either succumbed to infection, or expressed signs of severe morbidity, whereas WT mice remained viable for more than 20 weeks[294]. Another study using adoptive transfer for a model also suggested CD8+ T-cells play a role in controlling *M. tuberculosis* infection, particularly through mechanisms involving pro-inflammatory cytokine production as opposed to cytotoxicity[270, 292]. These findings were further confirmed using the CD8α$^{-/-}$ and other CD8+ T-cell impaired models[295].

1.5.5 Cytokines and Chemokines

The immune system is a complex network of cells and chemical messengers that interact to provide protection against many invading pathogens. One component of this network includes the chemical messengers, cytokines and chemokines, which are a diverse group of proteins that act as messengers between cells and direct cellular function[161, 296]. There are multiple categories of cytokines including monokines produced by mononuclear phagocytic cells, lymphokines produced by activated lymphocytes like T-helper cells, and interleukins (IL) that act as mediators between leukocytes[296].

Cytokine synthesis is initiated by gene transcription of short lived mRNA as cytokines are not stored as preformed proteins[296]. Cytokines are produced as a result of infection and dissipate upon resolution. Some cytokines are pleotropic and can be produced by multiple cell types, and can act on multiple cell types. In addition, many cytokines play a redundant role and perform many overlapping functions. Cytokines are able to act on cells displaying ligands for those specific cytokines, binding of cytokines to their ligands results in signal transduction which initiates a response in the cell like activation, or subsequent cytokine production in response[296]. Some cytokines can be antagonistic and oppose the functions of other cytokines such as IL-10, which is anti-inflammatory and can modulate the effects of pro-inflammatory cytokines[297, 298]. Conversely, cytokines can act synergistically to create a greater effect than cytokines working separately, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ synergy in a Th1 type immune response. Cytokines also play an important role in homeostasis of the immune system, creating a balance between immunity and immunopathology[299].

Resistance to *Mycobacterium tuberculosis* infection requires the interaction between antigen-specific T-cells, and macrophages, however, without cytokines these cells cannot traffic or interact[149]. Cytokine production in mycobacterial infection is a double-edge sword in that prolonged pro-inflammatory cytokine production is associated with chronic infection and pathology[114, 300], however, cytokines are absolutely necessary for a proper immune response for any pathogen, and *M. tuberculosis* is no exception[149].

Exposure to *M. tuberculosis* induces the production of a number of cytokines including: interleukin (IL)-1, IL-6, IL-10, IL-12, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β[149, 172, 180, 210, 263, 301-305]. Upon infection, alveolar macrophages begin to engulf the bacteria, and in some cases various virulence mechanisms of the organism prevent the macrophage from killing bacteria and replication occurs[52].

The immune response to the bacterium is complex, but begins in infected lung tissue where phagocytic cells such as macrophages and dendritic cells (DCs) take up bacterial

antigens and produce the pro-inflammatory cytokines IL-1, and IL-6 to recruit immune cells to the site of infection[149, 212, 297]. IL-1 has also been shown to play a role in protection against *M. tuberculosis* infection, and granuloma formation[306 177, 305]. DCs then take antigens to local draining lymph nodes to present them to T-cells on major histocompatability complex I, and II (MHC I and MHC II) molecules while producing IL-12[149, 163]. The T-cell then activates the DC and traffics to infected tissue to subsequently activate macrophages by secreting IL-2, TNF-α, and IFN-γ and other cytokines[149]. Once activated, macrophages acquire the ability to kill bacteria more effectively, and DCs become better able to present antigen to T-cells and activate them by expressing co-stimulatory molecules[203].

A Th1-type immune response and cell-mediated immunity is necessary for control of *M. tuberculosis* infection[220, 265]. A Th1 immune response is defined by pro-inflammatory cytokines, and is involved in controlling intracellular infections[259, 307, 308], whereas some Th2 cytokines are generally effective against extracellular organisms but are also responsible for symptoms of allergies[259, 308, 309]. The Th1 cytokine profile involves the production of specific cytokines by $CD4^+$ T-cells[258, 269], including: IL-2, IL-12, IL-18, IL-27, IFN-γ, TNF-α, and TNF-β[258, 310, 311]. Th2 cytokines include IL-4, IL-5, IL-10, and IL-13[258, 310, 311]. Further supporting evidence of the need for a Th1 response as opposed to a Th2 response was demonstrated by the experimental infection of *M. tuberculosis* in mice and observation of cytokine profiles in humans[192, 263, 265, 308]. In these studies, Th1 cytokines are produced in response to infection, and mice lacking these cytokines showed decreased survival compared to wild-type mice. Another study supporting a Th1 vs. Th2 response during infection is demonstrated by the observation that little amounts of IL-4 mRNA were present in infected mice and IL-4 deficient mice still remain resistant to infection[263, 312].

Type I interferons (IFN-α, and IFN-β) inhibit viral replication in cells and are produced by many cell types including macrophages, conventional DCs (cDCs), and plasmacytoid (pDCs)[313]. pDCs are major producers of Type I IFNs in response to viral infections and cDCs

specialize in antigen presentation to T-cells[314]. Type I IFNs also increase expression of MHC class I in infected cells that then makes them more easily recognized by $CD8^+$ cytotoxic T-cells (CTLs) as being infected. Type I IFNs have been shown in some cases to work antagonistically against IFN-γ and therefore may actually reduce resistance to *M. tuberculosis* infection[315], however; other studies have shown that Type I IFNs may play a role in cytosolic recognition of mycobacteria that disrupted the membrane of the phagosome[316]. Some studies have shown that Type I IFNs play a role in recognition and clearance of infection[317]. One possible pathway in which this could be utilized is by stimulation with CpG oligodeoxynucleotides that are recognized by toll-like receptor (TLR)-9 in antigen presenting cells (APCs) and in response, pDCs produce IFN-α[316, 318], resulting in protection.

Another important cytokine, **IL-6**[266], is produced by Th1 cells, fibroblasts, monocytes, macrophages and other cell types[319]. This pro-inflammatory cytokine facilitates hematopoiesis, activation of T-cells, and the differentiation of B-cells[319, 320]. When IL-6 $^{-/-}$ mice are challenged with *M. tuberculosis* infection, bacterial burden is significantly increased in the lung, and mice quickly succumb to infection[266]. It was also shown in the spleen cells of infected mice that IFN-γ production is significantly impaired and IL-4 is upregulated, one possible reason for this could be the critical importance of pro-inflammatory cytokine production stimulated by the presence of IL-6[266]. Conversely, in another study, after an immune response is initiated, IL-$6^{-/-}$ mice are capable of controlling infection and surviving[321]. That study however also confirmed the importance of IFN-γ production stimulated by the presence of IL-6 early in infection[321]. This difference in results could be due to the experimental models chosen, for example, in the first study H37Rv *M. tuberculosis* with intra-venous inoculation was used, but in the following study the Erdman strain was used via aerosol route.

IL-12 is a key cytokine in innate immunity and has been shown to play a critical role in controlling *M. tuberculosis* infection[171]. It is produced by activated macrophages, DCs, and B-cells; and stimulates the production of IFN-γ and causes the differentiation of naïve T-helper

cells into Th1 cytokine producing cells[322, 323]. The driving force for development of a Th1 response after exposure to the bacteria is the production of IL-12 by macrophages and DCs after bacteria has been phagocytosed[169, 201, 202, 310, 323-325]. It has been shown that IL-12 administration in mice results in a decreased bacterial load and increased survival time[301]. IL-12 is composed of multiple genetic subunits, the most bioactive of which are IL-12p40, and IL-12p70 which sometimes form a heterodimer with IL-12p35[326]. When IL-12p40-gene-deficient mice are challenged with *M. tuberculosis*, higher bacterial loads and decreased survival time were observed, thus supporting the critical need for this cytokine, and the role it plays in IL-12-dependent IFN-γ production[169, 301, 323].

TNF-α is produced by activated macrophages and is an important mediator of inflammation. Macrophages, DCs, and T-cells all produce TNF-α in response to *M. tuberculosis* infection[172, 192, 281, 323]; and TNF-α is a Th1 cytokine involved in many roles during infection such as inducing the expression of cell adhesion molecules involved in the extravasation of neutrophils and other macrophages to sites of infection[327]. It also initiates the inflammation and fever associated with infection, in conjunction with other cytokines. It has been shown that the absence of TNF-α is associated with reactivation of latent *M. tuberculosis* infection[328]. It is necessary for control of acute infection[321, 329], but in the same token may be implicated in immunopathology related to excessive inflammation[180]. TNF-α works synergistically with IFN-γ in multiple functions including: granuloma formation, mediating macrophage activation, as well as in the induction of NOS2 expression[89, 147, 180, 321, 329-332]. In TNF-α knockout mice, granuloma formation was impaired with mice unable to contain infection[180, 181, 329, 333], cellular recruitment for macrophages and lymphocytes was inhibited, and the deficiency ultimately led to the rapid death of infected mice[180].

The cytokine **IFN-γ** also plays a critical role in *M. tuberculosis* infection and studies have shown that infection cannot be controlled in experimentally infected animals when this cytokine is depleted[89, 334-336]. It plays a role in both innate and adaptive immunity and directs T-helper

cells towards a Th1 type immune response. IFN-γ is a pivotal cytokine for controlling infection and is produced predominantly by NK cells, $CD4^+$ and $CD8^+$ T-cells during infection[280, 310, 337]. IFN-γ production is IL-12 dependent, which is produced by *M. tuberculosis* infected alveolar macrophages[323]. IFN-γ plays a role in macrophage activation, restricting bacterial growth, granuloma function, and NOS2 expression in macrophages[89, 334-336]. Although IFN-$\gamma^{-/-}$ mice are still able to form granulomas, infected macrophages and arriving monocytes cannot be adequately activated resulting in tissue necrosis and severe dissemination of disease[184]. It has been shown that disruption of the IFN-γ gene resulted in the loss of ability to contain or control a normally sublethal dose of bacteria in mice, further demonstrating the necessity of this key cytokine for protective cellular immunity upon infection[184]. Further support of the necessity for IFN-γ was shown in studies undertaken to evaluate genetic mutations in related individuals that showed the inability to control *M. tuberculosis* infection. In this study, four children from a family all suffering from severe tuberculosis disease with the inability to clear infection were genetically tested for abnormalities relating to the immune response[328]. Microsatellite analysis revealed a defect in the gene for IFN-γ receptor-1[328], and verified by immunofluorescence analysis for this receptor. Macrophages no longer produced TNF-α in response to IFN-γ, suggesting that defects in IFN-γ signaling resulted in the loss of the ability for the immune system to control intracellular pathogens such as mycobacteria[328].

IL-10 is an anti-inflammatory cytokine produced by activated macrophages, monocytes, and T-helper (Th) 2 cells. Anti-inflammatory cytokines provide balance to an immune response that mediate excessive inflammation and immuno-pathology caused by chronic expression of pro-inflammatory cytokines such as TNF-α and IFN-γ[297, 338]. IL-10 downregulates the expression of MHC class II on macrophages, moderates macrophage activation, and inhibits IFN-γ production by down-regulating IL-12 secretion by macrophages[298, 323, 338-340]. IL-10 can also perform an auto-regulatory role where monocytes mediate their own functions[298]. This cytokine is important for later stages of infection to moderate excessive inflammation[339]. It is also

exploited by intracellular pathogens like *M. tuberculosis*, and can ultimately be detrimental to the host, used by virulence factors of the bacteria causing the downregulation of pro-inflammatory cytokines leading to further dissemination and exacerbation of disease[297].

TGF-β is another important anti-inflammatory cytokine that regulates the proliferation of T-cells and activation of macrophages. This enables a system of 'checks and balances' in the immune response which prevents immunopathology from occurring as a result of excessive inflammation, or it can be induced as a virulence mechanism of *M. tuberculosis*[341, 342]. TFG-β is produced by T-regulatory (T-reg) cells, T-cells, and monocytes from infected individuals as well as other cell types such as Langhans giant cells and epithelioid cells located in granulomas[343, 344]. Following exposure to *M. tuberculosis*, this cytokine inhibits macrophage activation by inhibiting NOS2 production induced by IFN-γ, and inhibits T-cell and B-cell responses[302, 345]. It displays auto-regulatory functions that limit T-cell clonal expansion, downregulates IL-1 and IL-2 dependent T-cell proliferation, and ultimately promotes tissue repair following infection or injury[341, 344, 345].

Chemokines are chemotactic cytokines produced by a number of cells including lymphocytes, monocytes, DCs, and epithelial cells in secondary lymphoid organs or infected tissue. These chemical messengers play a role in the trafficking of lymphocytes to sites of infection, as well as cellular activation. Chemokines are generally named CC for the two N-terminal cysteine residues followed by L or R depending if it is the ligand or chemokine receptor, or CXC denoting an amino acid residue separating the cysteins[161]. During infection, chemokines are induced by TNF-α activity to direct cellular trafficking of immune cells during both innate and adaptive immune responses[346]. Following exposure to bacteria, the chemokine CCL2 (monocyte chemoattractant protein-1 [MCP-1]) recruits immature DCs, macrophages, and monocytes to the site of infection and also directs cellular trafficking to the lymph node[347, 348]. It has been shown that without CCR2, which is the receptor for MCPs, mice quickly succumb to infection due to the impaired ability to recruit monocytes and macrophages to the site of

infection [347]. Following infection, alveolar macrophages produce the chemokines CCL3, CCL4, and CCL5 as well as cells expressing their receptor, CCR5[349, 350]. It has been hypothesized that CCR5 directs DCs to and from the lymph node during infection[346, 349]. Upon exposure to *M. tuberculosis* antigen Hsp70, naïve DCs become activated and begin producing the cytokine IL-12, which is mediated by CCR5[346, 351]. CCR7 is also expressed following infection and may be induced to recruit DCs to local draining lymph nodes[346]. MCP-1, produced by macrophages and monocytes, also recruits monocytes to the lungs in response to infection and assists with granuloma formation[352, 353]. CXCL-8 (IL-8) is an early chemokine, produced by macrophages in response to lipoarabinomannan (LAM), or following phagocytosis of bacteria[90]. IL-8 primarily attracts neutrophils to the site of early infection and also recruits T-lymphocytes and monocytes. Other cytokines like TNF-α and IL-1β influence production of IL-8[90, 352]. Early recruitment of inflammatory cells such as neutrophils, lymphocytes, and macrophages are involved with granulomatous lung inflammation and is the first step in the immune response against *M. tuberculosis*[354].

1.6 Vaccine Strategies

1.6.1 Background

The advent of vaccine development began in 1796 when Edward Jenner inoculated an 8 year old boy against smallpox by scratching cowpox virus (vaccinia) into the skin, which resulted in successful protection against challenge with virulent virus[355, 356]. Because of this successful demonstration, vaccination programs became widely used throughout the 19th century, and branched out to many other organisms[356]. By the early 70's the eradication of smallpox was in sight, and in 1974 the WHO began a program to take aim at other infectious diseases through vaccination including diphtheria, pertussis, tetanus, measles, poliomyelitis, and tuberculosis[356]. By 2012, the WHO's forecasted budget for immunization work that year reached $380 million[357] showing the ever increasing need for vaccination programs, and research and development. Vaccines have progressed since the first vaccination in 1796 from whole cell to live attenuated,

to inactivated or killed bacteria and viruses, recombinant, toxoid, and other subunit vaccines[355, 358].

1.6.2 Whole Organism Vaccines

Whole-organism vaccines consist of either live attenuated or killed bacteria or viruses[355]. Live-attenuated vaccines are generally created by producing a mutant strain of the original pathogenic microbe that has lost its virulence factors rendering it benign. For example, bacillus Calmette-Guérin (BCG) is a live-attenuated vaccine in which *Mycobacterium bovis* was subjected to 230 subcultures in potato-bile medium over the course of 13 years[359]. By the end of that 13 years, BCG was safe enough to administer to children, and has never reverted back to virulence to this day[359]. Other attenuated and killed viral or bacterial vaccines in use today include measles, mumps, rubella, and the polio vaccine[355, 360]. Inactivated vaccines contain viruses or bacteria that have been killed with heat, radiation, or chemicals[361]. The overall risk in using a live-attenuated vaccine is the possibility of reversion of the organism back to virulence in the form of a wild-type strain, and this poses a threat especially to immunocompromised hosts[355]. However, killed-vaccines cannot replicate and are non-infectious but they are not nearly as immunogenic as live-attenuated vaccines are, and thus require booster injections or the addition of an adjuvant, thus complicating inoculations[355].

Some whole-cell vaccines currently being tested as potential vaccine candidates include: VPM1002, a recombinant BCG expressing listeriolysin[362, 363], RUTI- a fragmented *M. tuberculosis* immunotherapeutic[364, 365], MTBVAC- genetically attenuated *M. tuberculosis*[366], and whole-cell *M. vaccae*[367] and *M. indicus pranii*[358, 368]. MTBVAC has made it to Phase I human clinical trials, RUTI and VPM1002 are in Phase II, and *M. vaccae* has progressed all the way to Phase III human clinical trials showing the most promise.

1.6.3 BCG

Bacillus Calmette-Guérin (BCG) is the only vaccine currently available for the prevention of *Mycobacterium tuberculosis* infection[369, 370]. BCG was prepared from live *Mycobacterium bovis* originally isolated by Nocard from the udder of an infected cow[359, 370]. In 1908 at the Pasteur Institute in France, Albert Calmette and Camille Guérin began sub-culturing virulent strains of *M. bovis* on media containing a mixture of glycerin, bile, and potato for the purpose of developing a vaccine[371]. In 1919 it was discovered that the bacteria, over many passages, were attenuated and would not cause disease in research animals[371]. The attenuation of the bacteria, it was later observed, occurred when various virulence factors of *M. bovis* were lost over multiple passages[372].

The vaccine was first tested in clinical trials from 1921 to 1927 in France and Belgium and showed protection against tuberculosis infection when administered to children[370]. Because of the success of BCG vaccination in children[369, 373], an oral vaccine given to children in milk[370] became popular in France until 1930 when the vaccine was contaminated with virulent *M. tuberculosis* in an event named the Lübeck disaster in which 67 babies succumbed to infection after being given the BCG vaccine [5, 370]. Initially a tuberculin skin test was required prior to vaccination to ensure individuals were not already infected with *M. tuberculosis,* because it was thought that vaccination after a previous exposure would result in adverse side effects. A tuberculin test, or Mantoux test, is performed by injecting a small amount of liquid containing protein derived from *M. tuberculosis*, called tuberculin, into the arm. A positive reaction will be reached in 48-72 hours indicated by induration and swelling at the site of injection. This increased the time and cost of vaccination until later studies confirmed the safety of BCG vaccination in previously infected individuals which then lead to the ability for mass vaccination campaigns including areas in which *M. tuberculosis* is endemic[5].

Today, BCG is the most widely used vaccine globally with more than 3 billion people having been vaccinated since 1921. Although the efficacy of the vaccine and the reasons for it

remain debated to this day, BCG has been at least partially credited for ending the *M. tuberculosis* epidemic in Europe. The United States and much of Canada are the only countries in which country-wide vaccination is not employed, this is due to the fact that the Mantoux test, which determines *M. tuberculosis* exposure, becomes positive after BCG vaccination[370]. Because the number of affected individuals is so low in these countries, only high-risk individuals like health-care workers are vaccinated[370]. Since the vaccine is a live-attenuated bacterium, individuals with compromised immune systems are advised against being vaccinated.

The attenuation of *M. bovis* and BCG is a result of the loss of multiple virulence factors over time. The genetic differences are characterized by the loss in various segments in the bacterial genome called regions of difference or RD, including RD1 through RD16, nine of these regions are completely absent in both BCG and *M. bovis* compared to the parent strain of *M. tuberculosis*[374, 375]. RD1 in particular is responsible for the original attenuation of BCG developed by Calmette and Guérin[374, 376]. While *M. tuberculosis* contains the RD1 region, which encodes for immunodominant antigens like 6kDa early secretory antigenic target (ESAT-6) and culture filtrate protein (CFP)-10, BCG does not. Reintroducing these genetic regions reverts BCG back to virulent tubercle bacilli[376]. The attenuation of BCG is thought to be due to a regulatory mechanism. Since some antigens, like ESAT-6 is present in *M. tuberculosis* but not in BCG, it can be used for a diagnostic test capable of distinguishing between BCG vaccination and *M. tuberculosis* infection.

The success of BCG vaccination for pulmonary *M. tuberculosis* infection has proved to be widely variable with an efficacy rate ranging from 0-80%[377, 378] and is generally most effective when given to children[359, 369]. When widespread vaccination campaigns were initiated, seed lots for BCG were not yet standardized and it has been hypothesized that differences in genetic and antigenic composition may contribute to the variable efficacy of the vaccine[369, 379]. The BCG vaccine does however have a higher rate of success when vaccinating against tuberculous

meningitis and miliary tuberculosis in children. The high variability of vaccine efficacy could be attributed to a number of factors that have been hypothesized to include the conditions in which the BCG was cultured and the various strains being used that were created by attenuation in varying conditions, genetic differences in a population and exposure to non-tuberculosis mycobacterial species[5, 359, 369, 375, 379, 380].

Although the efficacy of BCG for preventing *M. tuberculosis* is variable, the vaccine nonetheless has a very effective adjuvant effect[381]. This is likely due to the many immunostimulatory antigens that the bacteria is composed of that both target cellular and humoral immunity[382]. Upon stimulation with BCG a marked increase in antibody production and CD4+ T-lymphocytes is observed as well as pro-inflammatory cytokines like IFN-γ, and activation of macrophages[382, 383]. BCG administration has also been shown to be successful as a treatment for other diseases like bladder cancer[384], leprosy[359], Buruli ulcer disease[385], and Type I diabetes[386].

Among the many types of vaccine strategies are currently being developed, some strategies include BCG in their protocol. For example, prime-boost strategies might be employed, which involves vaccination with a sub-unit vaccine for one inoculation and BCG for the other[387]. This may increase efficacy by stimulating the immune response in different ways that BCG alone is unable to. In addition, recombinant BCG strains have been developed and are currently being tested which involve genetic modifications to BCG that may increase its efficacy through increased antigen presentation or modification of key regulatory molecules[387]. Other methods of administration, such as aerosol inoculation of BCG provide promising results[388]. Mucosal administration may provide superior protection than the parenterel route due to the nature of the organism[389, 390]. Since pulmonary *M. tuberculosis* infects upon inhalation of bacteria via respiratory route, cells are primed locally in the mucosa, thus providing a superior immune response by targeting surfaces where infection actually occurs[389, 390]. In this particular vaccine strategy however, BCG may pose a threat to immunocompromised individuals when

administered as a live vaccine due to its potential for dissemination[388]. To solve this issue, inactivation of the bacteria via heat-attenuation, or more recently tested, gamma-irradiated BCG may be employed[388].

Upon vaccination, BCG stimulates a potent cell-mediated immune response, which is required for mounting protection against *M. tuberculosis*, and has also been shown to stimulate humoral immunity. This response involves the recruitment and activation of proliferating T-cells[383] and improved T-cell homing to local lymph nodes. Various routes of inoculation can be employed to acquire the best immune response and intra-dermal inoculation is recommended and widely used[391]. It has been shown that mucosal vaccination results in similar efficacy[392], and may result in improved alveolar macrophage activation. One other caveat with the usage of BCG is that is causes a positive reaction to the Mantoux or tuberculin test. Since this makes distinguishing vaccination from *M. tuberculosis* infection difficult, other vaccine strategies not containing BCG may provide an overall diagnostic benefit. Despite the robust immune response generated by BCG, much improvement needs to be made in the field and it is possible the answer may lie in other vaccine strategies like sub-unit vaccinations involving individual antigens and stimulatory adjuvants.

