THESIS

MINIPIGS AS A NEONATAL ANIMAL MODEL FOR TB VACCINE EFFICACY

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ABSTRACT

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Currently, the only vaccine available to prevent tuberculosis (TB) is Bacillus Calmette-Guerin (BCG). The vaccine lacks efficacy against pulmonary disease or reactivation of latent TB but prevents disseminated TB in children and is thus widely used in countries with endemic TB as part of the neonatal vaccine regimen. There are several new vaccines that have shown efficacy against TB in adult animal models yet fail to protect infants from TB disease in clinical trials. Failure in the development of new pediatric vaccines may be due to incomplete knowledge in the elicited immune response to BCG vaccination and testing of vaccine efficacy in adult rather than neonatal animal models. In this novel approach, we used the mini-pig as a neonatal animal model for evaluation of immune responses to BCG vaccine. We demonstrate young mini-pigs are susceptible hosts to the highly virulent *Mycobacterium tuberculosis* (Mtb) strain, HN878 and that the pathological course of infection resembles that seen in human TB. In this study we longitudinally monitored the immune response of neonatal mini-piglets vaccinated with BCG until adulthood, with the same monitoring applied to a group of unvaccinated mini-piglets. Further, we challenged both vaccinated and non-vaccinated animals via the aerosol route with HN878 and we characterized important changes between the two groups in the course of immune responses following challenge. Based on comparison of immune responses to BCG in mini-pigs and infants, our findings suggest that mini-pigs have the potential to serve as an effective neonatal animal model for TB vaccine development.

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1.1 Introduction

Currently, the only vaccine available to prevent tuberculosis (TB) disease is Bacillus Calmette-Guerin (BCG), an attenuated *Mycobacterium bovis* strain. The vaccine is effective against childhood tuberculosis meningitis and disseminated disease but lacks efficacy against pulmonary TB or reactivation of latent TB. According to WHO, TB is a leading cause of human disease and death in developing countries alongside acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV). In 2014, there were an estimated 1.5 million deaths due to TB and 1.2 million due to AIDS (WHO, 2015c). In addition, the number of TB cases with antibiotic resistance, multiple drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB), are increasing and for most of those cases no treatment is available. In 2013, 17% of newly diagnosed TB cases were found to be resistant to one or more first-line antibiotic treatments(Cruz-Knight & Blake-Gumbs, 2013). In the last 80 years, many efforts have been made to create a vaccine to protect against pulmonary TB, but none have proved efficacy. BCG remains the best option to partially protect infants from disseminated TB disease. New vaccines and boosters have been tested for efficacy in adult animal models of TB with positive results, however, clinical phase IIb studies with infants and natural exposure to Mtb demonstrated lack of protection (Tameris et al., 2013; WHO, 2015c).

It is well established that the T cell mediated immune response differs in newborns in comparison to adults and that the exact differences have yet to be determined (Marchant & Goldman, 2005). The TB research community has openly admitted a vacuum of

knowledge about pediatric immunity in TB (WHO, 2015c). In particular, there is a lack of knowledge in the kinetics induced by neonatal BCG vaccination and the functional and phenotypic attributes of BCG-induced memory T cell responses (A. P. Soares et al., 2013). To reduce the incidence of TB in children, WHO recommends additional research in understanding the host-pathogen interaction and neonatal and infant immunity to Mtb infection (WHO, 2013b). It is therefore essential to study neonatal immunology and test vaccine efficacy against infant and childhood TB in appropriate animal models.

Our study in developing mini-pigs as a neonatal model intends to make progress towards WHO's recommendations. Many human applied studies, including immunity studies, have used the pig as an animal model (Meurens, Summerfield, Nauwynck, Saif, & Gerdts, 2012). There is great potential in developing the mini-pig for future vaccine studies against TB, since pigs have an immune system with an 80% similarity to that of humans, have large litters and there are commercial resources available to be used as animal models (Meurens et al., 2012). The first goal in this project was to demonstrate that aerosol infection with a highly virulent clinical strain of Mtb is possible in Sinclair mini-pigs: adults and piglets. The second goal was to vaccinate neonatal mini-pigs with BCG followed by studies to reveal the longitudinal immune response to BCG and compare it to previously reported studies monitoring immunity developed during BCG vaccination in infants. TB is not only difficult to prevent, it is also challenging to diagnose and to treat in many cases. Overall, this study will improve our understanding of TB disease progression and vaccine induced immunity from neonate to adulthood.