1.6.4 DNA Vaccines

Another vaccine strategy is the use of plasmid DNA. DNA vaccines typically contain an antigen encoding gene regulated by a promoter that is incorporated into the bacterial DNAs plasmid backbone[393, 394]. Organisms like *E. coli* are altered to encode for vaccine antigens, and when administered can produce large amounts of pure DNA *in situ*[355, 395]. Because DNA vaccines circumvent the need for live bacteria or virus, they are safe to administer[395]. Plasmid DNA can elicit potent humoral and cellular mediated immune responses through potent major histo-compatibility complex (MHC) I presentation[395]. Depending on antigen and dose, DNA vaccines could provide a robust Th1 type immune response, ideal for pathogens like *M. tuberculosis*[395-397].

Another benefit of inoculation with DNA vaccines is the inherent adjuvant effect they possess because of the robust stimulation of the immune response by unmethylated CpG oligodeoxynucleotides motifs[395]. CpG is a series of alternating cytosine and guanine residues mimicking bacterial DNA and potentiating a strong immune response both *in-vitro* and *in-vivo*[393, 398-401]. When administered, these motifs stimulate pro-inflammatory cytokine production like IL-6, IL-12, and IFN-γ, driving T-helper cell differentiation towards a Th1 response[393, 398, 402, 403].

1.6.5 Sub-unit vaccines

Chimeric antigens can be created by performing a genetic fusion between two or more immunogenic antigens[355, 404-406]. This creates a broader immune response by combining multiple antigens at a time into one subunit vaccine[355, 404-406]. This process avoids the need to couple the antigens chemically, which can alter the effects of the antigens, and it also simplifies steps for recovery of the proteins and increases purity[355, 404-406].

Subunit vaccines generally require the presence of an adjuvant to stimulate a robust immune response at the time of vaccination. Generally when an entire microbe is introduced as a vaccine, such as a whole cell vaccine, an adjuvant is not required because the microbe contains many antigens and immune stimulating molecules that stimulate a robust immune response.

The first subunit vaccine in the U.S. was the Hepatitis B vaccine in 1981[355, 407], and it contained a viral antigen obtained from the serum of infected individuals. It was later replaced with a vaccine that did not contain human serum in order to prevent accidental infection with HIV. In 1982 a new vaccine was licensed using recombinant yeast cells that created the antigen.

Some current subunit vaccines that have shown promise in animal models and clinical trials include M72 a subunit fusion protein expressing antigens Rv1196 and Rv0125; Hybrid 1 (H1+IC31) expressing Ag85B and ESAT-6; Hybrid 4/Aeras-404 (H4: IC31) expressing Ag85B and TB10.4; Hybrid 56/Aeras-456 (H56:IC31) with Ag85B, ESAT-6, and latency antigen

Rv3875; M72+AS01$_E$ containing antigens 32A and 39A in adjuvant[408-410]; and ID93 expressing four different *M. tuberculosis* antigens associated with both active and latent disease[358, 411]. These protein/adjuvant combination vaccines have shown some promise with ID93+GLA-SE in Phase I clinical trials; H1, H4, and H56 have all reached Phase II clinical trials, and M72+AS01$_E$ has shown the most promised reaching Phase III human clinical trials[411].

1.6.6 Adjuvants

An adjuvant is a substance or a compound that enhances an individual immune response and might, for example stimulate cytokine production or cellular recruitment to the site of vaccination. Once these immune cells are deployed, the antigen of choice is then taken up by phagocytic cells that travel to local draining lymph nodes where antigen presentation to naïve lymphocytes occurs. In order to achieve a memory immune response three signals need to occur. The first signal, requires an initial detection of non-self molecules by innate receptors called pattern recognition receptors (PRRs), for example, the toll-like receptor (TLR) present on the surface of professional antigen presenting cells[412]. The second signal requires the presentation of antigen on one of these receptors on a phagocytic cell, namely the dendritic cell (DC), which then presents this antigen to a T-cell on the major-histo compatibility (MHC) molecule. And lastly, a co-stimulatory signal is required for activation of this T-cell by the interaction of co-stimulatory molecules present on the DC and T-cell like CD80/86.

There are only a few adjuvants available today that are approved by the FDA in the United States. These adjuvants include alum (aluminum salts), monophosphoryl lipid A (MPL), and oil-in-water emulsions like AS03 found in the flu vaccine, which is a squalene derivative[413, 414]. Currently there are a number of vaccines that contain alum including DTP, Hepatitis, HPV, Anthrax and Rabies to name a few. These adjuvants are relatively safe, however, most of them only stimulate one particular arm of the immune system[414]. In particular they usually stimulate a B-cell, or antibody response, or a Th2 type response, which is a part of the CD4+ T-cell response that induces specific cytokine production[414]. However, this type of immune response

is not always optimal for protecting against every type of pathogen that might require a Th1 type immune response for example which has a different cytokine profile[414]. There are many adjuvants currently being designed, tested, and optimized to stimulate a variety of immune responses.

Unmethylated DNA is one example of an excellent adjuvant that has been shown to stimulate a potent immune response in mice and other research animals[415]. The PRR for bacterial DNA is TLR9, which is present in the endosomal compartment of professional antigen presenting cells[416]. CpG DNA and its subtypes are one particular category of unmethylated DNA that potently stimulate professional antigen presenting cells through TLR9 and results in activation of the Th1-type immune response[398, 417, 418].

Other components can be added to a subunit vaccine in order to make it more effective, particularly if the antigens being given can easily be engulfed by phagocytic cells without ever stimulating a memory immune response. For example, antigen carrier vehicles can be utilized such as liposomes or nanoparticles that are not cleared quickly and easily by innate immune cells. This creates a 'depot' effect which ensures that the antigen remains intact long enough to be presented in the lymph node[412-414].

1.6.7 Recombinant Proteins

Recombinant antigens can be tailored to target a specific arm of the immune response and to induce a potent immune response capable of creating lasting immunity. There are multiple ways in which these antigens can be obtained. The two most common ways are to culture the microbe in a controlled laboratory setting or a recombinant antigen can be produced by culturing a vector such as yeast or *Escherichia coli*, which will produce the antigen *in-vitro*. Since the production of recombinant antigens sometimes requires the cultivation of large amounts of pathogenic organism, there is an inherent risk and increased cost for producing these antigens[355].

Some antigens very commonly used in *M. tuberculosis* vaccination are 6-kDa early secretory antigenic target (ESAT-6), culture filtrate protein (CFP)-10, and antigen (Ag)85B because of their potent immunogenicity and ability to target cell-mediated immunity[419]. A recombinant ESAT-6 could, for example, be added to the BCG vaccine which lacks ESAT-6 for a potentially more immunostimulatory vaccine resulting in a recombinant BCG (rBCG)[420]. It has also been shown to be highly stimulatory in sub-unit vaccines also containing Ag85B providing at least partial protection against infection[419, 420].

1.6.8 Recombinant Vaccines and Vectors

Recombinant vaccines contain a vector such as an attenuated or harmless bacteria or virus that was genetically modified to produce the antigens optimal for targeting an immune response against a given pathogen. Some recent viral vector vaccines that have shown some promise include the BCG prime/boost vaccine MVA85A/Aeras-485, which expresses antigen (Ag)85A, which showed great promise until human clinical trials[421, 422], Crucell Ad35/Aeras-402 which uses adenovirus as a vector[423], and Ad5Ag85A another BCG booster which also expresses Ag85A[358, 423-426]. MVA85A, a viral vectored vaccine has advanced to Phase IIb clinical trials but failed in providing adequate protection in humans, Crucell Ad35 another viral vectored vaccine is in Phase I, and so has AdAg85A[411].

The gene encoding for the vaccine antigen of choice is first isolated from the organism, and is transferred to a non-pathogenic organism[355]. That antigen can then be produced and administered separately from the organism that produced it, as a subunit vaccine antigen, or the host can be inoculated with the organism which then constitutively produces the antigen[355]. This model is valuable in a number of ways, the antigen can be safely given separate from the pathogenic organism, or a strain which might be reactivated; this also reduces risks of contamination, reversion, and toxicity[355]. Some examples of these include protein immunogens which require an adjuvant, live bacterial or viral vectors, and nucleic acids[355].

Recombinant proteins for use in subunit vaccines can be made in a number of different types of host cells, and vaccine vectors can be created with a number of bacterial strains as well[355]. Bacteria, for example, can be genetically altered in order to produce a specific protein, and vaccine vectors can be derived by bacterial strains that contain their own immunostimulatory antigens acting as an adjuvant, while producing an antigen from the target pathogen for specific stimulation[427]. The most commonly used organism for this purpose is *E. coli* because the organism is well characterized and many genetic tools are readily available, but other organisms can be used like *Vibrio cholera* and *Shigella flexneri* to safely express components of LPS for immune-stimulation[427-430].

Other vaccine vectors are generally created in attenuated pathogens including *Salmonella typhimurium*, or even the BCG vaccine which are highly immunogenic and can also be used in various disease applications like AIDS, Lyme disease, and leishmaniasis[427, 431-434]. Other non-pathogenic strains of bacteria have also been used as vaccine vectors like *Lactobacillus lactis*, *Staphylococcus carnosus*, and *Streptococcus gordonii* which unexpectedly persist in the mammalian hosts despite their non-pathogenic nature, and are able to produce immunomodulatory vaccine antigens while in the host[427, 435-437].

1.6.9 Toxoid Vaccines

Other types of vaccines, include toxoid vaccines, are used to protect against pathogens that produce a toxin such as Diptheria or Tetanus[414]. The toxins from these pathogens are attenuated with a chemical treatment and administered as a vaccine that will stimulate antibody production against the toxin itself[414]. Instead of containing an entire microbe, subunit vaccines consist of only a portion of the microbe, particularly optimal immuno-stimulatory components like proteins or epitope antigens. These components target memory T-cells or antibody producing B-cells to create a memory response that will last over time[414]. There are a number of advantages to using this type of vaccine, particularly with the reduction of potential side effects or reversion back to virulent strains that cause disease.

1.6.10 Prime-boost Strategies

One way in which vaccines can become more immunogenic, or produce a better memory immune response, is by using a prime-boost strategy[438]. Prime-boost means that multiple inoculations occur for a vaccine whether it be a homologous prime/boost with the same vaccine given twice, or a heterologous prime-boost in which multiple vaccine formulations with the same antigen are given that complement each other[438]. Multiple homologous vaccines are common and are needed for different types of vaccines as well including live attenuated, like with the polio vaccine; and inactivated or recombinant subunits like for Hepatitis A, and B; and the polysaccharide vaccine for *Haemophilus influenza* type b[438]. In recent years, heterologous prime-boost vaccines have been the object of great interest and have been found to be superior in protection to their homologous counterparts[438]. Some commonly used heterologous prime-boost regimens involve combinations of: a DNA prime with a boost of recombinant protein[439, 440], inactivated vaccine[441, 442], viral vectors[443-445], and BCG[446]; also, a viral vector prime with a recombinant protein boost[447]; and BCG boosted by a viral vector[438, 448, 449].

1.6.11 Routes of Inoculation

Another way in which vaccines can be modified or optimized for better efficacy is by adjusting the route of inoculation. It has been shown that the same vaccine components, given with various doses, adjuvants, or routes of inoculation drastically change the outcome of protection[382, 392, 440, 450-452]. Since pulmonary *M. tuberculosis* infection is initiated through aerosol exposure via respiratory droplets, an aerosolized, or mucosally delivered vaccine may provide superior protection simply because the area subject to infection is directly immunogenically primed by vaccine stimulation[453]. When the route of natural infection matches the route of inoculation the vaccine efficacy may be improved, especially in the case of priming mucosal immunity[453]. It is estimated that there are more plasma B-cells in the intestinal mucosa than in the spleen and lymph nodes combined; and when B and T-cells are activated in the mucosa, they then express a homing receptor that directs those immune cells to return back to mucosal

tissue in which they are primed[454]. When a non-parenteral vaccine is given, mass-vaccination becomes safer, easier, and less costly[453]. Needles and syringes have an inherent risk with contamination, safe disposal, and needle sticks. There is more compliance among patients when painful needles are avoided, and less training is required for personnel administering an aerosol vaccine like a nasal mist for example than for administering an injection[453]. Another alternative to intra-nasal vaccination is an aerosolized vaccine given with a nebulizer for pulmonary delivery, which has been shown to be more effective than an injection for vaccination against measles[453, 455-457]. This method can also be used in prime-boost strategies in which one inoculation could be via parenteral route and one via nasal or pulmonary route. This method has also shown to be more efficacious for protection against measles infection when compared to parenteral route for both prime and boost[458, 459]. It has also been shown in influenza vaccinations as well as measles that mucosal administration is indeed more protective than sub-cutaneous inoculation[458-460]. Increased efficacy in mucosal vaccination with BCG as compared to parenteral vaccination has also been shown in protection against *M. tuberculosis* in mice, guinea pigs, and non-human primates[382, 392, 425, 426, 450-452, 461-466]. One study monitored tuberculin skin test conversion as an outcome for aerosolizing BCG in guinea pigs, school children, and medical students in 1968 but the mucosal administration of live BCG possesses contraindications particularly when being administered to immunocompromised individuals[467]. The first candidate vaccine given via the respiratory route in human clinical trials was MVA85A and has been shown to be safe and efficacious for stimulating protective immunity[468]. The advantages of mucosal vaccinations are numerous including ease of use, cost, and efficacy[453]. With the advancement of more effective and analogous animal models in the future, mucosal vaccination may prove to be a highly superior route of inoculation, especially in the context of *M. tuberculosis*.

2 In-vitro analysis of CpG-DNA

2.1 Introduction

The only available vaccine in use today for protection against *M. tuberculosis* is the bacillus Calmette-Guérin (BCG) vaccine derived from attenuated *M. bovis*. This vaccine was first used in 1921 and has a variable efficacy of between 0-80%[469]. The need for a novel and efficacious vaccine is tremendous. There are a number of vaccine strategies available for testing such as whole cell/ live vaccines derived from BCG, or recombinant BCG and viral vectored vaccines[355, 469], however the focus of this study is to better understand how a sub-unit vaccine functions against *M. tuberculosis* infection. We aim to explore the possibility that a sub-unit vaccine may prove to be more efficacious than a whole-cell vaccine like BCG because we hypothesize that the immune response can be more precisely targeted by focusing on just one or two immunostimulatory components as opposed to multiple[5, 470, 471]. It has been suggested that due to highly complex protein repertoire of BCG, multiple antigens compete for presentation and the antigens dominating this response may not result in optimal protection and it may not be continually expressed by the organism[419]. For the current studies the sub-unit vaccine design was formulated by choosing a known[419],highly immunogenic protein, the well-characterized *M. tuberculosis* antigen 6 kDa early secretory antigenic target (ESAT-6), and the adjuvant CpG oligodeoxynucleotide (ODN) to target toll-like receptor (TLR)9.

TLRs are highly stimulatory pattern recognition receptors (PRRs) that recognize a variety of bacterial and viral antigens and provide a key link between the innate and adaptive immune systems by recognizing pathogen associated molecular patterns (PAMPs) from bacteria and viruses on the surface of antigen presenting cells (APCs), which then present antigen to circulating T-cells[98-100]. Thus far ten TLRs have been identified in humans, and twelve in mice, each one exhibiting their own functions and ligands[98-100]. Some TLRs reside on the cell surface like TLR5, TLR2/1, TLR2/6, TLR11, and TLR4[108]. Others remain in the endosomal compartment including TLR3, TLR7/8, TLR9, and TLR-13[100, 108]. TLR1 recognizes

lipopeptides; TLR2 recognizes peptidoglycan, lipopeptides, and glycolipids, TLR3, 7, 8, 9, and 13 recognize RNA and DNA with TLR9 recognizing unmethylated bacteria DNA such as CpG ODN in particular; TLR4 recognizes LPS; TLR5 and 11 recognize flagellin; TLR6 recognizes lipopeptides; and TLR12 binds with profilin[472]. Although TLR2 and TLR4 have been shown to recognize BCG antigens; due to BCGs variable efficacy, stimulation through these particular TLRs might not be the most immunogenic targets possible[473]. Many TLRs signal through the adaptor protein, myeloid differentiation primary-response protein 88 (MyD88) which results in translocation of NF-κB into the nucleus and ultimately pro-inflammatory cytokine production[114]. One TLR that has been shown to signal through alternative pathways is TLR4 in response to LPS stimulation, although the mechanism is not fully understood[109, 474].

There are a number of available CpG ODN sub-types available for use, but the most characterized types are: ODN 2216 (A-class), ODN PF-3512676 (B-class), and ODN 2395 (C-class)[416]. Each have slight differences in their cytosine/guanine sequences or differences in their phosphate backbone, but most importantly perhaps are the differences in immunogenicity of each. CpG-A is particularly effective in stimulation of interferon (IFN)-α, CpG-B is highly effective at B-cell stimulation, and CpG-C has a broader immunostimulatory effect with stimulation of IFN-α production, B-cells, and more[416, 475]. For example, stimulation of TLR9 with CpG-C ODN has been shown to enhance T-cell responses by strongly enhancing dendritic cell (DC) activation and differentiation, and facilitates activation of T-cells[401, 416, 476]. For these reasons, CpG ODN was considered for the immunostimulatory adjuvant used in conjunction with other sub-unit components in an *M. tuberculosis* vaccine[401, 416, 475, 476].

Our hypothesis is that targeting TLR9 stimulation through CpG ODN produces a potent pro-inflammatory cytokine response that can then be optimized for use as a vaccine adjuvant in a sub-unit vaccine against *M. tuberculosis*. In order to test this hypothesis, we must first optimize the CpG ODN sub-type to be used by comparing them in various concentrations *in-vitro*. In this set of experiments we evaluated the pro-inflammatory cytokine profile elicited by

CpG ODN stimulation of bone marrow derived macrophages (BMMØs) and dendritic cells (BMDCs) *in-vitro*. Three different CpG subtypes, including CpG -A, -B, and -C were compared at different concentrations. Culture supernatants were then harvested and cytokine production was evaluated by enzyme-linked immunosorbant assay (ELISA), and cytometric bead array (CBA) assays. CpG-C ultimately delivered the most potent pro-inflammatory cytokine production and was chosen for evaluation *in-vivo* as the adjuvant portion of a sub-unit vaccine. These data confirm our hypothesis that CpG ODN is a potent stimulator of pro-inflammatory cytokines, and CpG-C appears to be the optimal sub-type for our application.

2.2 Materials and methods

2.2.1 Mice

Female C57BL/6 mice aged 6-8 weeks (5 animals/treatment group) were purchased from Jackson Laboratories (Bar Harbor, MA). Mice were house in a specific temperature and humidity controlled pathogen-free environment. All mice had unlimited access to sterile mouse chow and water. All experimental procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (CSU IACUC).

2.2.2 In-vitro bone marrow derived macrophages (BMMØ) stimulation assay

Bone marrow cells were harvested from C57BL/6 mice (see Appendix A: Izzo Lab BMMØ SOP) and added to complete medium containing L929 cell-line cultured supernatant to promote differentiation (RPMI-1640 with essential and non-essential amino acids, penicillin, streptomycin, and 10% fetal bovine serum (FBS)) (Invitrogen) (see Appendix A: Izzo Lab Media Prep SOP). Media was changed every 48 hours until the sixth day in which media was changed to exclude L929 and antibiotics. On day 7 cells were harvested and brought to a concentration of 2.0×10^5 macrophages per well in a 96 well plate for stimulation for 24 hours with CpG oligodeoxynucleotides (ODN) (Hycult Biotech) at varying concentrations based on the manufacturers recommendations.

2.2.3 In-vitro bone marrow derived dendritic cell (BMDC) stimulation assay

Bone marrow cells were harvested from C57BL/6 mice (see Appendix A: Izzo Lab BMDC SOP) and added to complete medium containing 20 ng/mL GM-CSF to drive DC differentiation (RPMI-1640 with essential and non-essential amino acids, penicillin, streptomycin, and 10% fetal bovine serum (FBS)) (Invitrogen) (see Appendix A: Izzo Lab Media Prep SOP). Media was changed every 72 hours until the eight day in which media was changed to exclude GM-CSF and antibiotics. On day nine cells were harvested and brought to a concentration of 2.0×10^5 dendritic cells per well in a 96 well plate for stimulation for 48 hours with CpG ODN (Hycult Biotech) at varying concentrations based on the manufacturers recommendation. Positive control wells were cells stimulated with Bacillus Calmette-Guérin (BCG) at a MOI of 1:5. Negative control wells included the addition of non-CpG DNA (Hycult Biotech), or cRPMI alone.

2.2.4 ELISA colorimetric cytokine analysis

Cytokine quantification in culture supernatants following stimulation was performed by enzyme-linked immunosorbant assay (ELISA). ELISA kits were purchased from Affymetrix/eBioscience Inc. (San Diego, CA) for the following cytokines: IFN-γ, TNF-α IL-10, IL-12, and IL-6. Plates containing cultured cells were centrifuged at 200xg for 5 minutes; supernatants were aspirated and stored at -80℃ until used. ELISA was performed on supernatants following the manufacturer's protocol. The color intensity in wells of 96 well plates was then read using the BioRad (Hercules, CA) iMark microplate absorbance reader. A standard curve was also used with each assay to determine cytokine concentration in pg/mL.

2.2.5 Cytometric Bead Array (CBA) Analysis

Multi-plex cytokine analysis in culture supernatants was performed by using BD™ cytometric bead array (CBA) mouse inflammatory cytokines kit (BD Biosciences San Jose, CA) following the manufacturer's protocol for the following cytokines: IL-6, IL-10, MCP-1, IFN-γ, and TNF-α. Plates containing cultured cells were centrifuged at 200xg for 5 minutes; supernatants

were aspirated and stored at -80℃ until used. Supernatants were then incubated with CBA beads and samples were analyzed by the FACS Canto II flow cytometer on FCAP Array™ CBA analyzing software (BD Biosciences). A standard curve was also used with each assay to determine cytokine concentration in pg/mL.

2.2.6 Mycobacterium species

Mycobacterium bovis BCG Pasteur (TMCC 1011) strain was grown to mid-log phase in Proskaur and Beck (P&B) medium containing 0.1% Tween 80 (Sigma, St. Louis, MO) (see Appendix A, Izzo Lab P&B/Seed Stock SOP). Aliquots were stored at -80C and were thawed then sonicated before use.

2.2.7 CpG Oligodeoxynucleotides (ODN)

CpG ODN was purchased from Hycult Biotech Inc. (Plymouth Meeting, PA). DNA arrived in 200nmol lyophilized vial containing approximately 20 mer CpG ODN. Manufacturer recommended concentration for *in-vitro* stimulation is between 0.01 to 10 μM after reconstitution in sterile distilled or deionized water. CpG ODN is then stored at 4℃ for short -term storage, and -80℃ for long -term storage.

2.2.8 Statistical Analyses

For multi-variate analysis, statistical differences between treatment groups were compared using one-way ANOVA analyses, and non-parametric testing when performed if normality test failed. For comparisons between two treatment groups, Student's *t* test was used. Statistical analyses were done using SigmaStat software. A p value <0.05 was considered statistically significant for these analyses and was signified by a star (*) in graphs.

2.3 Results

2.3.1 In-vitro comparison of CpG ODN subtypes in bone marrow derived macrophages (BMMØs)

Optimal concentration and sub-type for *in-vitro* CpG stimulation in macrophages was analyzed in 24 hours cultures of bone marrow derived macrophages (BMMØs) from C57BL/6

mice and then by performing cytokine analysis with ELISA and CBA assays. All major subtypes of CpG ODN were compared including sub-types A, B, and C, plus non-CpG ODN as a negative control. A range of concentrations was evaluated based on manufacturer's suggestions which include 5uM, 1uM, 0.2uM, and 0.04uM. Non-CpG ODN did not have a significant stimulatory effect in all cases (data not shown). With the highest concentration of 5uM of each CpG subtype, cytokine concentrations appeared to reach similar levels as shown in the line graph **(Figure 2-1)**. However, CpG-C stimulated higher cytokine levels at the lower concentrations compared to CpG-A and CpG-B, with the most marked increases at 1uM and 0.2uM CpG ODN compared to other sub-types **(Figure 2-2)**. Figure 2-1 shows TNF-α production with all sub-types at all concentrations to demonstrate the superior efficacy of cytokine stimulation by CpG-C at lower concentrations. Figure 2-2 includes the amount of cytokine present after stimulation with each CpG ODN subtype in the three lower concentrations, which include 1uM, 0.2uM, and 0.04uM. Stimulation at a concentration of 1uM resulted in the most consistent cytokine production. 1uM concentrations are also represented in individual graphs **(Figure 2-3)**. The same data as reported previously, but replotted to show the superior efficacy of CpG-C stimulation at lower concentrations of antigen (1uM), the consistency in results between groups, and the media-only negative control and non-CpG is also included **(Figure 2-3)**. Results were consistent with all pro-inflammatory cytokines evaluated including TNF-α, IFN-γ, MCP-1, and IL-6. The anti-inflammatory cytokine IL-10 also displayed a similar trend, with IL-12 below the limit of detection. Both TNF-α ELISA and TNF-α in CBA assay reached comparable levels indicating accuracy and consistency between assays.

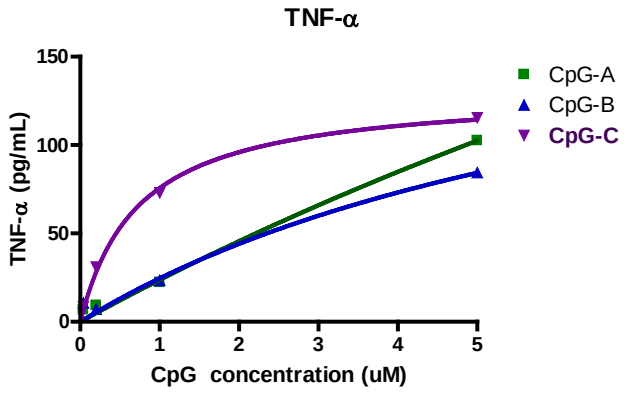


Figure 2-1: Line graph displaying TNF-α production in response to stimulations with all concentrations of CpG ODN stimulation including 0.04uM, 0.2uM, 1uM, and 5uM. CpG-C produces the most cytokine with the least stimulation. Data obtained by ELISA and CBA assays.

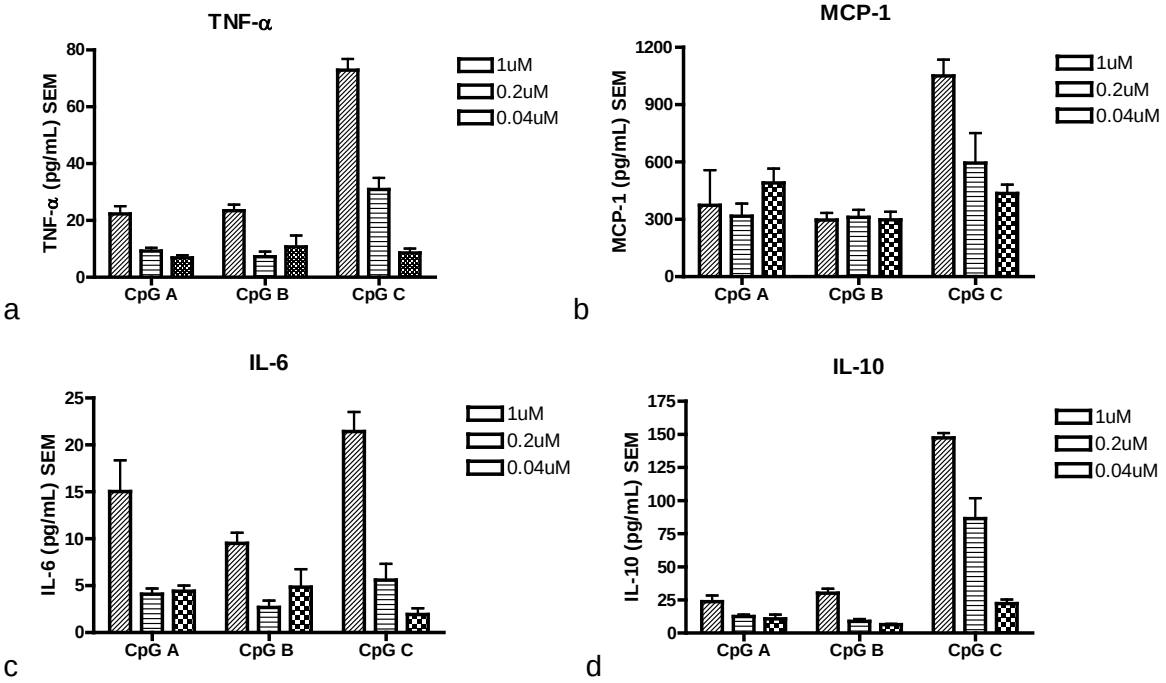


Figure 2-2: Bar graphs containing the same data a presented previously in line graph while excluding the 5uM concentration in order to better display cytokine production at lower amounts of CpG ODN stimulation. Other cytokines are also included. Data obtained by CBA assay.
(a) TNF-α graph (b) MCP-1 graph (c) IL-6 graph (d) IL-10 graph

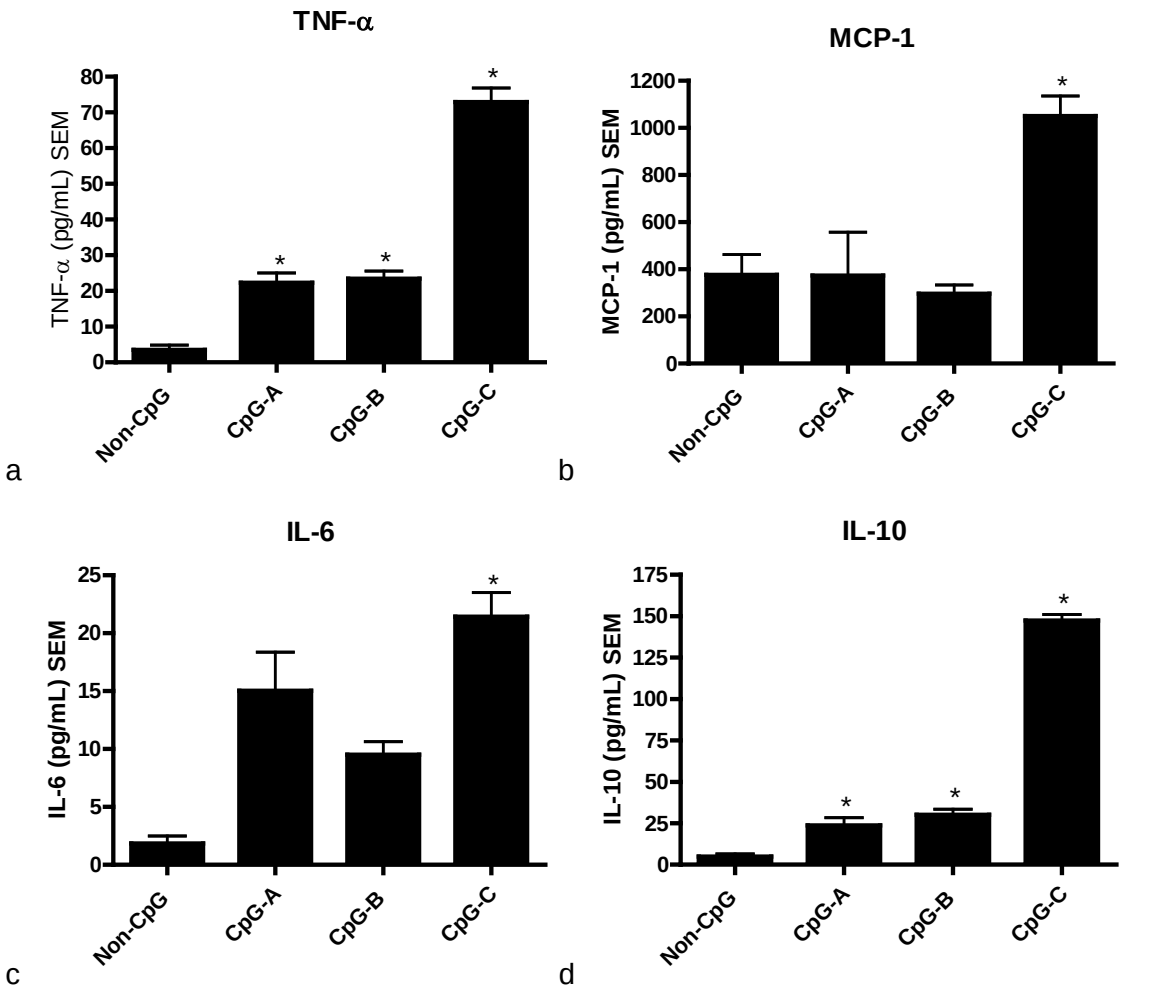


Figure 2-3: These graphs include the same data as displayed previously, however, with just the 1uM concentration of CpG ODN for stimulation. This is to show the efficacy of CpG-C ODN for stimulating pro-inflammatory cytokines with minimal stimulation. Star represents statistical significance compared to negative control group where $p < 0.05$. Data obtained by CBA assay. (a) TNF-α graph (b) MCP-1 graph (c) IL-6 graph (d) IL-10 graph

2.3.2 In-vitro comparison of CpG ODN subtypes in bone marrow derived dendritic cells (BMDCs)

Optimal concentration and sub-type for in-vitro CpG stimulation in dendritic cells was determined by performing cytokine analysis with CBA assay after stimulation of BMDCs. All major subtypes of CpG were compared as described previously. A range of concentrations was evaluated based on manufacturers suggestions and include 4uM, 2uM, and 1uM. Non-CpG ODN did not have a significant stimulatory effect. Figure 2-5 includes cytokine concentration after stimulation of BMDCs with each CpG subtype with all three concentrations. In the highest concentration of 4uM CpG of each subtype, TNF-α, IL-6, and IL-10 concentrations reached similar levels. However, CpG-C stimulated higher cytokine levels at lower concentrations, with the most marked increases at 1uM and 2uM CpG DNA compared to other sub-types. Stimulation of BMDCs at a concentration of 1uM resulted in the most consistent cytokine production by the BMDCs. 1uM concentrations are also represented in individual graphs **(Figure 2-4);** including the amount of cytokine present after stimulation with each CpG subtype in the three lower concentrations, which include 1uM, 0.2uM, and 0.04uM. Results were consistent with all pro-inflammatory cytokines evaluated including TNF-α, IFN-γ, and IL-6. The anti-inflammatory cytokine IL-10 also displayed a similar trend, although when TNF-α, IL-6, and IL-10 are compared directly, IL-10 levels are markedly lower than those of the pro-inflammatory cytokines **(Figure 2-5)**.

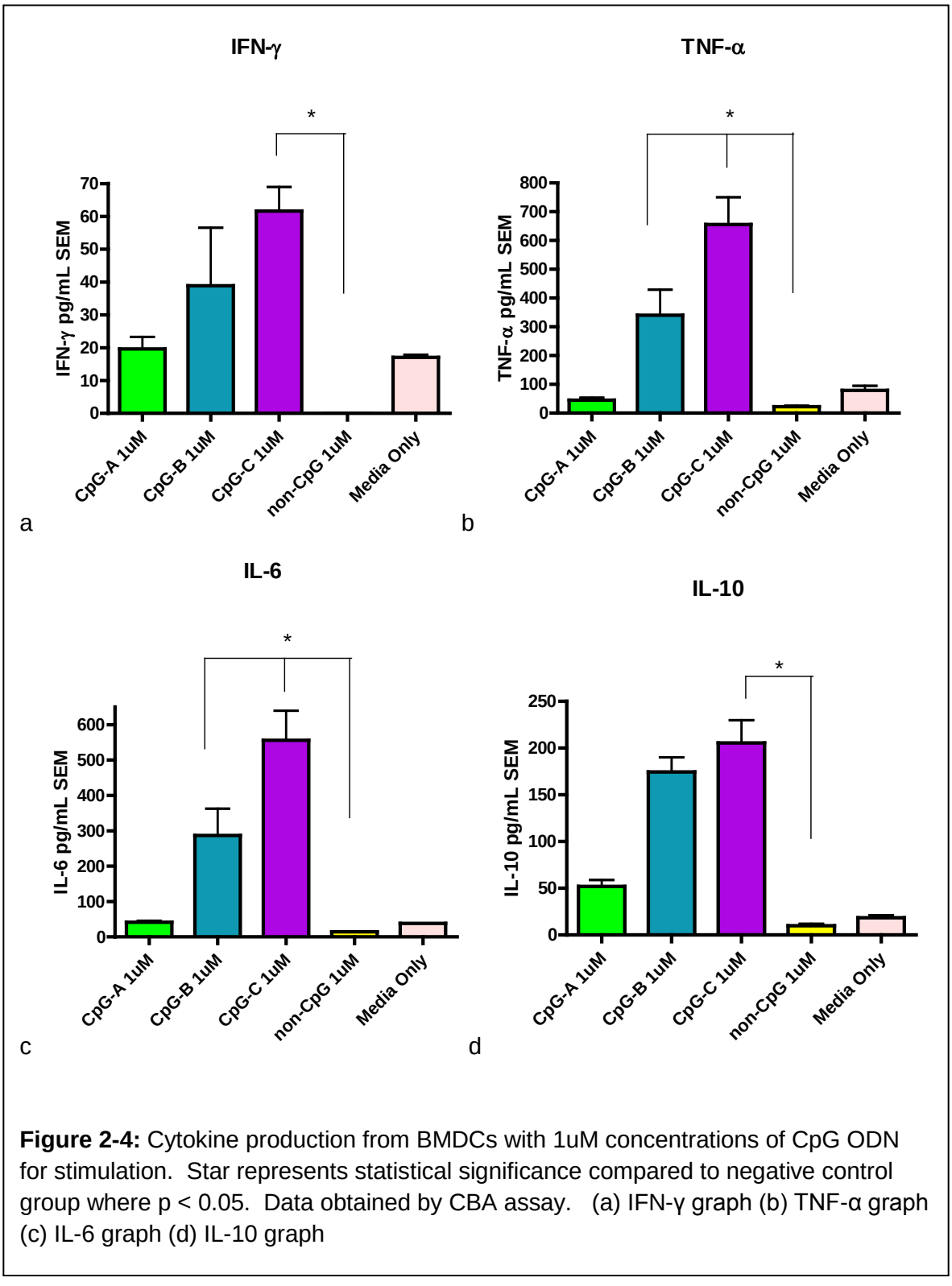


Figure 2-4: Cytokine production from BMDCs with 1uM concentrations of CpG ODN for stimulation. Star represents statistical significance compared to negative control group where $p < 0.05$. Data obtained by CBA assay. (a) IFN-γ graph (b) TNF-α graph (c) IL-6 graph (d) IL-10 graph

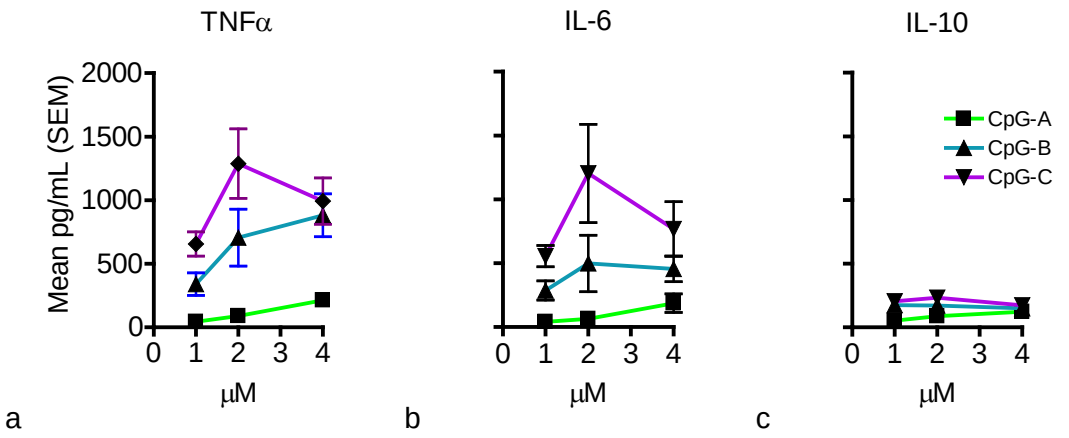


Figure 2-5: Line graphs directly comparing pro-inflammatory cytokines to IL-10 with same axes in order to demonstrate the relatively low amount of IL-10 being produced in comparison. Data obtained by CBA assay. (a) TNF-α graph (b) IL-6 graph (c) IL-10 graph

2.4 Discussion

The ability of CpG sub-types to induce pro-inflammatory cytokines was compared *in-vitro* by stimulating C57BL/6 BMMØs and BMDCs with three different CpG ODN sub-classes, CpG-A, -B, and -C[475]. Previous studies have shown *in-vitro* stimulation using other CpG ODN sequences in murine BMDC, PBMCs and lymphocytes results in cell activation and proinflammatory cytokine production[398, 477-479]. In order to evaluate the comparative efficacy of these three particular CpG ODN sub-classes, BMMØ and BMDC were stimulated with varying concentrations of each sub-class, and the culture supernatants analyzed for cytokines using ELISA and CBA assays. Since macrophages and dendritic cells play such a critical role in both innate immunity and bridging the gap between innate and adaptive immunity respectively[85, 133, 192, 201, 203], it was of great interest to determine which CpG ODN was the best at inducing the pro-inflammatory cytokines that are needed to induce a protective immune response.

Key pro-inflammatory cytokines that are critical for mounting an effective immune response against *M. tuberculosis* include TNF-α, IFN-γ, MCP-1, IL-6, and the anti-inflammatory

cytokine IL-10[8, 85, 149]. All of these cytokines were upregulated in response to CpG ODN stimulation at the highest concentrations tested in both BMMØs and BMDCs; however, as the concentration of CpG ODN stimulation was reduced, the CpG-C sub-type showed the highest levels of cytokine production at the lowest levels. This fact is of critical importance when considering minimizing the effects of immunopathology and cytotoxicity in the context of vaccination. The more efficacious the response, the lower the dosage is required and thus increased safety and reduced chance of cytotoxicity. The negative controls, non-CpG DNA as well as media-only addition to cells further confirmed the positive results elicited by CpG ODN stimulation. A cellular immune response and a Th1 type immune response are critical for controlling *M. tuberculosis* infection and the cytokines TNF-α, IFN-γ, and IL-6 play a key role in this type of immunity[8, 85, 149]. The monocyte chemoattractant protein (MCP)-1 also plays an important role in immunity by recruiting circulating monocytes to the site of infection for further differentiation into macrophages and dendritic cells in the lung[480], thus perpetuating a robust innate immune response following stimulation with CpG ODN.

The anti-inflammatory cytokine IL-10 was also up-regulated in response to stimulation, and this may not be a detriment if this effect is observed *in-vivo* as well. Previous studies have shown that IL-10 limits excessive release of IL-12 and IFN-γ in macrophages stimulated with bacterial DNA which was associated with increased morbidity in mice[481]. The relative amount of IL-10 when compared to the pro-inflammatory cytokines TNF-α and IL-6 is low IL-10 in comparison to the pro-inflammatory cytokines **(Figure 2-5)**. This may be an indication that although IL-10 is being produced, it may not have a detrimental effect *in-vivo* but may be a necessary component of balancing the inflammatory response, and maintaining a protective response while preventing immunopathology.

Consistent with previous studies, these CpG ODN sub-classes also stimulated pro-inflammatory cytokine production *in-vitro* in both macrophages and DCs[477, 482]. In this study, CpG-C ODN produced the highest concentrations of pro-inflammatory cytokines, with the lowest

concentrations. This data suggests that CpG-C ODN could produce a potent adjuvant effect when used as part of a sub-unit vaccine *in-vivo* thus confirming our hypothesis and identifying an optimal CpG ODN sub-type. In addition the data suggest that stimulation through a single TLR, in this instance, TLR9 provides a potent pro/anti-inflammatory response that may be sufficient to stimulate protective anti-tuberculosis immunity. Our next set of experiments will determine if this is true.

3 CpG-based vaccine, determining optimal route and subtype

3.1 Introduction

When considering vaccine design, the route of inoculation must also be taken into account. It has been shown that when designing a vaccine against a mucosally introduced pathogen, it should be considered that administering the vaccine via the same route in which infection occurs may produce a more robust immune response capable of containing infection better than an alternate route such parenteral vaccination[453, 483]. Activation of the mucosal immunity has been evaluated to determine if direct priming does indeed provide superior protection over for example a sub-cutaneous vaccination against a pulmonary infection[484]. In order to determine the optimal route of vaccination with CpG-C combined with liposomes and ESAT-6 **(Figure 3-1)**, C57BL/6 mice were vaccinated via various routes of inoculation and were subsequently challenged via aerosolized H37Rv *M. tuberculosis* infection. The routes of inoculation evaluated include the intra-nasal (i.n.), sub-cutaneous (s.c.), intra-peritoneal (i.p.), and intra-gastric (i.g.) routes. Our hypothesis is that CpG-C combined with liposomes and ESAT-6 provides superior protection against *M. tuberculosis* infection compared to CpG oligodeoxynucleotides (ODN) sub-types –A, and –B when given via the intra-nasal route.

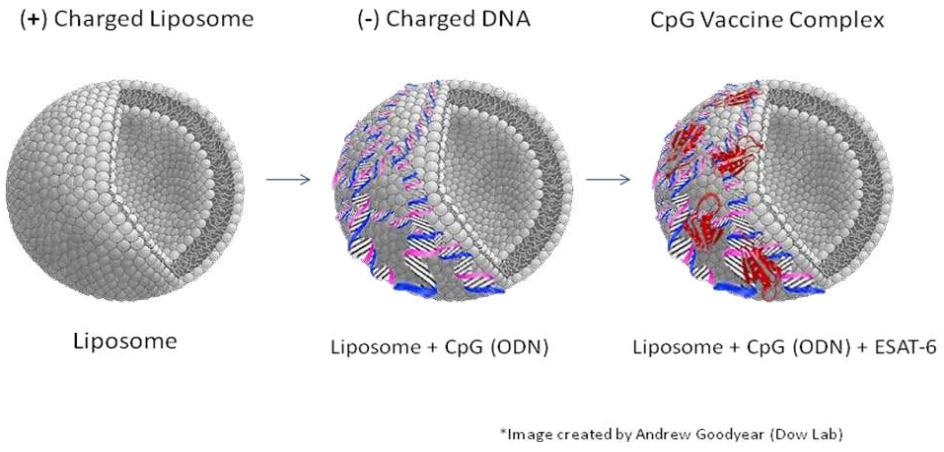


Figure 3-1: Diagram of CpG ODN/cationic liposome/ESAT-6 complex.

There are three main subtypes of CpG ODN including CpG-A, -B, and -C. With each CpG ODN specializing in promoting various types of immune functions[475], all three were compared *in-vivo* to determine the most efficacious subtype as a vaccine adjuvant against *M. tuberculosis*. Mice were vaccinated three times at two-week intervals with each sub-type separately, in combination with liposomes and ESAT-6 and were challenged with H37Rv *M. tuberculosis*, 30 days following vaccination. In another 30 days mice were sacrificed and the lungs and spleens were removed, homogenized, and plated on 7H11 agar to determine bacterial burden in infected organs. This was done following experiments with *in-vitro* stimulation of bone marrow derived macrophages and dendritic cells with *M. tuberculosis* antigens which demonstrated superior pro-inflammatory cytokine stimulation compared with the other CpG ODN subtypes CpG-A and CpG-B.