1.2 Tuberculosis

Tuberculosis (TB) is a human disease mainly caused by the bacilli of *Mycobacterium tuberculosis* (Mtb). Mtb is non-motile, an obligate aerobic bacterium and a facultative intracellular parasite (Cruz-Knight & Blake-Gumbs, 2013). The bacilli are easily transmitted through aerosol droplets from an actively infected individual and although the bacilli can be found in most tissues, infection by this bacilli and subsequent disease is most frequently associated to the lungs. In the lungs Mtb persists in a granuloma, a structure composed of macrophages, T cells, B cells, and fibroblasts (Flynn & Chan, 2001). A small number of bacilli are required to cause infection, however only 5-15% of infected individuals progress to active TB (WHO, 2015c). The symptoms of active TB can include coughing with or without sputum or blood, fever, weakness, weight loss, night sweats and chest pain (WHO, 2015f). Mtb also has the capability of infecting any organ system such as lymph nodes, central nervous system, liver, bones, genitourinary tract and gastrointestinal tract (Cruz-Knight & Blake-Gumbs, 2013). Mtb has a unique cell wall structure with high lipid content to protect itself from degrading host enzymes and impermeability to toxic macromolecules (Korf et al., 2005). In addition, Mtb can prevent phagosome-lysosome fusion and block apoptosis (Robinson, Orme, & Cooper, 2015). Mtb is also able to interfere with antigen presentation, function of CD8+ T cells, natural killer cells and the complement system (Munoz, Stagg, & Abubakar, 2015). Lastly, it is able to resist host-derived antimicrobials such as reactive nitrogen and oxygen intermediates (Russell, 2011). The progression of Mtb to disease is dependent on the amount of bacilli an individual is exposed to and their immunological state, as a high level of local bacterial burden can interfere with efficient protective immunity (Cooper, 2009).

1.2a Latent Tuberculosis Infection

Most exposures of Mtb become latent TB infections (LTBI), in fact one-third of the world's population is considered to have LTBI (WHO, 2015c). During LTBI, individuals do not have any symptoms of TB nor do they spread any bacilli; it is defined as showing presence of immune responses to Mtb antigens without clinical evidence of active TB (WHO, 2015c). Instead, the bacilli are contained within macrophages and extracellularly within granulomas (Getahun, Matteelli, Chaisson, & Raviglione, 2015). The state of the bacilli in this setting is not known (Flynn & Chan, 2001). Individuals affected by LTBI develop healed granulomas, in which they have central calcification with fibrotic encapsulation and no detectable bacilli (Fogel, 2015). By avoiding the innate and adaptive immune system, Mtb is able to persist for several decades or even for the lifetime of a host (Munoz et al., 2015). Cases of LTBI have a 5-10% lifetime risk of developing active TB with most occurring within five years of initial infection (WHO, 2015d). Reactivation of LTBI is to blame for the majority of new TB cases, especially in countries where the incidence of TB is low (Getahun et al., 2015). Targeting LTBI requires identification and preventative treatment of asymptomatic individuals to reduce the progression to active disease and transmission.

1.2b MDR-TB and XDR-TB

Multiple drug resistant tuberculosis results from infection with strains of Mtb which have become resistant to isoniazid and rifampin, the two most effective currently available drugs to treat Mtb (Cruz-Knight & Blake-Gumbs, 2013). In most instances resistance develops as a direct result of TB treatment mismanagement and person-to-person transmission. Lack of compliance with TB chemotherapy is a major reason attributable to