The antigen used as part of the sub-unit vaccine containing liposomes and CpG ODN as an adjuvant is the 6 kDa early secretory antigenic target (ESAT-6). ESAT-6 is one of multiple immunodominant antigens secreted by virulent *M. tuberculosis* bacteria[485]. There are a number of highly stimulatory antigens that are well characterized for anti-tuberculosis immunity, some of which are currently part of vaccines in clinical trials and include: Ag85A, Ag85B, TB10.4, 30-kDa major secretory protein, and ESAT-6[486]. ESAT-6 has been shown to be a dominant stimulator of cell-mediated immunity during early infection with *M. tuberculosis* in both human and animal models[419]. It has been shown by Andersen *et al*. that ESAT-6 in combination with the adjuvant monophosphoryl lipid A (MPL), and dimethyl dioctadecylammonium bromide (DDA) as a delivery system for the vaccine, induced protective immunity comparable to results attained from BCG[419]. Because of the reproducibility of ESAT-6 to induce potent cellular immune responses *in-vitro*, it was chosen as the antigen component to be used in the CpG-C ODN vaccine to dissect the immune response generated by CpG-C.

The final component of the CpG ODN vaccine, is the cationic liposome N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP). Both the CpG ODN

adjuvant and the antigen ESAT-6 were covalently bound to the surface of the cationic liposome attracted by opposite charges. This resulted in a liposome complex that when engulfed provides potent stimulation to the immune system with prolonged exposure by creating a depot effect[487-490]. Liposomes were utilized as a vaccine delivery system in order to provide sustained exposure of the vaccine components to the innate immune system for a long enough period of time to ensure that antigen presenting cells (APCs) would engulf the liposome complex and provide a robust immune response through TLR9 in response to CpG ODN, and thus present the antigen, ESAT-6 to circulating naïve T-cells, producing a potent cellular immune response, and triggering a memory immune response against *M. tuberculosis*.

In almost all experiments conducted *in-vitro* for evaluating the efficacy of CpG ODN, BCG was included as a positive control group. Despite the history of BCG with variable efficacy and inability to protect over sustained periods of time, BCG remains to be the gold standard as a comparison for vaccine efficacy and immune-stimulation in the animal model. With a general full $1Log_{10}$ reduction in colony forming units (CFUs) compared to saline controls, the CpG ODN vaccine administered intra-nasally (i.n.) provided protection in similar levels when compared to BCG vaccination. In most cases, there was not a statistically significant difference in CFUs between the BCG control group and the CpG ODN vaccine.

The data collected from this experiment confirms our hypothesis by showing that CpG-C vaccinated mice had a significant reduction in bacterial burden compared to saline controls and other CpG ODN subtypes when the vaccine was administered i.n.

3.2 Materials and Methods

3.2.1 Mycobacterium species

As described previously in 2.2.6

3.2.2 CpG Oligodeoxynucleotides (ODN)

As described previously in 2.2.7

3.2.3 Mice

As described previously in 2.2.1

3.2.4 Immunizations

Mice were inoculated intra-nasally (i.n.) with 20uL of vaccine containing 2ug of 6 kDa early secretory antigenic target (ESAT-6) protein complexed with 10ug CpG oligodeoxynucleotides (ODN) (Hycult Biotech) and cationic liposomes (Avanti) (see Appendix A: Izzo Lab Anesthesia SOP). Mice vaccinated intra-gastrically (i.g.), intra-peritoneally (i.p.), or sub-cutaneously (s.c.) were given 100uL vaccine containing 10ug of ESAT-6 protein complexed with 50ug CpG ODN. Control groups were mice, immunized with BCG, or monophosphoryl lipid-A (MPL-DDA) with ESAT-6, cationic liposomes without antigen, and non-CpG ODN. A group in which mice received pyrogen-free saline was included for each experiment. Cationic liposomes: N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) or 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), were kindly provided by S. Dow (Colorado State University, Fort Collins, Colorado). DOTIM and DOTAP liposomes were prepared in lyophilized cholesterol (Avanti) dissolved in chloroform and diluted in sterile 10% sucrose (see Appendix A, Dow Lab Liposome SOP). Vaccinations were administered three times, at two-week intervals (see Appendix A, Izzo Lab Animal Handling SOP). Intra-nasal vaccines were performed while mouse was anaesthetized by ketamine (see Appendix A, Izzo Lab Anesthesia for Mouse SOP).

3.2.4 Aerosol infection

Mice were infected with approximately 100 CFU of aerosolized *M. tuberculosis* H37Rv using a Glas-Col inhalation exposure system (see Appendix A: Izzo Lab Aerosol SOP). Mice were challenged thirty days after the third and final vaccination, and in long-term studies, mice were challenged 120 days following vaccination. Following infection mice were sacrificed and the number of viable organisms was quantified in lung and spleen by plating 10-fold serial dilutions of lung and spleen organ homogenates on Middlebrook 7H11 agar plates (see

Appendix A: Izzo Lab Viable Count SOP). Colonies were counted after incubating plates at 37℃ for 14 -21 days.

3.2.6 Cell preparation for flow cytometric and ELISpot analysis

Single-cell suspensions of organs were prepared by mechanical disruption through a 70µm nylon mesh screen. The cell-suspensions were further purified with ACK Solution for lysis of red blood cells (RBC) and were then resuspended in complete medium (RPMI-1640 with essential and non-essential amino acids, penicillin, streptomycin, and 10% fetal bovine serum (FBS)) (Invitrogen) (see Appendix A: Izzo Lab Media Prep SOP). Cells were counted using 1% Trypan blue solution to determine the number of viable cells and then diluted accordingly, depending on the analysis.

3.2.7 ELISpot assay analysis

Cytokine production by immune cells was assessed by determining the number of cells capable of cytokine production by ELISPOT (see protocol: eBiosciences) analysis for IFN-γ, TNF-α, and IL-2. Single cell suspensions were incubated overnight at a concentration of $5x10^6$ cells/mL in complete medium in the presence or absence culture filtrate protein (CFP) or ESAT-6 depending on immunization. Negative controls included cells from non-immunized mice and cells incubated without antigen, positive control cells were stimulated with Concanavalin A (ConA). Plates were then analyzed by quantifying the number of spots produced by cytokine producing cells using the Series 5 UV-Immunospot Analyzer/(C.T.L. Shaker Heights, Ohio).

3.2.8 Statistical analyses

As described previously in 2.2.8

3.3 Results

3.3.1 Comparison of delivery route for CpG-C vaccination: i.n., i.p., and s.c. delivery

Once the optimal CpG ODN sub-type was established *in-vitro*, various routes of inoculation were compared to determine the efficacy after pulmonary infection with *M. tuberculosis*. C57BL/6 mice were vaccinated intra-peritoneally three times, two weeks apart,

challenged with *M. tuberculosis* H37Rv via the pulmonary route, and sacrificed 30 days after challenge. Efficacy of i.p. vaccination was evaluated using CpG-C in conjunction with liposomes, and ESAT-6. Overall, i.p. injection protected mice with variable efficacy with the CpG-C vaccine at times appearing to provide protection with a significant reduction in CFUs compared to saline control mice, and in repeat experiments not providing protection at all. **Figure 3-2** shows lung CFUs from multiple experiments in which i.p. injection was used for vaccination that resulted in variable efficacy in which i.p. injection was used for vaccination. In one experiment, the vaccine resulted in an overall reduction in CFUs that was statistically significant, however in repeat experiments this was not the case, thus i.p. inoculation resulted in variable efficacy in the lung. Spleen CFUs did not exhibit a similar trend to the lung CFUs, nor did they result in any statistical differences between groups (data not shown).

Due to the variable efficacy of i.p. vaccination, other routes of inoculation were also evaluated. C57BL/6 mice were vaccinated 3 times, two weeks apart using multiple routes of inoculation and challenged with H37Rv *M. tuberculosis* via the pulmonary route. These routes of inoculation included further i.p. testing from that described previously as well as intra-nasal (i.n.), sub-cutaneous (s.c.), and intra-gastric (i.g.) to evaluate an additional mucosal inoculation site. A compilation of the data comparing i.n., i.g., and s.c. routes is shown in **Figure 3-3**. Control groups consisting of saline, liposomes alone, and CpG-C with liposomes were included but are not shown for clarity. Adjuvant/carrier control groups did not result in a significant reduction in CFUs compared to saline controls. For CpG-C/Liposome/ESAT-6, i.p. vaccination did provide a significant reduction in CFUs, although the consistency of efficacy was not reliable **(Figure 3-2)**. The i.n. administration resulted in almost a $1\log_{10}$ reduction compared to the saline control, and the i.g. inoculation provided no protection with any statistically significant reduction in CFUs compared to the saline control.

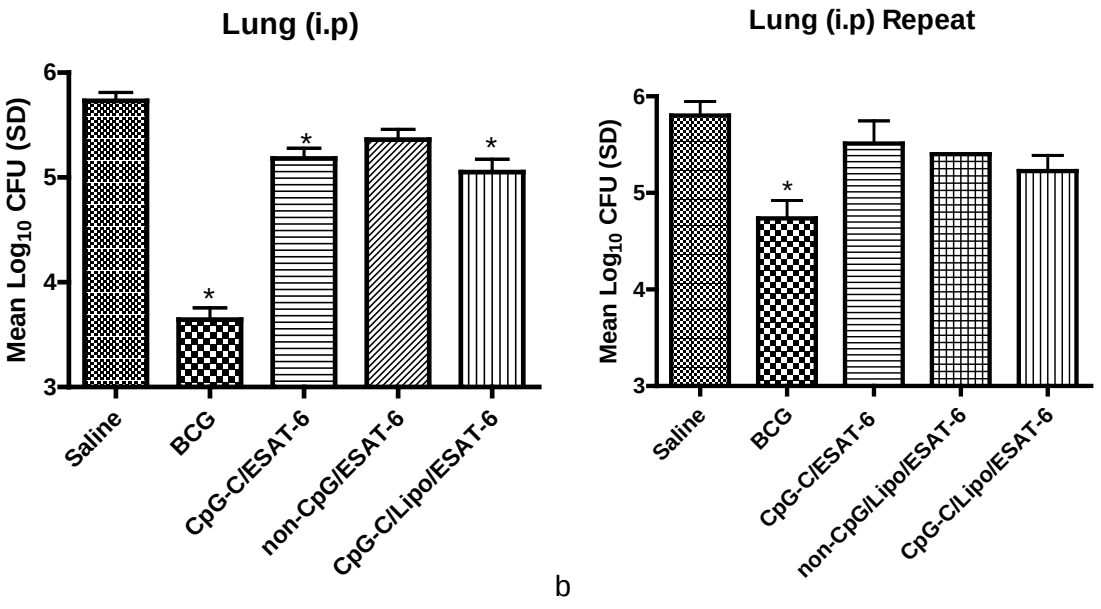


Figure 3-2: Results from repeat challenge experiments vaccinating mice with CpG-C based vaccine i.p. (BCG was administered s.c.) Star represents statistical significance compared to negative control group where $p < 0.05$ (a) CFUs from initial experiment vaccinating animals i.p. with CpG-C based vaccine. (b) Repeat experiments show i.p. vaccination did not provide protection consistently as shown by no significant reduction in CFUs compared to saline control

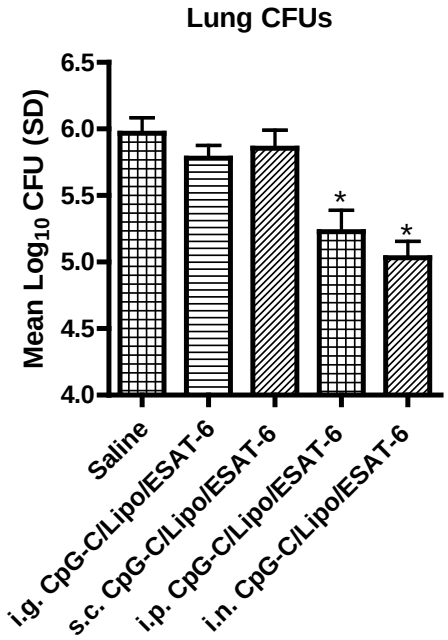


Figure 3-3: Compilation of CFU data displaying results from vaccinating with multiple routes of inoculation. Star represents statistical significance compared to negative control group where $p < 0.05$.

3.3.2 Comparison of intra-nasal vaccination with CpG-A, CpG-B, CpG-C subtypes

In order to determine the most protective CpG sub-type, all three were tested *in-vivo* by vaccinating animals with liposomes and ESAT-6 in conjunction with each sub-type and then challenging the mice after 30 days. After challenge the lung and spleen were harvested and homogenized for bacterial burden. Control groups tested but are not shown include liposomes/ESAT-6, and liposomes in combination with CpG-A, -B, and -C. None of these groups showed a statistically significant difference in CFUs when compared to the saline control. The bacterial burden in the lung 30 days post-infection, showed no significant difference in groups inoculated with either CpG-A, or CpG-B compared to the saline control. Vaccine containing CpG-C did provide protection by reducing bacterial burden in the lung significantly compared to the saline control **(Figure 3-4)**. Although bacterial burdens in the spleen exhibited a similar trend to lung CFUs, there was a decrease in CFUs in the CpG-C group compared to the saline control, although not statistically significant **(Figure 3-4)**.

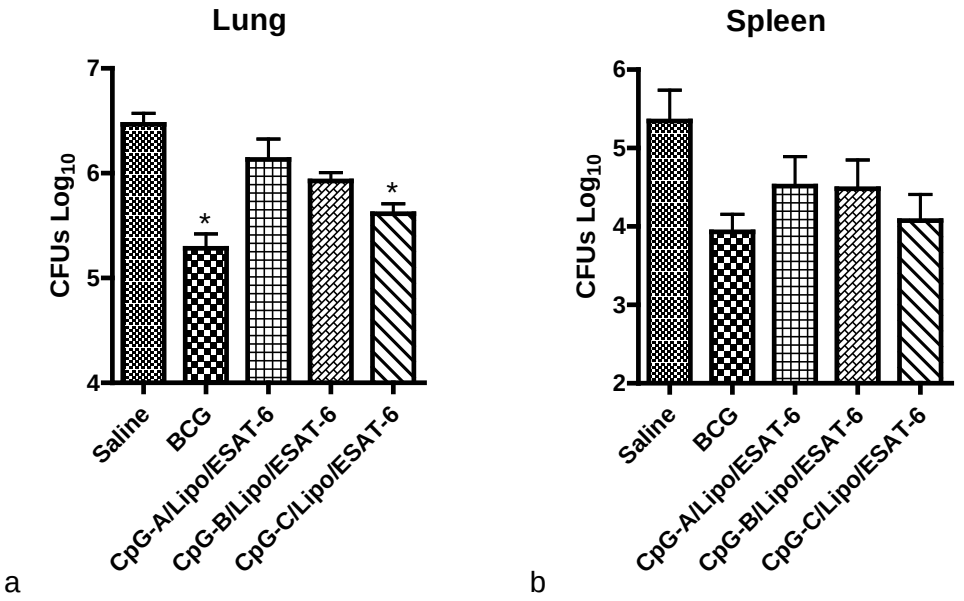
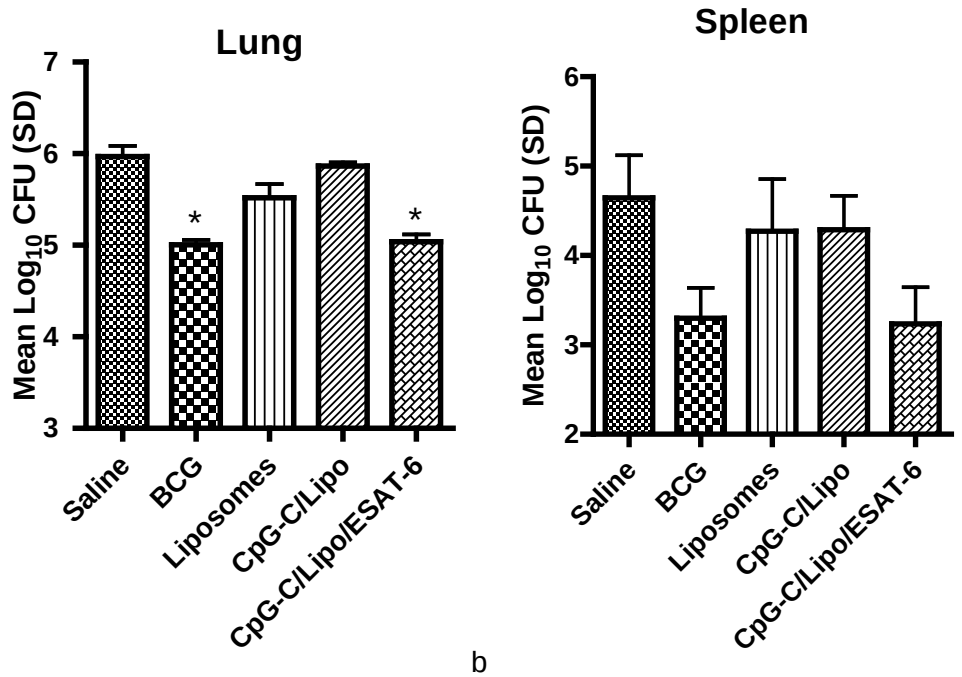


Figure 3-4: Comparison of CpG-A, -B, and –C subtypes combined with liposomes and ESAT-6 as an i.n. vaccine. Results displayed as bacterial burden in the lung and spleen as quantified by CFUs. Star represents statistical significance compared to negative control group where $p < 0.05$. (a) Lung CFUs (b) Spleen CFUs

3.3.3 Intra-nasal CpG-C vaccine in C57BL/6 mice infection study

The efficacy of CpG-C combined with liposomes given intra-nasally was evaluated by infecting animals 30 days after vaccination and sacrificing mice 30 days following infection to determine bacterial burden in the homogenized lung and spleen of infected mice. Intra-nasal delivery of CpG-C combined with liposomes and ESAT-6 consistently provided significant reduction in CFU when compared to saline treated mice in multiple repeat experiments **(Figure 3-5)**. Other control groups evaluated, but not shown include non-CpG ODN, CpG-C/ESAT-6, and liposomes alone. These control groups did not exhibit a protective response against bacterial burden and showed no significant difference when compared to saline controls. CpG-C based vaccine also reduced bacterial burden in the spleen compared to the saline control in a trend similar to the lung **(Figure 3-5)**. The results however were not statistically significant.



a b

Figure 3-5: Bacterial burden in the lung and spleen of infected mice after i.n. vaccination with CpG-C based vaccine. Star represents statistical significance compared to negative control group where $p < 0.05$. (a) Lung CFUs (b) Spleen CFUs

3.4 Discussion

Once the optimal CpG ODN sub-type was determined *in-vitro*, the optimal route of inoculation was tested to observe the effect of combining CpG-C with liposomes and ESAT-6 as a vaccine and administering the formulation to mice through various routes of inoculation. Since many vaccines are administered parenterally, mice were vaccinated via intra-peritoneal (i.p.) and sub-cutaneous (s.c.) routes. Also, because it has been shown that matching the route of infection with the route of inoculation results in greater efficacy of the vaccine administered[453, 483], mucosal administration of the vaccine was also evaluated in the form of an intra-nasal (i.n.) vaccine. In order to evaluate mucosal immunity further, mice were also vaccinated intra-gastrically (i.g.). Mice were all vaccinated three times, two weeks apart and were infected with *M. tuberculosis* 30 days following the last vaccination. After another 30 days mice were sacrificed and the lung and spleen were removed and homogenized in order to determine the bacterial burden in the organs.

Mice were first vaccinated via i.p. injection in repeat experiments. Although the CpG-C vaccine resulted in an overall reduction in bacterial burden in some cases, these results were not consistent. This effect may have to do with the skill by which the mice were vaccinated, or more likely, the immune response may not have been primed as effectively with i.p. injected which lead to variable efficacy. Although a systemic immune response may have been initiated, a local response in the lung where infection occurs was not reproducible.

Next, other routes of inoculation were then tested and compared including i.n., i.g., and s.c. In some plots, data were compiled from multiple experiments in order to demonstrate differences between treatment groups. In repeat experiments, i.g., i.p., and s.c. routes of inoculation consistently failed to provide protection against bacterial burden in the lung and spleen of infected animals. The i.g. vaccination confirms that mucosal administration in general is not efficacious in providing protection, and the actual site of infection had the most robust immune response when stimulated directly. The i.n. route of vaccination routinely resulted in a

statistically significant reduction in CFUs compared to saline controls and other routes of inoculation with the vaccine. I.n. vaccination was therefore chosen for subsequent experiments.

In order to confirm the differences in efficacy between CpG sub-types in-vivo, mice were vaccinated via i.n. route with the three main types of CpG ODN, CpG-A, -B, and –C. Mice were vaccinated using the same model as described previously. Vaccination resulted in almost a full $\log_{10}$ reduction in CFUs compared to saline controls with CpG-C but not CpG-A or CpG-B. Reduction in CFUs in the spleen followed a similar trend however the data was not significant. This is an indication that CpG-C in combination with liposomes and ESAT-6 provide superior protection against *M. tuberculosis* compared to other subtypes. This may be due to the upregulation of IFN-α from plasmacytoid dendritic cells (pDCs) in response to vaccination, or because CpG-C possesses a combination of qualities that CpG-A, and –B exhibit and the combination of effects results in a more robust and well-rounded immune response targeting multiple cell types.

To replicate the protection of the CpG-C vaccine in providing protection against infection in mice, mice were vaccinated once again via the i.n. route with the CpG-C vaccine in a standalone experiment to reproduce the result. Mice were vaccinated in the same manner as described previously. In repeated experiments the CpG-C vaccine provided a significant reduction in CFUs compared to the saline control group. Our conclusion is that the CpG-C vaccine is efficacious for protection against *M. tuberculosis* infection in C57BL/6 mice for at least up to 30 days following infection. This protection is presumably due to CpG-C stimulation through TLR9 in response to potent activation of adaptive immunity initiated by pro-inflammatory cytokine production from antigen presenting cells. Our hypothesis is that CpG-C is also stimulating IFN-α production by pDCs which stimulates cell-mediated immune responses. Our next step was to determine the mechanism of action by which immunity is generated, and efficacy of the vaccine over a longer period of time.

4 Mechanism of action induced by CpG-C and memory immunity

4.1 Introduction

Once the optimal CpG ODN sub-type and route of inoculation was established in the C57BL/6 mouse, we wanted to determine the mechanism of immunity, and the ability of the vaccine to establish a memory immune response. In order to evaluate this, C57BL/6 mice were inoculated intranasally with CpG-C combined with liposomes. Our hypothesis is that CpG-C combined with liposomes stimulates a potent innate immune response shortly after vaccination as indicated by cellular activation markers and pro-inflammatory cytokine production. The ESAT-6 antigen was not added in order to fully evaluate the adjuvant effect created by CpG-C and its effect on innate immunity as others have shown that ESAT-6 can activate immunity[491, 492]. The CpG-C/liposome was administered once in order to see the effect on innate immunity without triggering an adaptive immune response. To examine the effect on innate immunity we examined cytokine production in the lungs by performing broncho-alveolar lavage (BAL) at specific time-points. At 6, 24, and 48 hours post-vaccination, mice were sacrificed and broncho-alveolar lavage fluid was collected from the lungs of vaccinated mice. Cells were separated from BAL and analyzed for cellular markers via flow cytometry, and the supernatant was retained for cytokine analysis of specific cytokines by ELISA assay. Although much of the data was not significant, an apparent trend was revealed with various DC markers were upregulated after 6 hours including mPDCA, Ly6c, CD11c, and I[A]-b. In addition, pro-inflammatory cytokines were upregulated after 24 hours including IFN-α, TNF-α, and IL-12, although this data was not statistically significant, a trend seemed to emerge indicating that an early immune response was being generated just hours after vaccination. This data supports our hypothesis that CpG-C combined with liposomes may induce proinflammatory cytokine production soon after vaccination.

In order to further determine the mechanism by which the CpG-C vaccine results in protection and reduction of bacterial burden, the role of IFN-α production was evaluated. IFN-α

is produced by plasmacytoid dendritic cells in response to TLR9 stimulation, and is generally produced in response to viral infection[493, 494]. Despite the controversial role IFN-α plays in bacterial infections, we hypothesize that CpG-C provides protection against H37Rv *M. tuberculosis* infection in mice by stimulating IFN-α production by pDCs resulting in restricted bacterial growth within infected macrophages, and increased bacterial killing[493]. CpG-A stimulates copious amounts of IFN-α production, and CpG-C is known to also stimulate IFN-α production in moderate amounts[478, 495]. The role of IFN-α in CpG-C mediated immunity was evaluated by vaccinating wild-type (WT) and IFN-αR1$^{-/-}$ mice three times, two weeks apart, and then challenging the mice with H37Rv thirty days after vaccination. The lung and spleen were plated on 7H11 agar to determine the bacterial burden 30 days following infection. Vaccinated IFN-αR1$^{-/-}$ mice did not result in a significant reduction in CFUs compared to saline controls suggesting that vaccine mediated protection was lost without the presence of IFN-α signaling. There was a significant statistical difference between CpG-C vaccinated WT and knockout (KO) groups which may indicate that IFN-α plays a role in mounting the immune response following vaccination with CpG-C ODN. This data supports our hypothesis that IFN-α plays a role in providing protection against infection when vaccinated with the CpG-C/liposome vaccine.

MyD88 is a cytosolic adaptor protein involved in TLR signaling[496]. Although there are some TLRs that can signal through a MyD88 independent pathway, TLR9 is generally not considered one of them[472]. In order to determine the mechanism by which CpG-C stimulates immunity, C57BL/6 (WT) mice and MyD88$^{-/-}$ mice with C57BL/6 background were vaccinated with CpG-C ODN combined with liposomes and ESAT-6 three times, two weeks apart and then infected with *M. tuberculosis* H37Rv after 30 days. Thirty days after infection mice were sacrificed and the lung and spleen were plated on 7H11 agar to quantify bacterial burden with CFUs. In contrast to previously published reports[474], CpG-C vaccinated MyD88$^{-/-}$ mice showed comparable CFUs to WT mice with no significant detriment due to the lack of MyD88. MyD88$^{-/-}$ mice also displayed a significant reduction in CFUs compared to KO saline controls with no

statistically significant difference between CpG ODN vaccinated groups comparing KO and WT. This data suggests that the CpG-C/liposome vaccine may be signaling through an alternative mechanism not yet described in the literature.

Lastly, the ability of the vaccine to provide long-term protection in mice was assessed. The experimental model used for this study is similar to the one described previously with one exception **(Figure 4-1)**. Mice were vaccinated three times, two weeks apart, and 120 days following vaccination mice were either sacrificed and organs were harvested for immunology studies or mice were challenged with *M. tuberculosis* H37Rv **(Figure 4-1)**. For challenge studies, mice were then sacrificed 30 days after infection for quantification of bacterial burden as performed previously. This experimental model evaluates a longer time-frame from vaccination to evaluation of cytokine or bacterial burden to determine the ability of the vaccine to provide protection over an extended period of time. Bacterial burden in the lungs of mice vaccinated with the CpG-C/liposome vaccine resulted in a statistically significant reduction in CFUs compared to saline controls. CFUs in the spleen displayed a similar trend although the reduction was not statistically significant. These results may be an indication that this vaccine is capable of providing long-term protection in mice resulting from an enhanced population of long-lived memory T-cells in vaccinated animals. This hypothesis is further supported by cytokine analysis of cellular supernatants from homogenized lung with sustained production of pro-inflammatory cytokines including IFN-γ and IL-2 a full 120 days following vaccination.

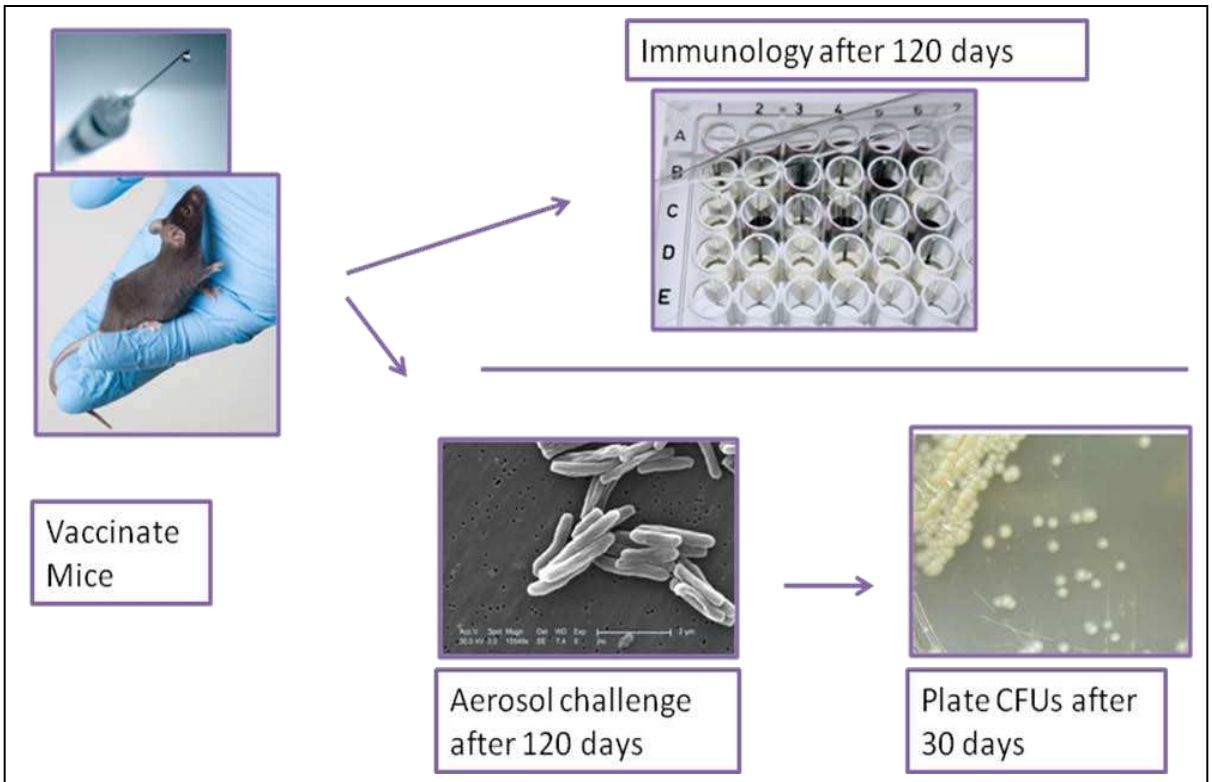


Figure 4-1: Experimental diagram – Mice were vaccinated three times, two weeks apart, after 120 days mice were either sacrificed for immunology or received aerosol challenge. Mice that were challenged were then sacrificed after 30 days and lung and spleen were removed to quantify bacterial burden.

4.2 Materials and Methods

4.2.1 Mycobacterium species

As described previously in 2.2.6

4.2.2 CpG Oligodeoxynucleotides (ODN)

As described previously in 2.2.7

4.2.3 Mice

Female C57BL/6 mice aged 6-8 weeks (5 animals/ treatment group) were purchased from Jackson Laboratories (Bar Harbor, MA). MyD88$^{-/-}$ and IFN-αR1$^{-/-}$ mice bred with C57BL/6 background were also obtained from Jackson Laboratories (Bar Harbor, MA). Wild-type

C57BL/6 mice were used as a control. Mice were house in a specific temperature and humidity controlled pathogen-free environment. All mice had unlimited access to sterile mouse chow and water. All experimental procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (CSU IACUC).

4.2.4 Immunizations

As described previously in 3.2.4

4.2.5 Aerosol infection

As described previously in 3.2.5

4.2.6 Bronco-alveolar lavage (BAL)

BAL cells were obtained by cutting the trachea of euthanized mice and inserting a catheter tube into the trachea and securing with sutures. The lung was then flushed with 1mL of PBS containing 5% FBS, rinsed an additional time, and fluid containing cells was retained for analysis (see Appendix A: Izzo Lab Bronchoalveolar Lavage SOP).

4.2.7 Cell preparation for flow cytometric and ELISpot analysis

As described previously in 3.2.6

4.2.8 In-vitro bone marrow derived macrophages (BMMØ) stimulation assays

As described previously in 3.2.6

4.2.9 In-vitro bone marrow derived dendritic cell (BMDC) stimulation assays

As described previously in 2.2.3

4.2.10 Antibodies and flow cytometric analysis

Antibodies directly conjugated with fluorochromes were used for flow cytometry (eBiosciences; BD Biosciences). The following antibodies were used: anti-CD8a (Alexa700; clone 53-6.7), anti-CD11c (PE-Cy7; clone N418), anti-I[A]-b (PE; clone AF6-120.1), MACS Miltenyi Biotec anti-mPDCA-1 (Clone: JF05-1c2.4. - FITC) – MACS Miltenyi Biotec anti-Ly-6C (Clone: 1G7.G10 - APC).

Antibodies were diluted in fluorescence-activated cell sorting (FACS) buffer (PBS with 0.05% sodium azide) for staining. Staining was done by incubating with 25uL of fluorochrome for 20mins at 4℃ followed by washing in FACS buffer. Cells were then fixed with 4% paraformaldehyde for 25 minutes at 4℃ and were stored in FACS buffer prior to analysis (see Appendix A: Izzo Lab 4% PFA SOP). Flow cytometry was performed using a FACS Canto II flow cytometer (BD Biosciences) for eight-color analysis. Cellular responses were analyzed by focusing dot-plot gates to include live lymphocytes, based on forward- and side-scatter characteristics of lung and spleen cells. Approximately 100,000 total events were collected, and data analysis was done using FloJo software (Tristar Inc, OR).

4.2.11 ELISA colorimetric cytokine analysis

As described previously in 2.2.4

4.2.12 ELISpot assay analysis

As described previously in 3.2.7

4.2.13 Statistical analyses

As described previously in 2.2.8

4.3 Results

4.3.1 Innate immune response generated by CpG-C stimulation

In order to determine the adjuvant effect of CpG ODN and its effect on innate immunity, mice were vaccinated with CpG-C and liposomes just one time. This was to determine the immune response of CpG-C without the presence or influence of ESAT-6. Mice were vaccinated only once in order to observe the innate immune response by avoiding triggering adaptive immunity. Broncho-alveolar lavage (BAL) fluid was removed from animals at 6, 24, and 48 hour time points to characterize dendritic cell (DC) differentiation, kinetics, and recruitment following intranasal inoculation with saline, liposomes, or liposomes with CpG-C. Cells were harvested and flow cytometry was performed on BAL cells for various DC markers

including mPDCA, Ly6c, CD8, and CD11c and supernatants were retained for ELISA. ELISA was performed for cytokine analysis on BAL supernatants for IFN-α, IL-12, and TNF-α. Flow cytometry data revealed the presence of mPDCA, Ly6c, CD8, CD11c, and I-A^b (MHC II) in low levels in most groups, but each marker was markedly elevated following liposome/CpG-C stimulation at the 6 hour timepoint, with CD11c having a statistically significant increase **(Figure 4-2)**. Cytokine analysis revealed no significant differences between groups; however, certain trends were established. IFN-α showed production of cytokine after 24 hours following inoculation with liposomes combined with CpG-C **(Figure 4-3)**. The production of TNF-α was elevated following CpG-C/liposome stimulation after 24 and 48 hours **(Figure 4-3)**. IL-12 was also elevated following CpG-C/liposome stimulation after 24 and 48 hours **(Figure 4-3)**.

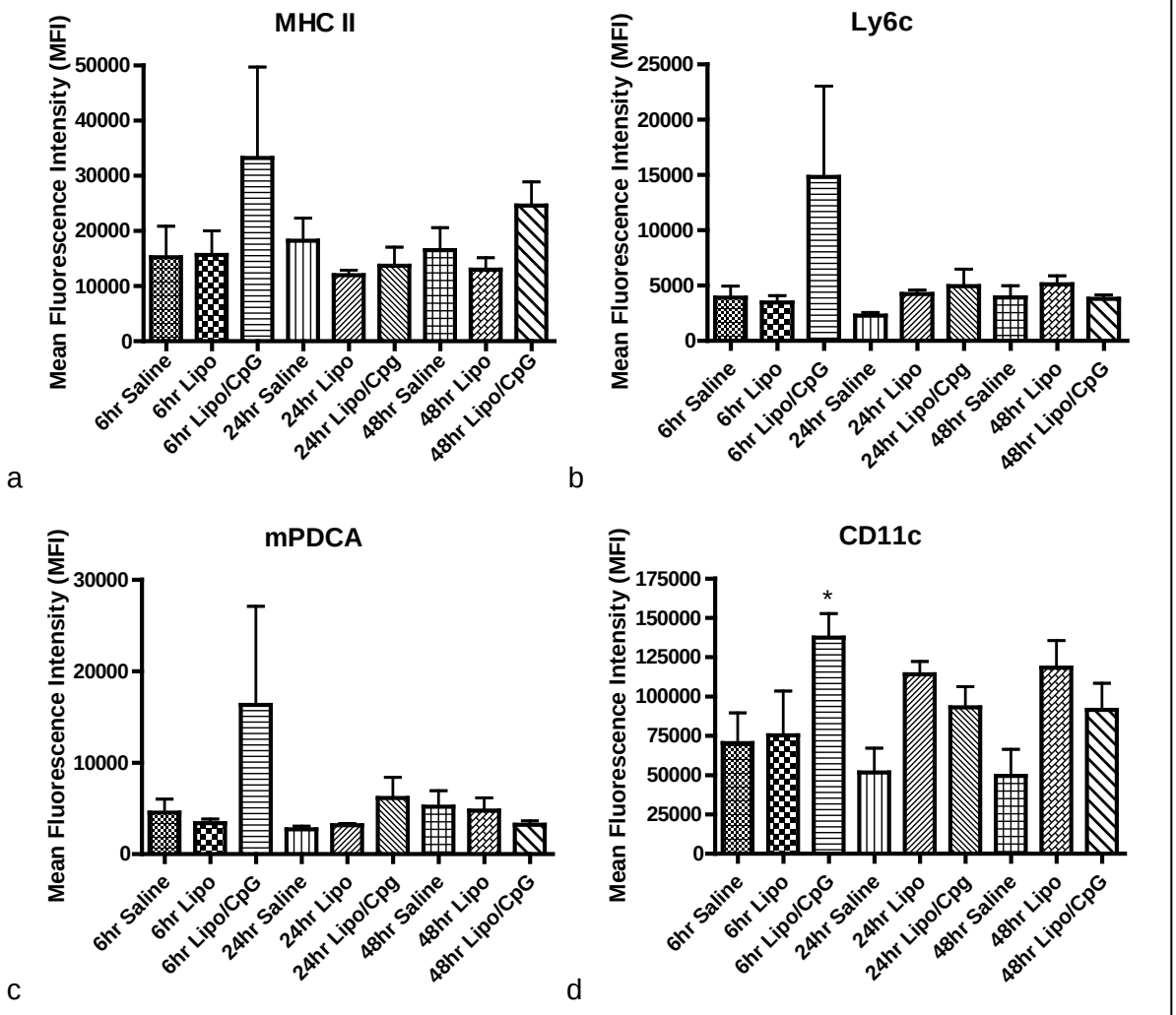


Figure 4-2: Upregulation of markers in BAL fluid indicative of cellular influx and activation 6hr after vaccination with CpG-C/liposomes. Star represents statistical significance compared to negative control group where $p < 0.05$. Data obtained by flow cytometry. (a) MHC II graph (b) Ly6c graph (c) mPDCA graph (d) CD11c graph

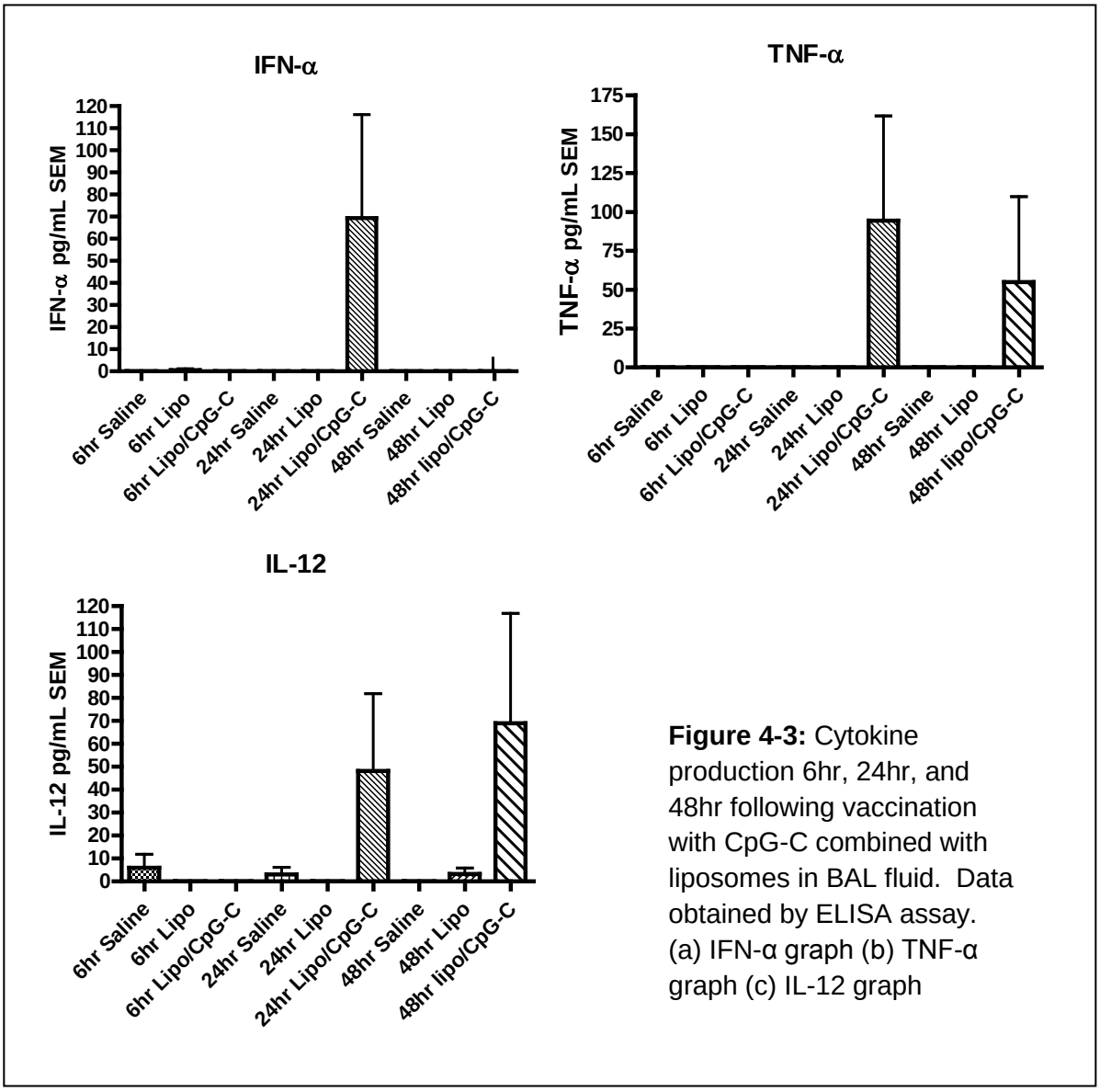


Figure 4-3: Cytokine production 6hr, 24hr, and 48hr following vaccination with CpG-C combined with liposomes in BAL fluid. Data obtained by ELISA assay. (a) IFN-α graph (b) TNF-α graph (c) IL-12 graph

4.3.2 Immune response generated 30 days post-intra-nasal vaccination with CpG-C

The immune response generated by intranasal vaccination with CpG-C vaccine was evaluated by sacrificing mice 30 days following vaccination and performing ELISpot assay on spleen cells and evaluating the presence of the following cytokines: IFN-γ, TNF-α, and IL-2. Other control groups, not shown include mice inoculated with CpG/ESAT-6, and liposomes/ESAT-6, which produced negative results for cytokine production. Following

vaccination with CpG-C/liposomes/ESAT-6, a significant increase in number of spot forming units for all three cytokines was observed after 30 days **(Figure 4-4)**. These key cytokines are produced 30 days after inoculation with CpG-C/liposomes/ESAT-6, suggesting the potent nature of the CpG-C to activate T-cells.

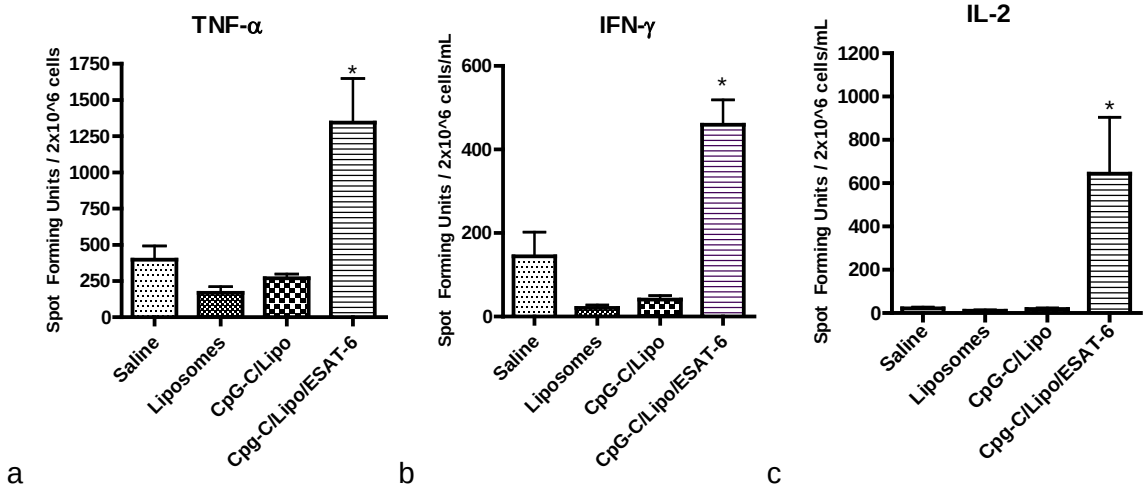


Figure 4-4: Pro-inflammatory cytokines generated 30 days after vaccination with CpG-C based vaccine as shown by ELISpot assay. Star represents statistical significance compared to negative control group where $p < 0.05$. (a) TNF-α graph (b) IFN-γ graph (c) IL-2 graph

4.3.3 Intra-nasal vaccination of IFN-αR1$^{-/-}$ mice

The role of IFN-α was evaluated during vaccination and infection by inoculating IFN-α receptor 1 (IFNR1) knockout (KO) mice with CpG-C/liposomes/ESAT-6. Saline and liposomes/ESAT-6 treated mice were used for controls. Bacterial burden was evaluated in lung and spleen homogenates, 30 days following infection. In wild-type mice vaccinated with CpG-C/liposomes/ESAT-6, a significant reduction in CFUs was observed in the lung compared to saline controls **(Figure 4-5)**. In addition, the WT mice inoculated with CpG-C/liposomes/ESAT-

6 had a significant reduction in CFUs compared to IFN-αR1$^{-/-}$ mice as well **(Figure 4-5)**. The differences in spleen CFUs between groups were not statistically significant **(Figure 4-5)**.