the continuing rise of MDR-TB cases (WHO, 2015c). It is estimated that 3.3% of new cases and 20% of previously treated cases have MDR-TB; more than half of these patients are in endemic countries namely India, China and the Russian Federation (WHO, 2015c). Globally only 50% of patients with MDR-TB were successfully treated with current TB chemotherapy in 2015 (WHO, 2015c). Inadequate treatment of MDR-TB results in increased treatment failure, increased mortality and emergence of new Mtb strains with even more complex resistance profiles (Cruz-Knight & Blake-Gumbs, 2013). Extensively drug resistant tuberculosis is even more difficult to treat, as the Mtb strain is resistant to at least four of the core anti-TB drugs (WHO, 2015b). It is resistant to rifampin and isoniazid, fluoroquinolones and at least one of the three injectable second line drugs: amikacin, kanamycin, capromycin profiles (Cruz-Knight & Blake-Gumbs, 2013). XDR-TB has been reported in 105 countries and there are an estimated 9.7% of people with MDR-TB who have XDR-TB (WHO, 2015c). MDR-TB and XDR-TB require a treatment time of up to two years or more as well as second-line anti-TB drugs which are painful injections and more expensive, less potent, and have more side-effects than first-line anti-TB drugs (WHO, 2010a). Cure for XDR-TB is possible yet the success rate is lower than in patients with drug susceptible TB or MDR-TB. Successful treatment is dependent on the extent of the drug resistance, the severity of disease and the immune state of the patient (WHO, 2010a). Treatment regimens in children for MDR-TB or XDR-TB is the same as in adults, however, treatment durations have not been optimized and there is a higher risk of poorer treatment outcomes and higher mortality (WHO, 2016). In order to prevent the rise of MDR-TB and XDR-TB, diagnostics must improve and treatment regimens must be easier to follow to increase patient compliance.

1.3 Host Immune Response

Initially, TB was known as consumption, due to symptoms caused by infection, until 1882 when German microbiologist, Robert Koch, discovered Mtb was the etiological agent of TB; this finding aided developments in diagnostics, prevention and treatment (Fogel, 2015). Upon Mtb infection there are two types of immune responses, an innate response and delayed-type hypersensitivity to the bacilli; a dynamic equilibrium is established between these two responses and can lead to either active TB or latent TB (Munoz et al., 2015). Initially, Mtb bacilli bind to pathogen recognition receptors (PRR) located on macrophages, monocytes, dendritic cells and neutrophils found in the lungs (Hawn et al., 2014). These PRR recognize pathogen associated molecular patterns on Mtb. The innate immune system detects Mtb by phagocytic receptors such as Toll-like receptors, NOD-like receptors, C-type lectin receptors, mannose receptor, dendritic cell-specific intracellular adhesion molecule-3, grabbing nonintegrin, and DNA sensors (Hawn et al., 2014). This detection initiates inflammatory cytokines: tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-10, IL-1β, and IL-12 (Hawn et al., 2014). Mtb targets alveolar macrophages and by evading the innate cell's antimicrobial activity as a phagosome it is able to establish an intracellular niche in which to replicate (Armstrong & Hart, 1971). By secreting early secreted antigenic target (ESAT)-6 Mtb is able to escape from the alveolar macrophages before the cell is killed. The dying cells trigger inflammation and thus recruit more macrophages to the site which then become infected (Houben et al., 2012). Eventually, CD4+ T cells enable a pro-inflammatory response signal cascade of more cytokines: interferon gamma (IFN-γ), IL-2 and TNF-α (Fogel, 2015). This cascade attracts macrophages, neutrophils, monocytes, T lymphocytes and dendritic cells to build a

granuloma (Fogel, 2015). In addition, IFN-γ stimulates macrophages to kill Mtb (Hawn et al., 2014). While the adaptive immune response is establishing , the granuloma starts maturing and lymphocytes begin to surround the infected macrophages (Ehlers & Schaible, 2012). Eventually the granuloma is deprived of oxygen and the pH lowers, causing tissue necrosis to occur internally and rupture of the structure drains viable Mtb into the alveolar space enabling it to complete its life cycle by spreading through an aerosol producing cough (Lerm, Sweden, Netea, & Netherlands, 2016). In some cases, Mtb can induce a granuloma in which foamy and epithelioid macrophages along with T and B cells and a fibrotic encapsulation can co-exist for decades (Fogel, 2015).