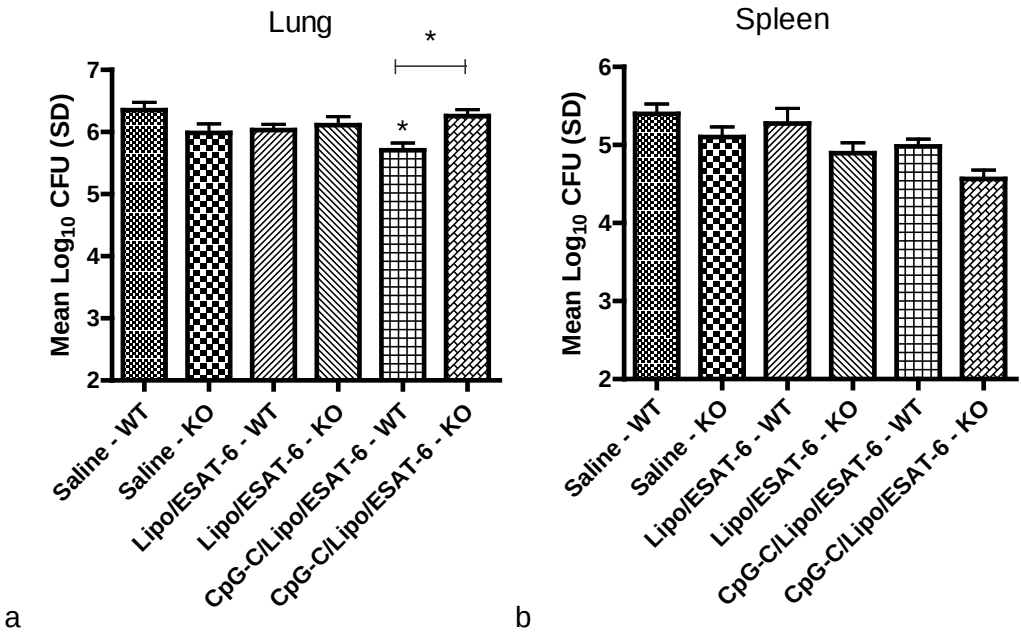
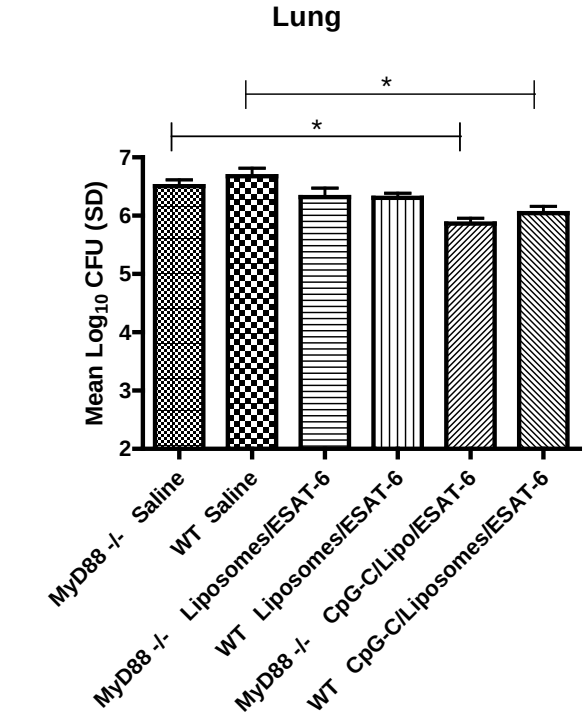


Figure 4-5: Bacterial burden in the lung and spleen of WT and IFN-αR1$^{-/-}$ mice vaccinated with CpG-C based vaccine i.n. – Star represents statistical significance compared to negative control group where $p < 0.05$. - Bar represents statistical difference between adjacent groups where $p < 0.05$. (a) Lung CFUs (b) Spleen CFUs

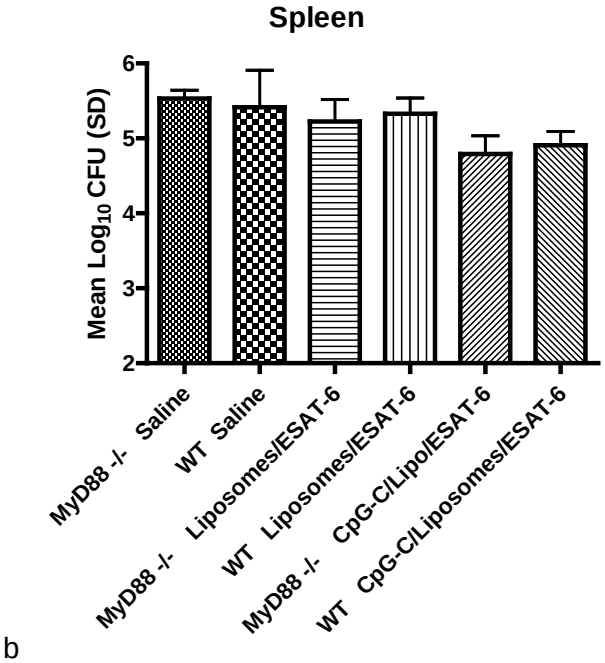
4.3.4 Role of MyD88 in intra-nasal vaccination with CpG-C based vaccine

The mechanism of immunity created by the CpG-C/liposomes/ESAT-6 was evaluated by vaccinating and then infecting myeloid differentiation primary response gene 88 (MyD88)$^{-/-}$ mice and observing the change in bacterial burden after 30 days. The bacterial burden in the lungs of CpG-C/liposomes/ESAT-6 vaccinated mice was significantly reduced compared to the saline control **(Figure 4-6)**. In addition, vaccinated MyD88$^{-/-}$ mice also resulted in a significant reduction in CFUs compared to the saline control group. There was no significant difference in the bacterial burden in the CpG-C/liposomes/ESAT-6 vaccinated group between MyD88$^{-/-}$ mice

and wild-type mice indicating comparable protection with or without the presence of MyD88. Although a trend was observed in reduction of the bacterial burden in the spleen there was no significant difference between groups.



a



b

Figure 4-6: Bacterial burden in the lung and spleen of WT and MyD88$^{-/-}$ mice vaccinated with CpG-C based vaccine i.n. - Star represents statistical significance compared to negative control group where $p < 0.05$. - Bar represents statistical difference between connected groups where $p < 0.05$. (a) Lung CFUs (b) Spleen CFUs

4.3.5 Long-term protection of intra-nasal vaccination with CpG-C

In order to determine if CpG-C/liposomes/ESAT-6 is capable of inducing long-term protection, mice were vaccinated three times, two weeks apart. Mice were then infected 120 days later, and sacrificed 30 days following infection to evaluate the bacterial burden in the lung and spleen. The bacterial burden in the lung following infection was significantly reduced in the CpG-C/liposomes/ESAT-6 vaccine group compared to saline control mice **(Figure 4-7)**. In the spleen, a similar trend in bacterial burden was observed, but the results were not statistically significant. Cytokines were also evaluated in order to determine immune response sustained by vaccinating **(Figure 4-8)**.

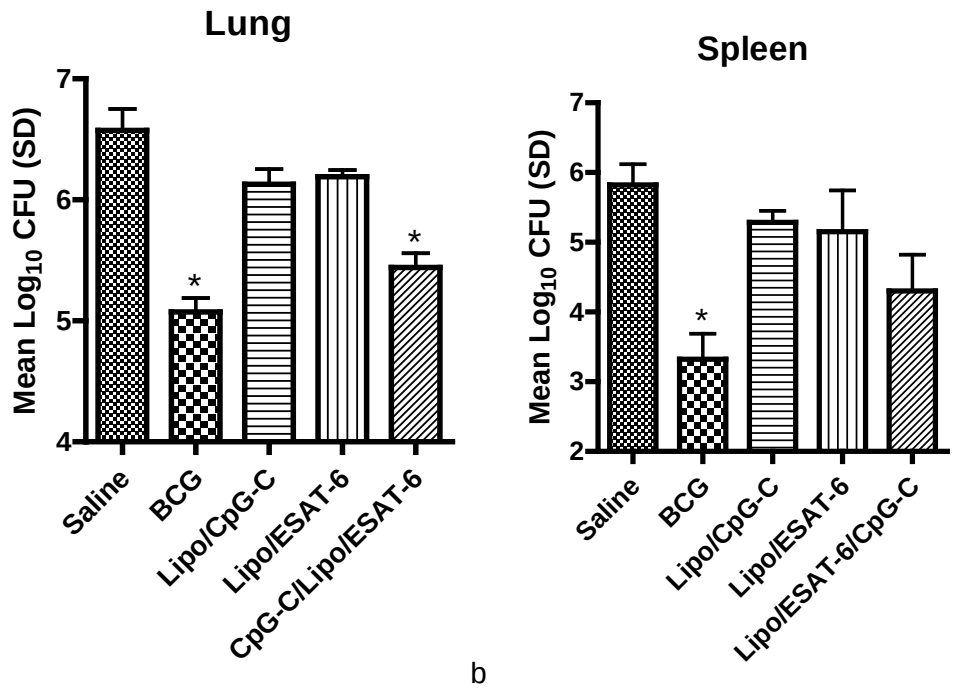


Figure 4-7: Bacterial burden in the lung and spleen when mice were challenged 120 days after vaccination. Star represents statistical significance compared to negative control group where $p < 0.05$. (a) Lung CFUs (b) Spleen CFUs

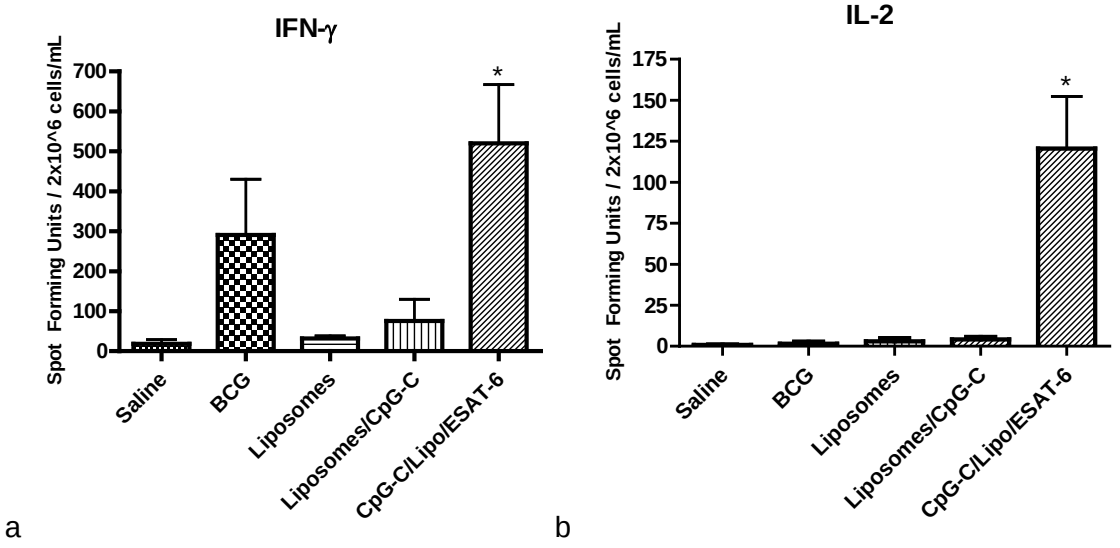


Figure 4-8: Pro-inflammatory cytokine production in the lung 120 days after vaccination shown by ELISpot assay. Star represents statistical significance compared to negative control group where $p < 0.05$. (a) IFN-γ graph (b) IL-2 graph

4.4 Discussion

In order to characterize the local innate immune response being generated by CpG-C, without the influence of ESAT-6, mice were vaccinated only one time, and were sacrificed at 6, 24, and 48 hours post-vaccination. Contents from within the lung were then removed via broncho-alveolar lavage (BAL) and the cells were isolated cytokine analysis with ELISA assay for the cytokines IFN-α, TNF-α, and IL-12; cells were also analyzed by flow cytometry for the following cellular markers: mPDCA, Ly6c, CD8, MHCII (I[A]-b), and CD11c.

IFN-α, TNF-α, and IL-12 are all pro-inflammatory cytokines crucial for driving cellular immunity and developing a protective Th1 type immune response against *M. tuberculosis*. Although none of the results for cytokine analysis were statistically significant, a definite trend was established which indicated at 24 and 48 hours, these pro-inflammatory cytokines peaked. Cells stimulated with CpG-C in combination with liposome produced an upregulation of these pro-inflammatory cytokines with a marked difference from negative controls. Levels of cytokine

from the negative control groups, which include saline and liposomes resulted in almost completely undetectable production of cytokine. With pro-inflammatory cytokine production after just one vaccination, it is clear that CpG-C has an immunostimulatory effect. If mice had been inoculated with a booster vaccine, the response most certainly would be more potent, and longer lasting. These data however do show the effect of CpG-C on the innate immune response and the quickness in which it begins to initiate immunity.

Flow cytometry analysis was also performed to determine the upregulation of various cellular activation markers. Although some data are analyzed as single-parameter histograms instead of dual parameter for clarity of output, a definite trend was observed with the data. The plasmacytoid dendritic cell marker, mPDCA was shown to be upregulated 6 hours post-vaccination with CpG-C and liposomes, although this increase was not statistically significant compared to saline, this could be an indication of early recruitment of plasmacytoid dendritic cells in the lung recruited just a few hours following vaccination. This increase showed that CpG-C elicited a potent innate immune response early after intranasal inoculation. The cellular marker Ly6C was also upregulated in lavage cells after 6 hours although this result was not significant. Ly6C is found on macrophages, monocytes, granulocytes, and some subsets of lymphocytes. Although it is not clear which particular subtype is being upregulated, this does reinforce a trend developing that CpG-C elicits an innate immune response soon after vaccination. The upregulation of MHC II was also evaluated by the marker I[A]-b, which was similarly upregulated in lavage cells 6 hours after vaccination although the change was not statistically significant. This may be an indication that CpG-C is priming antigen presenting cells and may be capable of upregulating MHC II expression to more effectively present antigen to circulating T-cells. This may be one mechanism of action by which the CpG-C vaccine confers immunity. The conventional DC (cDC) marker CD11c was also evaluated to determine if other DC types are being stimulated in addition to pDCs. After 6 hours, the CpG-C/liposome component of the vaccine produced a statistically significant increase in the presence of

CD11c+ cells in the lung environment compared to saline controls. This could be an indication that the CpG-C is recruiting this powerful antigen presenting cells soon after vaccination, and the upregulation of MHC II for example could be making them better suited for antigen presentation.

The immune response generated by the vaccine was further evaluated *in-vivo* by observing the adaptive immune response. This was done by vaccinating mice with CpG-C combined with liposomes and ESAT-6 and sacrificing uninfected animals after 30 days to observe cytokine production by memory T-cells established in spleen cells following vaccination. Cytokine analysis was done by ELISpot assay in which cells were stimulated for 24 hours after harvesting with ESAT-6 antigen. The cytokines evaluated were IFN-γ, TNF-α, and IL-2. In each of these pro-inflammatory cytokines evaluated, the cells from mice vaccinated with the CpG-C vaccine produced significantly more cytokine compared to the saline controls. These pro-inflammatory cytokines are an indication of cellular recruitment, cellular activation of antigen presenting cells and T-cells, as well as T-cell proliferation in the case of IL-2. This shows that the vaccine is inducing a potent pro-inflammatory cytokine response, and a cellular immune response with Th1 type characteristics. This type of immune response is critical when priming the immune response for protection against *M. tuberculosis*.

Because we have shown that CpG-C may be stimulating IFN-α production by pDCs, we wanted to further examine the role this cytokine plays during infection. Although it has been shown in some studies to be detrimental to protecting against *M. tuberculosis* infection, other studies have shown this cytokine to play a positive role[493]. In order to evaluate the role of IFN-α during vaccination and infection following vaccination with CpG-C we vaccinated IFN-α receptor 1 sub-unit (IFN-αR1$^{-/-}$) mice with the CpG-C vaccine and infected and sacrificed following the same method described previously. As expected, CpG-C vaccinated wild-type (WT) mice resulted in a statistically significant reduction in CFUs compared to saline controls, however, bacterial burden in the lungs of vaccinated KO mice were not significantly reduced. In addition,

there was a statistically significant difference in CFUs when comparing CFUs in WT and KO mice which not only indicates that IFNαR1$^{-/-}$ mice are not protected from infection, but they resulted in significantly more CFUs than the WT mice. CFUs in the spleen did not result in any significant differences. These data suggest that not only does the CpG-C vaccine target IFN-α production in presumably pDCs following vaccination, but this cytokine production results in protection against infection and bacterial burden in the lungs of infected mice and the cytokine is also important for generating a protective immune response following infection. This study may provide valuable insight into the mechanism of action by which this vaccine elicits protection.

Next, we wanted to further elucidate the mechanism by which the vaccine protects by observing the role of the adaptor protein MyD88 during vaccination and infection. MyD88 $^{-/-}$ were vaccinated and infected following the same protocol as described previously. Surprisingly, our data revealed that MyD88 $^{-/-}$ still resulted in a significant reduction in CFUs compared to the MyD88 $^{-/-}$ saline group. There was no statistical difference in the CpG-C vaccinated KO and WT groups indicating equal protection as a result of the vaccine with or without the presence of the MyD88 adaptor molecule. Since CpG-C ODN is generally observed to signal through TLR9, which is thought of as a MyD88 dependent pathway, these results could indicate an alternative pathway is in play. This has not been shown previously in the literature, but LPS stimulation of TLR4 has shown alternative signaling bypassing the MyD88 adaptor[497]. This signaling occurs through the TRIF molecule, and it is possible that CpG-C may be somehow inducing a similar alternative pathway.

Lastly, we wanted to evaluate the ability of the vaccine to protect over a longer period of time. Both bacterial burden and cytokine production were evaluated in mice that were infected 120 days following vaccination. Mice were vaccinated with the CpG-C based vaccine three times, two weeks apart, and were challenged with H37Rv *M. tuberculosis* 120 days following vaccination instead of 30 days. Mice were then sacrificed after 30 days and the lung and spleen were harvested to determine bacterial burden in these organs in the form of CFUs, and also

cytokine production in cells from infected organs. The bacterial burden in the lungs of infected mice did show a significant reduction in CFUs when compared to the saline control. The spleen showed a similar trend however, results were not significant. In addition, pro-inflammatory cytokine production was also increased in CpG-C vaccinated mice with a statistically significant difference compared to saline controls. These results show promising evidence that a CpG-C based vaccine delivered intransally may be capable of conferring long-term protection against *M. tuberculosis* infection. Not only has this vaccine been shown to provide a robust pro-inflammatory and cellular immune response with protection following infection, this response is sustained for an extended period of time. This model may show potential in other animal models to provide long-lasting protective immunity against *M. tuberculosis* infection.

5 Concluding remarks

It was once thought that through widespread vaccination of *M. tuberculosis*, education, drug-compliance, and the plethora of tuberculosis control strategies being employed across the globe since the announcement of tuberculosis being a global health emergency in 1993 that we would have seen a steady and substantial decline in rates of infection and disease by now. Unfortunately, with increasing rates of multi-drug resistant (MDR) and extensively drug-resistant (XDR) TB, and HIV co-infection this dream has not yet come to fruition.

This further emphasizes the need for a reliable and efficacious vaccine against *M. tuberculosis* that prevents infection before any of these other issue can take hold. It has long been known that a replacement for BCG is of utmost importance in the field of infectious disease and yet after almost a century, one has yet to be developed. Our hypothesis is that by targeting alternate routes of inoculation, and designing a sub-unit vaccine focusing on only the most robust and effective immune responses, we could design a superior formulation capable of providing protection against *M. tuberculosis* by using CpG ODN as a TLR9 agonist.

With *in-vitro* cytokine analysis (Chapter 2) we showed that the CpG-C sub-type provides better stimulation of pro-inflammatory cytokine production from bone marrow derived macrophages and dendritic cells compared to CpG-A, and CpG-B. This sub-type comparison was confirmed *in-vivo* with challenge studies showing that mice vaccinated with CpG-C had significantly reduced bacterial burdens in the lung following infection with *M. tuberculosis* H37Rv compared to CpG-A, and CpG-B. Since CpG-C has an immune response encompassing the effects of both CpG-A and CpG-B, it is possible the more broad stimulation of the immune response results in a more protective vaccine.

By comparing various routes of inoculation (Chapter 3) we showed that the CpG-C vaccine administered mucosally provided superior protection with more reliable and consistent results over other routes of inoculation. This confirms our hypothesis that targeting the initial

site of infection primes the local lung environment more effectively than parenteral or intra-gastric routes of vaccination.

The immune response generated by this vaccine and the mechanisms of action by which its efficacy is derived was evaluated as well (Chapter 4). We showed that the innate immune response is triggered soon after vaccination with a trend displaying increased numbers of dendritic cells, both plasmacytoid (pDC) and conventional (cDC), as well as increased MHC II expression in broncho-alveolar lavage fluid as soon as six hours following vaccination with CpG-C combined with liposomes. After 24 hours a similar trend developed in which pro-inflammatory cytokines critical for controlling *M. tuberculosis* infection are upregulated in response to only one round of vaccination.

The immune response generated in uninfected mice after three rounds of vaccination were indicative of a memory immune response being initiated as evidenced by pro-inflammatory cytokine production in activated T-cells in the lung and spleen 30 days after vaccination. This result was repeated in an experiment observing the immune response in infected mice up to 120 days after vaccination, showing great promise in the ability of this vaccine to provide long-lasting immunity (Chapter 4).

The role of IFN-α was also demonstrated (Chapter 4) in vaccination and subsequent infection by observing IFN-α receptor 1 knockout mice (IFN-αR1$^{-/-}$) following the same experimental procedures as described previously. The hypothesis for this experiment was that CpG-C ODN provided protection against infection at least in part by stimulating IFN-α production by pDCs. This hypothesis was confirmed when, following infection, bacterial burden in the lungs of IFN-αR1$^{-/-}$ mice were significantly higher than wild-type (WT) mice and these mice showed no significant protection compared to saline controls.

It is clear that further studies need to be performed in order to fully understand the mechanism by which this vaccine affords protection as evidenced by results from vaccinating mice lacking the adaptor protein Myeloid Differentiation Primary Response 88 (MyD88).

Surprisingly, MyD88$^{-/-}$ mice were also protected from infection after challenge with H37Rv *M. tuberculosis* in much the same way WT mice were (Chapter 4). Since CpG ODN is believed to signal through TLR9 in a MyD88 dependent pathway, this hypothesis seems to have been disproven with this experiment. Future studies need to be performed in order to confirm this result and also further elucidate possible alternative pathways that may be activated following vaccination in these mice.

All of these data suggest that our hypothesis is correct in that using the TLR9 agonist CpG-C ODN in combination with liposomes with *M. tuberculosis* antigen ESAT-6 provides a robust Th1-type immune response in the lungs of infected mice capable of providing long-lasting protection against infection and bacterial burden when administered intra-nasally. This vaccine, or one similar, could be a stepping stone in one day creating an effective and reliable vaccine providing sustained protection against *M. tuberculosis* infection in other animal models, and ultimately one day in humans.

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7 Appendix A: SOPs

Aerosol infection of mice with *Mycobacterium tuberculosis*

-Twenty-five mice can fit in each section of the aerosol basket. Pathogen use is associated with proper ACUC project approval # only. Confirm the cage cards ACUC protocol # permits use of the pathogen you intend to expose those animals to.

-Risk Assessment

Safety checks on the equipment before and after running an exposure study is required to minimize pathogen releases into the aerosol room.

-Check the lid-gasket's integrity

-Confirm the incinerator is properly connected

-Inspect incinerator hoses for cracks and holes

Safety Considerations

-The technician must be wearing appropriate safety equipment (a RACAL safety helmet with HEPA filters is required.

-Surgical gown

-All disinfecting materials are bagged and autoclaved.

Signs

Turn on 'IN USE' sign on Room 110D.

Equipment

-Middlebrook Airborne Infection apparatus

-BSL-2 hood

Materials

-Glas-Col venture Nebulizer

-Glas-Col Airborne Infection Apparatus (Middlebrook)

-Mycobacterial Suspension

-PAPR hood (personal HEPA filtered breathing apparatus)

-Surgical gown

-5 ml syringe fitted with 18G needle

-5% Lysol wash bottle

-70% Ethanol wash bottle

-50 mL Screw-cap tube

-Paper towels

-Autoclave bags (large and extra-large)

Procedure

Note: Extreme care must be exercised when laboratory personnel are using the Glas-Col Airborne Infection Apparatus. BRB Room 110D area housing this instrument should be off limits to all except those personnel who are involved with its operation. Animal Care staff should be notified as to the experimental schedule for this instruments operation.

-Prepare inoculum in Necropsy Room BSL-2 hood using approved method. The concentration of bacteria should be 4 logs/ml. higher than the amount you want delivered in the lungs. 4-5 ml. for this suspension must be aerosolized from the nebulizer at the end of operation of the

instrument. (Example: If you want to deliver 1.0E+02 cfu in the lungs of mice, you will need 5 ml. @ 2.0E+06 cfu/ml (1.0E+07 cfu in Total)). Draw up 5 mL of inoculum into a 5 mL syringe fitted with an 18 ga. needle. Leave the inoculum in the hood until you are ready to load it into the nebulizer.

-Secure nebulizer to Airborne Infection Apparatus by clamping to inlet ports. Ensure that the nebulizer is secure to avoid the possibility of the aerosol being released into the room. Fill the plastic container from the nebulizer half-full with 5% Lysol.

-Unlatch and raise lid to the infection apparatus fully. Place a basket stand on the floor of the infection chamber. Place a basket on top of the stand. Load animals into basket and secure lid to basket. Use caution when loading animals through the lid opening, as the area around the lid can be sharp. If loading different groups of mice, mark the tail of each mouse with a sharpie designating which group it belongs to. Mice may be placed on the side of the basket and allowed to climb their way to the bottom of the basket. Place no more than 25 mice into each chamber of the basket. When turning the lid to access another chamber, check to ensure that there are not any mice in danger of being caught between the lid and the top of the chamber. Close lid to infection chamber. Press firmly next to each of 4 latches until each one snaps with an audible click.

-Don PAPR. Retrieve inoculum from necropsy room. Place the syringe inside a zip-lock bag when transporting. Notify surrounding personnel that you will be carrying inoculum.

-Remove nebulizer cap. With a 5 ml. syringe fitted with an 18 gauge needle,slowly add inoculum to nebulizer by introducing inoculum slowly down side of venturi and avoiding delicate parts within; watch inoculum as it fills the nebulizer chamber. Inoculum should move through the capillary tube in the center of the venturi. If capillary tube appears to be blocked, abort procedure and use a new nebulizer. When inoculum has been added remove syringe and replace nebulizer cap. Screw on cap securely. Draw volume of Lysol equal to that of inoculum

into syringe from nebulizer container. Allow syringe to sit in container until the run is over to ensure decontamination.

-Turn “POWER” switch on Airborne Infection Apparatus to “on” position.

-When LED screen reads “GLAS-COL APPARATUS CO.”, turn on UV LIGHT switch and PROGRAM switch

-When LED screen reads “ENTER WHEN READY,” press ENTER

-When LED screen reads “ENTER PREHEAT TIME… 900,” press ENTER to accept.

-When LED screen reads “ENTER NEB TIME… 1800,” change value to 2400 and press ENTER.

-When LED screen reads “ENTER CD TIME… 1800,” change value to 2400 (4800 for multi-drug resistant *M. tuberculosis* strains) and press ENTER.

-When LED screen reads “ENTER DEC TIME… 900,” press ENTER to accept LED screen will read “PREHEAT CYCLE IN PROCESS”. Right hand (vacuum) gauge should read 40-60 psi; if it does not, adjust upward with corresponding control beneath.

-Leave the room.

-After 900 seconds (15 minutes), check that left hand (compressor) gauge reads ~10 psi. by viewing through glass window in door. If gauge needs to be adjusted, don PAPR and quickly adjust till psi is in correct position. Avoid being in the room as much as possible when the run is going.

-After the cycle is finished (alarm will sound and UV lights will go off), don PAPR and enter room. Inspect nebulizer to see if inoculum went through the cycle. If it did not then aerosol needs to be repeated, but is recommended that it is done on the same day as to avoid stressing the mice.

-Turn off all switches and open Infection Apparatus to let fresh air into the chamber for the mice.

-Remove syringe from nebulizer container and place in sharps container. Remove nebulizer and pack into (half Lysol-filled) container; wrap and cover liberally with paper towels. Pack

container in red biohazard bag, seal with autoclave tape and very clearly mark “FRAGILE.” Wipe down area of Infection Apparatus adjacent to nebulizer interface with Lysol.

-Remove animals from Infection Apparatus and return them to their cages (large blunt forceps may be used to aid in removing mice); remove basket and stand. Pack basket, stand, cover, and handle in extra large autoclave bags and seal with autoclave tape. Basket must be DOUBLE BAGGED.

-Wipe inside of Infection Apparatus with Lysol twice; repeat process with 70% ETOH. Bag and autoclave paper towels used in the cleaning process. Do not allow debris to fall into aerosol port on the bottom of apparatus, as the debris will cause the port to become clogged. Sweep up and dispose of any debris from animal cages on the floor.

-Turn off “IN USE” light switch in airlock. Leave the room and replace PAPR. Leave packed nebulizer and aerosol basket in anteroom for autoclaving.

Injectable anesthesia using Ketamine/Xylazine for the mouse

Materials and Equipment Needed:

100 mg/ml Ketamine-HCL

20 mg/ml xylazine

Sterile saline or phosphate-buffered saline (PBS)

1 ml syringe with 26 ½ -G needle

Sterile conical tube (optional)

Warm heating pad

Identification of "Critical Steps" in Procedure

-Determine the weight of the mouse in grams.

-Draw up 0.1 ml of Ketamine-HCL (100 mg/ml) into a 1-ml syringe with a 26 ½ -G needle (or add to sterile conical tube for larger volumes).

-Draw up 0.075 ml of xylazine (20 mg/ml) with same syringe (or add to sterile tube).

-Draw up 825 ul of sterile PBS with same syringe and mix well (or add to sterile tube).

-Final concentration of xylazine is 10.0 mg/ml and ketamine is 1.5 mg/ml.

-Load Ketamine/xylazine/PBS solution into a 1 mL syringe with new 26 ½ -G needle.

-Administer mixture intraperitoneally #009M-IMO_ACUC-6/95 at a dose of 0.005ml/gm. Equivalent to 50.0 mg/kg of Ketamine and 7.5 mg/kg of xylazine (Rhompin). For a 20.0 gm mouse use 0.1 ml.

-The length of anesthesia will vary from 15 to 45 min. The Ketamine/xylazine combination provides analgesia with muscle relaxation. Hypothermia can be a problem and use of a heating pad set on low/medium setting to maintain body temperature is recommended.

Preparation of bone marrow-derived Macrophages (BMDM)

-Sacrifice mouse via CO_2 asphyxiation.

-Place mouse on dissection board covered with a paper towel, soak with 70% ETOH.

-Using scissors, clip the skin at mid-stomach and peel the skin with your fingers over the hind legs, soak with 70% ETOH.

-Separate leg from the body at the hip joint and remove the foot, place in a culture dish containing ice cold incomplete RPMI on ice.

-Separate the tibia and the femur with scissors, and remove the epiphyses.

-Using a 3 ml syringe with a 26 gauge needle attached, expel the marrow by pushing incomplete RPMI through the center of the bone, continue until the bone is clean.

-Draw the marrow in and out of the needle and syringe to obtain a single cell suspension.

-Wash cells in incomplete RPMI.

-Lyse red cells using ACK lysis buffer, add 3 mls to the cell pellet and swirl for 3-5 minutes, wash with complete media.

-Resuspend cells in RPMI-1640, + 5% FBS, L-Glut, Pen/Strep and 30% L929 condition media.

-Count cells.

-Culture at $2x10^6$ cells/ml at 37^0 C, 10% CO_2 for 7-9 days, changing the media every 3 days.

Directions for CO_2 Euthanasia of Rodents

-Secure a euthanasia chamber, ideally using the home cage. Animal density and mixing of different animals from separate cages in the euthanasia chamber should be minimized to decrease pre-euthanasia anxiety.

- The volume of the euthanasia chamber (in liters) should be calculated and then divided by 5 in order to determine the appropriate CO_2 flow rate (20%).

If using one of the LAR standard cages the volumes are as follows:

#1 Mouse Thoren cage: 5.8L, flow rate of 1.2 L/min*

#9 Mouse Thoren cage: 6.5L, flow rate of 1.3 L/min

OptiMice cages: 6.2L, flow rate of 1.2 L/min

#2 Rat cage: 10.4L, flow rate of 2.1 L/min

- The cover should be placed over the euthanasia chamber/cage, and the flow meter should be checked to ensure it is in the off position. The CO_2 canister valve is then turned on (A) and the pressure gauge closest to the tank should register a pressure. The second gauge (B) should then be turned on. After both valves have been turned on, the flow meter can be adjusted to the appropriate flow rate (liters/min) in order gradually introduce 100% CO_2 to result in the replacement of 20% of the cage volume per minute. This flow rate has been shown to produce a loss of consciousness without apparent pain.

- Following the induction of unconsciousness (this will take approximately 3-4 minutes), the CO_2 flow rate can be raised to 3-4 times the initial flow rate to accelerate the process. Following apparent clinical death of the animal, gas flow should be maintained for at least one minute.

- Following death the flow meter and two gauges should all be turned to the off position.

- Cervical dislocation or bilateral thoractomy should be performed to assure the animal will not regain consciousness. Euthanized animals should be placed in a sealable bag and discarded on the lower shelves of the necropsy cooler unless there is some other need for the animals or

their tissues. Biohazardous or otherwise hazardous carcasses should be discarded using procedures established for the study in question.

NOTE: Neonate rodents (“pinkies”) will become anesthetized with CO_2. However, due to their high levels of fetal hemoglobin they are resistant to death via CO_2. Cervical dislocation or decapitation (following C02 narcotization) is the best method of euthanasia for these neonates.

Growth of virulent *Mycobacterium tuberculosis*

Preparation of Seed Stocks

From one vial of *M. tuberculosis* (Mtb)

Transfer 1ml of the Mtb to a glass tube containing 9ml of Proskaur & Beck (PB) medium

Incubate at 37C without agitation

Check the culture to ensure the formation of a layer of organisms growing on the top of the liquid, this is the pellicle

The remaining medium below the pellicle should be clear

Streak culture onto 1 or 2 slants of Lowenstein-Jensen media

Incubate slants at 37C until confluent

Transfer to -80C.

Passage 1:

When pellicle is confluent

Using a concentrically coiled loop transfer one loop-full of the pellicle to a 75cm^2 flask containing PB medium

In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (this is a "drop back")

Incubate at 37C without agitation

Examine the cultures – as the pellicle grows the medium below should remain clear with some organisms visible.

Streak a loop of the medium onto blood agar (or TSA)

Incubate 37C, for 48-96hours

Passage 2:

When pellicle is confluent

Using a concentrically coiled loop transfer one loop-full of the pellicle to a 75cm^2 flask containing PB medium

In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (this is a "drop back")

Incubate at 37C without agitation

Streak a loop of the medium onto blood agar (or TSA)

Incubate 37C, for 48-96hours

Examine the cultures – as the pellicle grows the medium below should remain clear with some organisms visible.

Passage 3:

When pellicle is confluent

Using a concentrically coiled loop transfer one loop-full of the pellicle to a 75cm^2 flask containing PB medium

In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (this is a "drop back")

Incubate at 37C without agitation

Examine the cultures – as the pellicle grows the medium below should remain clear with some organisms visible.

Streak a loop of the medium onto blood agar (or TSA)

Incubate 37C, for 48-96hours

Bottling seed stocks

When pellicle is confluent

Using a concentrically coiled loop transfer as much of the pellicle from the 75cm^2 flask to a grinding tube containing approximately 10ml of PB medium

In addition, transfer one loop-full into a new glass tube containing 9ml of PB medium (this is a "drop back")

Incubate at 37C without agitation

Streak a loop of the medium onto blood agar (or TSA)

Incubate 37C, for 48-96hours

Grind the solution for approximately 30-60 seconds

Place tube on ice for 10-15min to allow clumps to settle

Transfer organisms from the top layer (avoiding large clumps) to vials

Seal vials

Label vials as seed stock with the strain name/number, initials of RA and the mm/yy

Store seed stocks at -80C

Growth of Working Stocks for Virulent *Mycobacterium tuberculosis*

-From one vial of Seed Stock aliquot 1 ml into 9ml of Proskaur & Beck (PB) medium containing 0.1% Tween 80

-Incubate at 37C

-Every day: check culture by shaking tube – look for white swirl

-After shaking a frothy layer is noticeable above the liquid, this is the Tween.

-As the Mtb grows the amount of Tween is reduced and it may be necessary to add new Tween.

-To add new Tween: add 1 ml of a 20% Tween-80 in PB solution to 1000ml of culture.

-Over time the culture will turn a milky white color – this indicates growth

-At approximately 10 to 14 days transfer 5ml into 50ml of PB+0.1%Tween

Repeat as above.

-At approximately 10 to 14 days of incubation transfer 50 ml of culture to 500ml of PB+0.1%Tween.

Handling and Restraint of Animals Infected with Mycobacterium tuberculosis

Materials

Wear double disposable gloves when handling animals in the following protocols. Also, all work with Mycobacterium tuberculosis and Mycobacterium tuberculosis infected animals should be done in the BSL3 facility with appropriate facility clothing and personal safety equipment. All work should be done under the BSC.

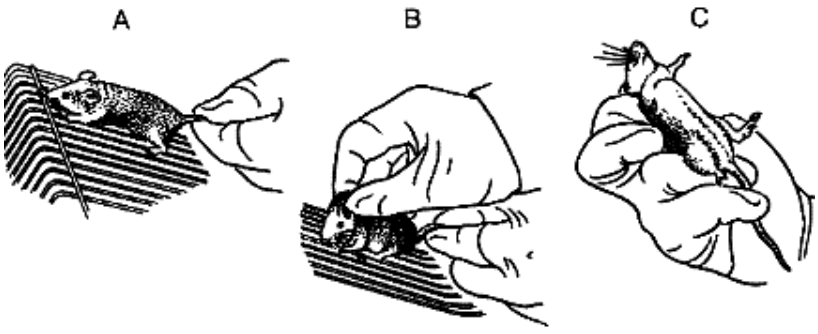
Basic Procedure - Mouse Handling and Manual Restraint

-Remove the mouse from the cage by gently grasping the tail (with preferred hand) at the base. Place the animal on a wire-bar cage lid to permit grasping (Figure A).

-Avoid exciting the mouse with loud noises or sharp movement. Slight, rearward traction on the tail will result in instinctive grasping on the wire-bar cage lid. The manual restraint can also be done on a flat surface if the restraint needs to be more secure for Oral gavage or SQ injections. For these procedures limited head movement is essential.

-Approach the back of the neck from the rear with the free hand. Firmly grasp the skin behind the ears with the thumb and index finger (Figure B).

-Transfer the tail from preferred hand to beneath the little finger of the hand holding the scruff of the neck (Figure C).

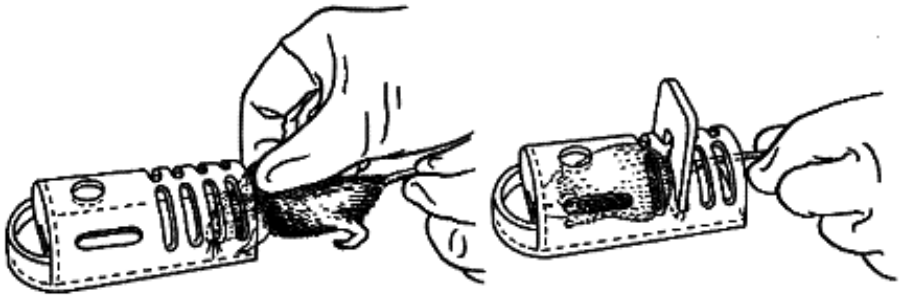


-Observe or inject the restrained mouse.

Alternate Procedure - Rodent Restrainers

Plastic restraining devices of various sizes, shapes, and designs are available. An example of the most common is depicted in below. When placing an animal in a plastic restrainer, consideration should be given to the size of the restrainer relative to the size of the animal. A restrainer that is too large will permit the animal to turn around, while one that is too small may not allow adequate respiration. The length of time in the restrainer is also a concern. Most restrainers do not allow for adequate dissipation of body heat, causing the animal's core temperature to rise. Plastic restrainers are particularly useful for intravenous injection and sampling because both hands are free for tail immobilization and syringe manipulation. This method of restraint is most useful for bleeding the mouse and rat. It is also valuable for intravenous injection of the mouse.

For IV work there should be no restriction of the tail. This can impede the injection.



-Prepare a clean, open restrainer, with all parts readily accessible.

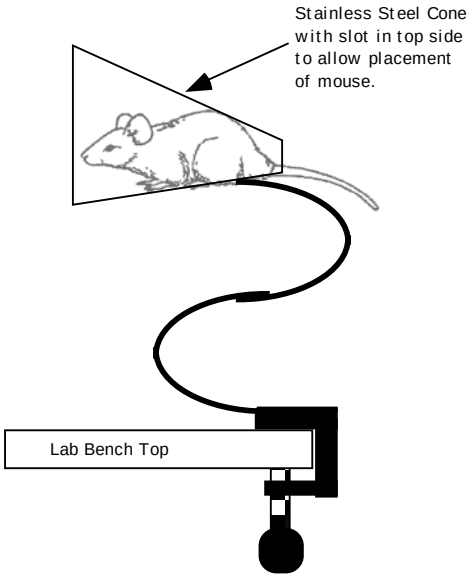
-Manually restrain the mouse, rat, or hamster as described in the basic protocols.

-Holding the tail at the base, place the animal's head near the entry of the restrainer.

-As the animal's thorax is released, maintain tension with the tail.

-Insert the securing block when the animal is fully in the device.

Note: We now utilize a less cumbersome stainless steel injection "cone" which clamps onto the workbench and is illustrated below. Cone’s can also be acquired made of Plexiglas with rubber suction cups to be secured to the bench.


Stainless Steel Cone
with slot in top side
to allow placement
of mouse.
Lab Bench Top

Bronchoalveolar Lavage Procedure for Mouse

Materials

- Sterile PBS plus 5% FBS

-1 mL luer lock syringes

-3 inch segments of suture

-Lavage tubing (Intramedic polyethylene tubing ID 0.58mm, OD 0.965mm) cut into 8 inch segments fitted onto a 22Gx 1 in. needle (attached to 1 mL syringe w/ 1 mL 5% FBS) Do not autoclave.

-Scissors (fine/sharp with pointed tip)

-Forceps

-15 mL conical tubes

-Ice

Procedure

-Euthanize mouse and place head towards you on the necropsy mat, douse with 70% EtOH.

-Snip away skin on neck to expose muscles. Gently retract the upper muscles that lies above the esophagus and carefully remove tissue from around either side of the esophagus. *Remember, don't cut the jugular

-When esophagus is exposed, insert the fine/sharp scissors under the esophagus to allow the suture to be slid under the esophagus. Tie suture loosely around esophagus. Carefully nick the upper side of the esophagus and insert lavage tubing into the esophagus about ¼ inch. Tighten suture snugly around the tubing and the esophagus.

- Inject 1 mL 5% FBS into the lungs and remove slowly. Place lavage fluid in 15 mL labeled conical tube on ice. Repeat wash procedure one additional time with 1 mL of 5% FBS and retain in 15 mL conical tube.

Preparation of DOTIM Liposomes (Dow Lab)

-Use a sterile glass pipette and a sterile glass 16x25mm tube with black cap, prepare the liposome in the fume hood as follows

Liposome Amount	DOTIM (20mg/mL)	Cholesterol (20mg/mL)
60mM	2.4mL	1.5mL

Note: Turn on the lyophilizer before preparing the liposome-(power button in back, press auto). DOTIM and cholesterol (Avanti polar lipids – CH-500 98% pure, 5g, 700,000) stocks are @ 20 mg/mL in HPLC grade chloroform, pentene stabilized from Fisher Scientific (stock #022881 UN1888 C607-1). The DOTIM is in the -80℃ , 3rd shelf in the back and the cholesterol stock is in the -20℃ in the liposome box. USE GLASS PIPETTES ONLY when pipetting the chloroform and DOTIM/Cholesterol stocks. Store stocks upright at -20℃. (Long -term storage should be at -80℃). Parafilm a ll caps before storage.

-Lyophilize overnight, leaving caps on loose. Be sure to introduce the vacuum slowly to ensure the liquid stays in the tube. In 24 hours, remove tubes from lyophilizer, turn off machine, and attach drain hose. If needed, lyophilized liposomes can be stored at 4℃ under argon.

-Re-hydrate liposome with sterile 10% sucrose. To the 60mM liposome, add 6mL of 10% sucrose. The liposomes with now be at a concentration of 10 mM.

-Place the tube in 50℃ H_2O bath for 50 minutes. Mix after 2 minutes and periodically about every 5 minutes thereafter.

-Remove from H_2O bath and set out at room temperature 1-3 hours.

-Transfer the liposome to a sterile 50 mL conical tube. Reheat liposomes for 2 minutes in the 50℃ H_2O bath to facilitate the filtering steps.

-Filtering supplies:

--2 - sterile luer-lock syringes

--2 - 18g needles

--3 – sterile 50 mL conical tubes

--Syringe filters

--1 – 1.0 µm (non-sterile Whatman puradisc- 6821-1310)

--1 – 0.45 µm (non-sterile Whatman puradisc – 6789-1304)

--0.2 µm (Sterile Pall acrodisc- 15247-2006-10)

-In a sterile hood use a 10 mL syringe and 18g needle, remove the liposomes from the tube and filter through a 1.0 µm filter into a new sterile 50 mL conical tube, heat back up, using the same 10 mL syringe and 18g needle, heat back up, remove the liposome and filter through a 0.45 µm filter into a new sterile 50 mL conical tube, heat back up. Use a new sterile 10 mL syringe and a new sterile 18g needle to remove the liposomes and then filter through a final 0.2 µm filter into a new sterile 50 mL conical tube. (This will be difficult to push through, ensure filters are tightly secured.) At the end of each filtration step clear the filter of liposomes by passing air through the filter, this will minimize the loss of liposomes during the filtration steps.

-If storing liposomes, aliquot 3 mL of liposomes per sterile vial. Add argon gas on the top of each vial, wrap top with parafilm, label, and store at 4℃.

Lung Digest

Materials:

-Book Necropsy and TC Hood at BHRB

-ICE

-Surgical kit (Lg and Sm scissors, two tissue forceps)

-Mice (Lungs)

-Dissection Board with pins

-Sterile 15 ml centrifuge tubes for lung collection

-50 ml conical tube with EtOH

-One 10-ml Syringe, filled with cold Heparin Solution, per lung, fitted with 26 gauge needles

-small Petri dishes

-Sterile razor blades or long-sharp scissors

-Nylon strainer (70□m)

-Collegenase/DNase (150/50 units/ml) solution thawed and on ice (2X) (for final 1X concentration)

-Cold Tissue Culture Media (DMEM or RPMI) and incomplete media

-Gey's Solution

-Hemacytometer and 3% Glacial Acetic Acid in PBS or Trypan Blue Counting Solution

-Water bath at 37^{o}C

-Pipet Boat

-Do/Make before entering barrier.

Procedures:

-Reserve Necropsy and TC Hood at BHRB for necessary time.

IN NECROPSY @ BRB:

-Sacrifice mouse by CO_2 asphyxiation. Place mouse on a paper towel and soak with 70% EtOH.