Mtb will continue to proliferate through the innate and adaptive response, as it does so it is released from dying macrophages. During this time, Mtb can develop genetic mutations and effectively change their surface antigens to successfully avoid T cell recognition (Fogel, 2015). Infected macrophages are able to kill or limit Mtb replication by synthesizing antimicrobial molecules, activating autophagy and apoptotic cell death after activation by CD4 T cells producing IFNγ (Hawn et al., 2014; Murphy, 2011). Apoptotic cell death is better for the host as necrotic death from granuloma buildup favors Mtb replication and spread to neighboring cells (Hawn et al., 2014). Despite the macrophages efforts to inhibit Mtb, the bacilli are able to employ mechanisms to support its survival. It can modify phagolysosomes, inhibit apoptosis, and induce traffic of cells to granulomas in order to expand the number of cells it can infect (Hawn et al., 2014). In addition, spread of Mtb bacilli is further supported by the delayed early T-cell response which takes two weeks as opposed to one week as seen in other pathogens such as influenza virus (Hawn et al., 2014). This delay offers the bacilli a protected cellular location from recognition and killing.

It also inhibits IFN-γ activated pathways in macrophages and development of regulatory T cells (Hawn et al., 2014). Although a robust immune response develops in the host upon Mtb infection, there are still many unknown factors of the immune system and the host-pathogen interaction that are halting progress towards global control and potential eradication of this disease.

1.4 Epidemiology of TB

1.4a Prevalence and Risk Factors

Currently, WHO acknowledges 22 countries as highly burdened by TB, which account for 80% of cases (WHO, 2015c). Southeast Asia and Western Pacific Regions accounted for 58% of TB cases worldwide while the most severe burden relative to population according to WHO is in India, Indonesia and China (WHO, 2015c). Mtb kills or debilitates more people aged between 15 and 59 years of age than any other disease in the world (WHO, 2004). Groups at risk include young adults, health workers surrounded by the disease, smokers, and the immunocompromised such as children and HIV+ individuals. Increased risk of infection is associated with long exposure periods to an infected individual or individual with a high bacterial load, poor housing ventilation, lack of exposure to ultraviolet light and susceptibility of the newly infected individual (Getahun et al., 2015). Genetics may play a role in susceptibility due to polymorphisms in vitamin D receptors and polymorphisms in genes of IL-12 and IFN-γ (Fogel, 2015). Additional factors, such as deficiency in IL-12 promoting a T helper 1 response are also to blame for TB susceptibility (Fogel, 2015). Malnutrition is a major risk factor as it can affect the key host defense against TB through cell-mediated immunity (Gupta & Katoch, 2005). Long term use of corticosteroids and TNF-α blockers also put individuals at risk (Fogel, 2015). Despite

knowledge of risk factors associated with TB, the TB research and clinical community cannot explain why some Mtb infected people progress to disease while others do not, even when sharing the same risk factors.

1.4b TB in children

TB in children is considered in those patients who are under the age of 15 years old (WHO, 2015c). The incidence of TB in children is difficult to assess for several reasons. For one, TB in children is challenging to be confirmed using bacteriology in sputum smears because children's cough is sputum-free and the Tuberculin Skin Test (TST) suffers from poor diagnostic performance (WHO, 2015c). As such it is believed that children are rarely infectious and thus contribute little to the spread of disease (Seddon & Shingadia, 2014). Therefore diagnosis is based on signs and symptoms that are not specific to TB and vary among countries (WHO, 2015c). Additionally, TB in children is not considered a public health priority and in cases where there is a high burden of TB in adults, reporting and diagnosing TB in children is deficient(WHO, 2015c). In 2013, WHO estimated that up to 80,000 children die of TB each year and there were over half a million new cases in children alone annually. These estimates were made in HIV negative children; the burden of TB in children is likely higher if HIV positive children were accounted for (WHO, 2015a). Factors that increase the likelihood of a child becoming infected or becoming diseased with TB include living in TB prevalent and highly populated areas, the length of time spent around adults, housing aeration-ventilation and vitamin D levels (Seddon & Shingadia, 2014). The age of a child also determines the likelihood of disease progression. It is estimated that infants less than 12 months of age have a 50% risk of disease, children from 1-2 years old have a 20-30% risk, 3-5 year olds have a 5% risk, 5-10 year olds have a 2%

risk and those from 10-15 years have an adult-like risk of 5% (Seddon & Shingadia, 2014). Disease is likely to develop within 12 months of infection, although if more than one year passes after infection the risk of progression to active TB disease lowers (Seddon & Shingadia, 2014). Around 90% of infected children will remain infected with Mtb in a latent stage (WHO, 2013b) . Most cases of TB become pulmonary disease while disseminated disease occurs in about 20-30% of cases (WHO, 2013b). Extra pulmonary TB occurs mostly in children younger than 5 years of age or those who are immunocompromised; children between the age of 5-10 year who are immunocompetent have a lower risk of disease progression (Marais et al., 2006). TB diagnostics and treatment in children need to be prioritized as children will continue to act as a reservoir for future cases, without this commitment it is unlikely that global TB control will be achieved (Seddon & Shingadia, 2014).

1.5 Disease Control and Vaccination

1.5a Diagnosing LTBI

TST is the standard testing method to determine prior exposure to Mtb. It detects a cellular immune response when the number of bacilli in the infected individual reach 1,000 to 10,000 which usually occurs within 2 to 12 weeks after infection (Cruz-Knight & Blake-Gumbs, 2013). The Tuberculin material is injected through the intradermal and consists of a standardized cell-free purified protein fraction obtained from Mtb. The Mtb antigens stimulate a type IV delayed hypersensitivity response via T lymphocytes (Fogel, 2015). A positive result is interpreted by the diameter of an induration of the skin on the site of injection after 48-72 hours. Factors such as immunosuppression as well as prevalence of TB in the region determine whether a result is considered positive. For example, a 5mm

induration is considered positive for an immunosuppressed individual but negative for a healthy individual living in a highly prevalent area; in some areas a more uniform cutoff is applied (Munoz et al., 2015). False positive results are common due to the low specificity of TST. It can result in a positive result in individuals vaccinated with BCG or infected with non-tuberculous mycobacteria since those antigens are part of the Tuberculin mixture. In addition, it has even poorer sensitivity in individuals who are immunocompromised for whom TST may appear as false negatives. Another drawback to TST is that it requires two visits as it must be administered and read by a trained professional. For some individuals living in remote areas seeking a TST can be burdensome.

Interferon-Gamma Release Assays (IGRAs) use blood samples from individuals to measure T-cell release of IFN-γ in vitro after stimulation with specific Mtb antigens. Two different IGRAs have been developed, Quantiferon-TB Gold in Tube (QFT) and T-SPOT.TB, which both use the early secreted antigenic target 6 (ESAT6) and culture filtrate protein 10 (CFP10). QFT includes an additional antigen, TB7.7. Since BCG and other NTM do not contain ESAT6, CFP10 or TB7.7, patients vaccinated with BCG or infected with NTM should test negative. The QFT is an enzyme-linked immunosorbent assay (ELISA) which uses whole blood to measure IFN-γ in the supernatant of a cell suspension. T-SPOT.TB is an enzyme-linked immunospot assay (ELISPOT) which uses separated T lymphocytes to determine the number of IFN-γ producing T cells. In addition to the specificity of IGRAs, they can also differentiate a negative result due to anergy by using a positive control, phytohemagglutinin (PHA), to stimulate production of IFN-γ (Munoz et al., 2015). Since IGRAs will exclude false positives, excessive use of treatment to prevent progression will be avoided as well as decrease the possibility of more antibiotic resistance.

While these tests are valuable in determining host sensitization to TB, they cannot accurately predict progression nor differentiate between LTBI or active TB, making it difficult in deciding who should be treated. In addition, they cannot detect TB in its first stages as it measures the adaptive immune response which takes approximately eight weeks to develop after infection (Munoz et al., 2015). Because of TST and IGRAs ability to only measure the adaptive immune response, they cannot determine whether the infection is recent or old. The expenses of both of these tests must also be considered; IGRAs are generally more costly than TST. As expected not every diagnostic test is perfect. In the case of using IGRAs or TST they can be of most benefit to prioritize treatment in the immunocompromised such as young children and HIV infected individuals.