-In a BioSafety Cabinet Level II, place mouse on dissection board covered with a paper towel and pin “hands” and “feet” to dissection board so that mouse is vertically stretched out with its feet towards you.

-Place surgical kit, containing Lg and Sm scissors, and 2 forceps, in a 50-ml conical tube with 70% EtOH.

-Make a 1 cm V-shaped midline incision with the larger dissection scissors.

-Retract the skin above the head and below the thighs by pulling it with gloved fingers vertically in opposite directions. Pin the upper portion of the skin over and above the head.

(Optional step: Open abdomen and cut the descending aorta with the scissors)

-Grasp the Sternum with the forceps, and make a small V-shaped incision starting at the Xyphoid process of the sternum (don’t cut deep or will cut the heart or lungs). Insert pointy scissor end into cut and slide laterally down both sides to open the thoracic cavity. Make a lateral cut towards the head at both sides of the ribs to further open the thoracic cavity. Lungs should be readily visible on either side of the heart. (If you prefer, at this point you can open the abdomen and cut the descending aorta.)

-Hold the heart at the auricles level with the forceps. Using a 10-ml Syringe filled with ice-cold Heparin Solution (per lung) insert the needle into the ‘dark spot’ on the right ventricle of the heart and inject the heparin until the lungs turn white and inflate.

-Remove all lung lobes and place in 2ml ice-cold incomplete media.

Grasp the collection of Lungs on the (your) left of the heart and pull out, cut away any connective tissue and remove the lungs. Next, move the heart to the (your) left and grasp the collection of lungs on the (your) right with the forceps and pull out, again cutting any connective tissue to free the lungs.

-Mouse carcasses should be double wrapped in autoclave bags.

IN IMMUNOLOGY SUITE @ BRB:

-Pour lungs from one group into separate small petri dishes. (you can also use the dish covers to save cost).

-Use sterile razor blades (*if you use EtOH to sterilize, rinse in incomplete-media or make sure the EtOH has dried before using on tissues*) to mince lungs, rotate dish and chop in multiple directions to create a crossing pattern.. Carefully return the minced lung back into same 15 ml tube. (*Lysol each dish and place in biohazard bag and razors go into sharps container)* (*You can also use long-sharp scissors and mince the lungs directly in a 15-ml conical tube prior to adding the digest solution - make sure to request long-sharp scissors*.)

-For 2X Collagenase/DNase add 2 ml to make a 1X dilution aliquot per organ.

-Incubate in rocking water bath at 37^{o}C for 30 (APJK 45) minutes.

-After incubation, place on ice and neutralize the enzyme on the cells by adding 5 ml of media to each tube.

-Create single cell suspension using plunger and mashing lungs through 70 µm nylon strainers.

-Place one nylon strainer in small petri dish and pour lung into screen. Use 3 ml syringe plunger (rubber or plastic end) to push cells through the strainer. Some tissue will remain in strainer. --- Rinse strainer with 3 ml media. Pipet cell solution back into the 15 ml tube. Lysol each dish and place into biohazard bag.

-Centrifuge 5 minutes at 1200 rpm, 4^{o}C.

-Pour off supernatant into pipet boat.

-Lyse the Red Blood Cell's:

--Resuspend cell pellet via repeated flicking

--Add 2 ml Gey's Solution for 5 minutes at RT (room temperature)

--Add 5 ml TC medium to neutralize Gey's Solution.

-Centrifuge 5 min. 1200 rpm, 4^{o}C.

-Decant supernatant and resuspend pellet via flicking and add

--1ml TC medium to each tube for small experiments

--2ml TC medium for larger experiments (Intracellular Staining + other)

-Place cell suspensions back on ice.

-Counting Cells: Using a non-sterile 96-well plate add 20 ul of cell solution to180 ul 3% Glacial Acetic Acid in PBS for a 1:10 dil. Transfer 20 ul of the 1:10 solution to another 180 ul counting solution to make 1:100 dil.

** [Important step for Flow Cytometery, so that you may say that the lymphocytes were X% of the total number of cells.

** [Normal lungs have about 1-2x10^6 cells, infected lungs can get 2-3x10^7 cells later on in infection]

-Cells now ready for FACS preparation or for Tissue Culture, etc.

GM-CSF+ c RPMI Media for Dendritic Cells

Materials:

-One bottle, 500 ml (1X) RPMI Medium 1640 w/o L-glutamine (Invitrogen, Cat. No. 21870-076)

-45.0 ml Heat-Inactivated Fetal Bovine Serum (10% FBS) (5% FBS use 22.5 ml)

-5.0 ml, 1M HEPES buffer (Sigma, Cat. No. H-0887)

-5.0 ml, 200 mM L-Glutamine (Sigma, Cat. No. G7513)

-5.0 ml, (100x) non-essential amino acids (Sigma, Cat. No. M-7145)

-5.0 ml, 100mM Sodium Pyruvate (Sigma, Cat. No. S-8636)

-5.0 ml antibiotic (100ug/ml streptomycin; 100U/ml penicillin) (Sigma, Cat. No. P0781)

-βMercaptoethanol

-100 ul Aliquot GM-CSF (20ng/ml) (Granulocyte Monocyte-Colony Stimulating Factor, Peprotech, Cat. No. 315-03)

-0.2 um filter unit

-Clean 37^{o}C water bath (as needed)

-Pipettes and pipette man

-Pipette boat

-Clean hood

Procedures:

-Prepare sterile working environment using 5% Lysol then 70% ETOH. Turn hood on 10 minutes prior to starting.

-Thaw any frozen materials in clean 37^{o}C water bath: FBS, L-glutamine, Penicillin/Streptomycin

-Using sterile technique add each “stared” solution, except the FBS, to a 0.2 um filter unit and filter.

-Add the filtered solutions and the FBS to the 500 ml RPMI bottle. Gently swirl to mix (harsh mixing will damage FBS).

-Store at 4^{o}C

-When ready to use for Dendritic Cells, add 100 ul aliquot of GM-CSF (20ng/ml) to 100 ml cRPMI for culture.

BMMØ Media or L929-conditioned DMEM Media for MØ

Materials:

-337 ml Dulbecco's Modification of Eagle's Medium 1X (MOD.) aka DMEM (Cellgro, Cat. No. 15-017-CV)

-45 ml FBS

-45 ml L-929 medium

-4.5 ml 1M Hepes buffer (Sigma, Cat. No. H-0887)

-4.5 ml MEM non-essential amino acids (Sigma, Cat. No. M-7145)

-4.5 ml, 200 mM L-Glutamine (Sigma, Cat. No. G7513)

-4.5 ml antibiotics (optional) (100ug/ml streptomycin; 100U/ml penicillin) (Sigma, Cat. No. P0781)

-β-Mercaptoethanol (Optional)

-Clean 37^{o}C water bath (as needed)

-0.2 µm filter unit

--Pipettes and pipette man

--Pipette boat

--Clean hood

Procedure:

-Prepare sterile working environment using 5% Lysol then 70% ETOH. Turn hood on 10 minutes prior to starting.

-Thaw any frozen materials in clean 37^{o}C water bath: FBS, L-glutamine, Penicillin/Streptomycin.

-Add all components, except FBS and DMEM, into a 100ml 0.2□filter unit.

-After filtration pour into DMEM bottle and add the FBS.

-Store at 4^{o}C.

Complete DMEM Media

Materials:

-337 ml Dulbecco's Modification of Eagle's Medium 1X (MOD.) aka DMEM (Cellgro, Cat. No. 15-017-CV)

-45.0 ml filter-sterilized Heat-Inactivated Fetal Bovine Serum (10% FBS) (5% FBS use 22.5 ml)

-4.5 ml, 1M HEPES buffer (Sigma, Cat. No. H-0887)

-4.5 ml, 200 mM L-Glutamine (Sigma, Cat. No. G7513)

-4.5 ml, (100x) non-essential amino acids (Sigma, Cat. No. M-7145)

-4.5 ml antibiotic/antimycotic (optional) (100ug/ml streptomycin; 100U/ml penicillin) (Sigma, Cat. No. P0781)

-β Mercaptoethanol (optional)

-45ml L-929 cell supernatant (in -20C freezer)

-Clean 37^{o}C water bath (as needed)

-Pipettes and pipette man

-Pipette boat

-Clean hood

-Add each solution into a 500 ml DMEM bottle

Procedures:

-Prepare sterile working environment using 5% Lysol then 70% ETOH. Turn hood on 10 minutes prior to starting.

-Thaw any frozen materials in clean 37^{o}C water bath: FBS, L-glutamine, Penicillin/Streptomycin

-Using sterile technique add each "stared" solution to 500 ml DMEM bottle. Gently swirl to mix (harsh mixing will damage FBS).

-Label: Your initials, Date, DMEM.

-Store at 4^{o}C

Complete RPMI Media

Materials:

-One bottle, 500 ml (1X) RPMI Medium 1640 w/o L-glutamine (Invitrogen, Cat. No. 21870-076)

-filter-sterilized Heat-Inactivated Fetal Bovine Serum (10% FBS)

-5.0 ml, 1M HEPES buffer (Sigma, Cat. No. H-0887)

-5.0 ml, 200 mM L-Glutamine (Sigma, Cat. No. G7513)

-5.0 ml, (100x) non-essential amino acids (Sigma, Cat. No. M-7145)

-5.0 ml, 100mM Sodium Pyruvate (Sigma, Cat. No. S-8636)

-5.0 ml antibiotic (optional) (100ug/ml streptomycin; 100U/ml penicillin) (Sigma, Cat. No. P0781)

-500 β-Mercaptoethanol [50 uM stock] (optional)

-Clean 37^{o}C water bath (as needed)

-Pipettes and pipette man

-Pipette boat

-Clean hood

-Add each solution into a 500 ml RPMI bottle

Procedures:

-Prepare sterile working environment using 5% Lysol then 70% ETOH. Turn hood on 10 minutes prior to starting.

-Thaw any frozen materials in clean 37^{o}C water bath: FBS, L-glutamine, Penicillin/Streptomycin

-Using sterile technique add each “stared” solution, except the FBS, to a 0.2 um filter unit and filter.

-Add the filtered solutions and the FBS to the 500 ml RPMI bottle. Gently swirl to mix (harsh mixing will damage FBS).

-Label: Your initials, Date, 10% (or 5%) cRPMI.

-Store cRPMI at 4^{o}C

Preparation of Proskauer and Beck Liquid Medium

Materials:

-1-liter Erlenmeyer flask

-0.22 µm sterile filter

-KH_2PO_4 (Mallinckordt #7100)

-Asparagine (Difco #214410)

-$MgSO_4 \cdot 7H_2O$ (Fisher #M63-500)

-Magnesium Citrate (ICN #212256)

-Glycerol (Fisher #G33-500)

-Tween 80 (optional for growth of dispersed cultures) (Mallinckrodt #2744)

-ddH_2O

Procedure:

-Prepare the following:

KH_2PO_4, 5.0 g

Asparagine, 5.0 g

$MgSO_4 \cdot 7H_2O$, 0.6 g

Magnesium Citrate, 2.5 g

Glycerol, 20.0 ml

Tween 80, 0.5 ml

ddH_2O (Q.S.), 1,000 ml

-Dissolve the above ingredients one at a time in 1-liter ddH2O.

-Adjust the pH to 7.8 using 40% NaOH (about 3.5-5.0 ml per liter)

-Autoclave at 121ºC for 15 minutes. Be sure to use the slow exhaust cycle.

-The pH after autoclaving will be about 7.4.

-Filter the medium through a 0.22µm filter to remove any precipitate.

Preparation of 4% Paraformaldehyde

-8% (w/v) paraformaldehyde stock solution: Dissolve 8 g of powdered paraformaldehyde in 100 ml of ddH_2O . Heat and stir to 55-60˚C (do not go higher than 60˚C). Add a few drops of 2 M sodium hydroxide until the solution clears. Make fresh each day. Alternatively, 8% paraformaldehyde can be purchased from Electron Microscopy Sciences

-Throw away whatever is not used in one day.

4% (v/v) paraformaldehyde is a 1:1 solution of 8% paraformaldehyde stock solution and 0.2 M PBS. Make up on the day of use.

*When using 4% paraformaldehyde solution to fix tissue infected with Mtb, tissue needs to be completely submerged in 4% paraformaldehyde for at least 48 hours before all bacteria are killed.

Determination of colony forming units (CFU) in mouse organs

Purpose. To define a procedure for performing viability count upon animals challenged with mycobacteria.

Supplies

Supplies are designated on the Set-Up sheet, which will accompany the technician to site. Pestles and plates for use at BHRB will be placed in autoclave bags for easy transfer.

-Prior to entering the barrier place the pan containing all the required material immediately outside the material transfer door (BHRB) or in the air-lock (Painter).

-Enter the barrier.

-Transfer pestles from transport cooler to barrier cooler. Transfer plates from transport container to barrier container. Transfer the remainder of supplies to a cart inside the barrier; proceed to tissue processing room. *Note: No items taken into the barrier may be taken outside of the barrier unless they have been decontaminated by approved methods, therefore do not bring outside coolers or containers into the barrier.*

Hood set-up

Note: All processing of tissues from Mycobacterium tuberculosis infected animals must be done in the biosafety cabinet and glove box. Ensure that the blower, power, and light switches of the biosafety cabinet are in proper working order before starting any laboratory techniques in this instrument.

-It is important to have all items immediately required for work within the hood. For the hood to function correctly and safely it is essential that the airflow is not restricted. The number of items that are placed into the hood are to be minimized; items must not be placed immediately in front of the vents.

-The viable count can be divided into 3 independent procedures (necropsy, homogenizing, plating) and after each procedure the hood can be cleaned and restocked with the required items.

-Ensure that an ample supply of correctly sized latex gloves, paper towel, and a wash bottle containing 5% Lysol are adjacent to work area.

-Place a necropsy board, located in a box adjacent to the cabinet (BHRB) or beneath the cart (Painter), into the hood in a central location without blocking any of the air vents. Cover the board with absorbent paper.

-Fill beaker with 70% ethanol and place inside the hood. Place the surgical kit into the beaker.

-Place a small biohazard bag inside the hood.

-Place one rack containing labeled homogenization tubes inside the hood (several racks may be required for the VC however only one rack should be in the hood at any given time).

-Place any additional supplies inside the hood (PCR tubes, histology cassettes, syringes, etc.).

-Necropsy

Depending upon the requirements of the study, removal of all organs is optional.

-Sacrifice animals in a humane manner (usually 4 to 5 at a time). Using a wash bottle thoroughly wet the fur with 70% ethanol to sterilize the area and reduce the possibility of fouling HEPA filters with animal dander.

-Transfer the animals to the hood and place them on clean, dry, absorbent paper on the necropsy board. Place the animals on their backs and pin them to the board using syringe needles.

-Remove surgical kit from beaker and air-dry.

-Make a midline incision(s) with large surgical scissors.

-Retract the skin above the head and below the thighs by pulling it with gloved fingers to expose the peritoneum. At this stage the animal is still not considered infectious as all the organs are

contained. Once the peritoneum integrity is compromised the animal must be treated as infectious.

-Cut the peritoneum using small surgical scissors and retract it to expose the internal cavity.

-Remove the spleen. The spleen is attached to the greater curvature of the stomach by connective tissue. Grasp as much of the spleen as possible with forceps. Gently pull the spleen free of the peritoneum, tearing the connective tissue behind the spleen. Alternatively, the top and bottom portions of the spleen can be grasped and torn from the connective tissue one part at a time. It is usually more efficient to remove the spleen all at once, particularly if large numbers of animals will be used. Avoid cutting into the intestine as this organ is not sterile.

-Uncap the appropriate tube and place the spleen into the tube. Place the spleen as far down the tube as possible to avoid any tissue touching the cap once it has been replaced. Cap the tube. The cap should be removed and replaced carefully, avoid touching the area that will be placed inside the tube.

-Remove the liver. The liver is the largest organ in the peritoneum. With small forceps grasp underneath the mouse's right lateral lobes of the liver to a point across the inferior vena cava. With scissors, gently flip these lobes back over forceps so that the top portion of forceps can grasp these lobes as well as the common bile duct. When the forceps have a firm hold, make an incision with the scissors between the liver and the diaphragm and cut the inferior vena cava. This then frees the liver; with a quick but gentle upward motion the liver should be extricated as one organ.

-Uncap appropriate tube; place the liver in designated tube. Cap tube.

-Remove the lungs. Make an incision through the thoracic cavity with surgical scissors and reflect the thoracic wall. The lung lobes should be readily visible in either side of the heart. With small forceps grasp the bronchial tree close to the heart and cut the lung lobes away. Individual lung lobes can also be removed for PCR/formalin fixation.

-Uncap appropriate tube; place the lung in designated tube. Cap tube.

-If collection of tissue for PCR is required, proceed to the glove box to homogenize tissue. Formalin fixed tissue may be infused, placed in cassettes, and stored.

-Mouse carcasses should be placed into the biohazard bag situated in the hood.

-Remove gloves and place them in the biohazard bag. Exit the hood and place on a fresh pair of gloves. Proceed to the next experimental group. To prevent the possibility of a spill, racks of completed tubes should be placed at the back of the bench to ensure that they cannot be tipped over.

-At the completion of necropsy, place all mice and trash in the small biohazard bag and tape is closed. Place the entire bag inside another bag and tape closed. Label the bag Trash (BHRB) or Mice (Painter). Remove the bag from the hood and store for autoclaving. Rinse the necropsy board with 5% Lysol, wipe, and place the board back in its container. Wipe the entire hood with 5% Lysol, followed by 70% ethanol and discard the paper towel in a biohazard bag for disposal.

-Each time any item is removed from the hood it is essential that soiled gloves are removed and fresh gloves worn. No items should be removed from the hood unless they are placed into autoclave bags, have caps on, or have been wiped with 5% Lysol.

-Homogenization of Organs

-Half fill a pestle boat with a 5% Lysol solution. Place the surgical kit from the necropsy step into the pestle boat. Open the outer access door of the airlock entry port for the glove box chamber and place the pestle boat within the airlock chamber. Close and secure the outer door of the airlock and then place hands in the glove ports and open the inner access door of the airlock entry port. Remove the pestle boat from the airlock and place it within the glove box chamber, with the lid off. Close the inner access door.

-Transfer a tube rack with 10 homogenization tubes containing organs through the airlock as described above. Transfer some paper towel that has been soaked in ethanol into the glove box.

-With hands in the glove ports carefully remove the foam test tube stoppers from the homogenization tubes and place them off to the left side of the hood on a paper towel.

-Transfer a pack of sterile chilled pestles into the glove box. Carefully open the pestle pack and place the chilled pestles into the homogenization tubes, taking care not to splash or spill the tube contents. Pestles can be placed in the tubes one at a time before homogenizing, or in groups of 10 (remember that the pestles warm up rapidly in the tubes).

-Remove the homogenization tubes one a time from the test tube rack and attach the shaft of the pestle into the chuck of the grinder. When secured, firmly grasp the homogenization tube with both hands and then actuate the grinder via the foot pedal. It is essential that a firm grasp of the homogenization tube is maintained while performing this technique as the pestle will rotate in the tube at a rather high rpm. If the tube is not securely held the contents will spatter within the glove box chamber and create a tremendous aerosol. While the pestle is rotating the tube is slowly moved up and down 3-4 times to thoroughly homogenize the organs. Ensure that the pestle can be seen at the bottom of the tube demonstrating that all of the organ has been homogenized.

-After homogenization is complete, remove pestle from the chuck (keeping it inside the tube). Hold the tube and pestle over the pestle boat and gently pull the pestle out and place it into the pestle boat. Return tube to the test tube rack and repeat until all tubes with organs have been homogenized.

-After homogenization, recap the tubes with foam plugs and allow the chamber to purge for a period of 5 minutes (to allow the decay of the aerosol that may have developed during the homogenization process). Remove the rack from the glove box. Place another rack of tubes inside the glove box and repeat steps 2-6.

-Pass paper towels and wash bottles containing 5% Lysol and 70% ethanol into the glove box. Wipe the interior surfaces of the glove with 5% Lysol followed by 70% ethanol. Place all used

paper towels and pestle packs into the pestle boat. Cover the pestle boat with a lid, remove from the glove box, and place into a red biohazard bag contained within a pan.

-Plating of infected tissues

-Half fill a pipette boat with 5% Lysol and place it in the hood either near the back or at the side. Ensure that airflow is not compromised. Leave the pipette boat lid outside the hood.

-Fill a rack with 8-10 tubes containing organ homogenates, and sufficient dilution tubes necessary to perform the required number of serial dilutions. Place the rack inside the hood.

-Place a pack of 1ml pipettes into the hood and open the pack. Place the pipette-man inside the hood.

-Place a pack of agar plates inside the hood. The agar plates are light sensitive, therefore only place those plates immediately necessary for use inside the hood. Remove the plates from their zip-lock bag and place them on the hood surface. Take one plate and face the lowest dilution (pre-labeled by the Work Studies) facing north.

-Carefully remove the cap from a homogenate tube. Using a 1ml pipette, draw up 600ul of homogenate. Replace the cap. Remove the lid from the agar plate and pipette 100ul onto the North quadrant. Replace the lid. Remove the cap from a dilution tube and place the remaining 500ul of homogenate into the tube. Pipette

-Homogenize into the saline, not down the side of tube. Refrain from creating bubbles. Replace the cap. Place the pipette into the pipette boat, draw up approximately 600ul of 5% Lysol solution, and discard the pipette into the boat.

-Gently swirl dilution tube to mix the suspension; refrain from creating bubbles. Using a 1ml pipette, draw up 600ul of the dilution. Replace the cap. Remove the lid from the agar plate and pipette 100ul onto the next quadrant. Replace the lid. Remove the cap form a dilution tube and place the remaining 500ul of suspension into the tube. Replace the cap. Discard the pipette as previously described. Repeat this process for all 4 quadrants.

-When all 4 quadrants are finished, gently swirl the plate to spread the innoculum, and place the plate to one side. Repeat the method for the remaining plates in the bag. Gently stack the plates and place inside the zip-lock bag. Label the bag. Place the bag of plates into a container with the lid on. Repeat these steps for all samples.

-When all the samples have been plated, place any remaining pipettes into the boat. Place the lid on the boat, remove from the hood, and place inside an autoclave bag, contained in a pan. Remove all racks and tubes from the hood. Wipe the hood with 5% Lysol, followed by 70% ethanol. Place used paper towel in an autoclave bag for disposal.

-Transport agar plates to the 37^{o}C incubator within a closed container.

-All materials that have been used (pestle boat, pipette boat, tubes) should be placed inside an autoclave bag that is contained within a pan, ready for autoclaving.

-Alternative plating method.

-Dispense 800ul of sterile saline into 24 well plates using an automatic pipettor; leave the first row empty.

-Dispense undiluted homogenate into the first row of a 24 well plate using a 1ml pipette. Discard the pipette in the pipette boat as described above.

-Using a multi-channel and sterile tips (with a wide bore to prevent blockage) carefully make serial 200ul dilutions across the plate. Change tips with every row as mycobacteria are very sticky and will remain in the tips resulting in carry-over and inaccurate dilutions. Dispense tips into the pipette boat.

-Using a pipette, dispense 100ul of each homogenate dilution onto the agar plates. This procedure should be carried out immediately after the dilutions have been prepared as over time mycobacteria will stick to the plates and dilutions will not be accurate.

-At the completion of a single 24 well plate, dispense 5% Lysol into each well using a pipette. A wash bottle must not be used, as this will generate an aerosol. Place plate inside a small autoclave bag, seal, and place into a pan for disposal. Wipe multi-channel with 5% Lysol and

remove from the hood. Ensure that no homogenate has entered the barrel of the pipette. In the event that this occurs, dismantle and clean pipette.

-Bag, label, and incubate plates at 37^{o}C as described above.