1.5b Diagnosing TB

In 2014, there were approximately 9.6 million incident cases of TB: 5.4 million in men, 3.2 million in women and 1.0 million in children (WHO, 2015c). Diagnosing these newly developed cases could not have been done without sputum smear microscopy. This method, which was developed more than 100 years ago, persists as the most common method used worldwide to diagnose TB as the bacilli can be observed under a microscope (WHO, 2015c). Due to mycobacterium's unique cell walls with thick mycolic acid lipids, the bacilli is able to retain biochemical gram positive stains (carbon-fuchsin and auramine-rhodamine) even after being decolorized by acid-containing reagents (WHO, 2015e). Although, it lacks sensitivity and can leave positive TB cases undiagnosed, it is simple, inexpensive and can identify the most infectious patients in various populations with different socio-economic levels in conjunction with TB associated symptoms (Desikan, 2013). Treating smear-positive patients is of upmost importance as they are ten times

more likely to be infectious than smear-negative patients (Initiative, 2013). The problems with smear microscopy are numerous. Under WHO guidelines, patients must provide at least two sputum samples as it has been conclusively shown that two consecutive sputum specimens will identify 95-98% of smear positive TB patients (Initiative, 2013). This proves to be a problem for some as it may be difficult to return to the diagnostic center on a different day (Desikan, 2013). In addition, it becomes less sensitive if there are not at least 5,000 bacilli per ml of sputum and has minimal power in detecting extra pulmonary TB or diagnosing children and patients co-infected with HIV (WHO, 2015e). Lastly, microscopy staining cannot distinguish Mtb from NTMs nor can it distinguish viable from non-viable organisms (WHO, 2015e). These issues debilitate treatment as patients may find it too inconvenient for the actual perceived benefit.

Due to sputum smear microscopy's lack of sensitivity and inability to distinguish drug-susceptible strains from drug-resistant strains other tests have been developed. Sputum culture increases specificity to Mtb and can aid in determining drug susceptibility (WHO, 2015e). According to WHO, it has been shown to increase number of TB cases identified by 30-50% when compared to smear microscopy (WHO, 2015e). However, it can be limited by the type of media used for culture and can take 4-8 weeks to get a result (Cruz-Knight & Blake-Gumbs, 2013). In addition, it requires samples to be decontaminated, which can be harmful to Mtb (WHO, 2015e). A more promising test to diagnose TB is Xpert MTB/RIF, a fully automated, cartridge based nucleic acid amplification test (NAAT) which detects TB and resistance to rifampicin using three specific primers and five unique molecular probes to ensure specificity (WHO, 2014). Its sensitivity and specificity to Mtb is similar to that of liquid culture, however, its strength is that it can take less than two hours

to retrieve a result and can use sputum, processed sputum sediment and select extra pulmonary specimens from adults and children (WHO, 2015e). In addition, it is much more sensitive to samples that have been smear negative but culture-positive and has been more useful in identifying cases in HIV positive individuals (WHO, 2015e). The major disadvantages to Xpert MTB/RIF are logistical. It requires a stable electrical supply, specific operating and storage temperatures and adequate scheduling, as cartridge shelf lives need to be considered (WHO, 2015e). These logistics are easily available in high but not in low resources settings which is the case for most TB endemic countries. However, thanks to recent efforts by international funding agencies, the Xpert MTB/RIF test is now being used to diagnose TB in many endemic countries with low resource settings (UNITAID, 2016).

Regardless of each diagnostic test flaws, it is important to recognize that each serves a vital purpose in combating TB. Sputum smear microscopy helps diagnose people with infectious TB, monitor treatment progression, and provide confirmation of cured cases (Initiative, 2013). It is especially necessary in areas where Xpert MTB/RIF testing is not available. Sputum culture is unique in its ability to distinguish drug susceptibility of anti-TB agents other than rifampicin such as isoniazid and second-line antibiotics (WHO, 2014). Lastly, Xpert MTB/RIF detects Mtb at a much higher specificity than microscopy and is not affected by individuals with HIV co-infection (WHO, 2014). Together or individually, these tests assure treatment is started and facilitate choice of chemotherapy to be initiated.

1.5c BCG and vaccine development

Albert Calmette and Camille Guerin began their research for an anti-tuberculosis vaccine in 1900. By 1919 they had developed a vaccine that failed to produce progressive TB when injected into guinea pigs, rabbits, cattle and horses (Luca & Mihaescu, 2013).

Calmette and Guerin developed BCG by isolating a live strain of *M.bovis* from cattle. From there *M.bovis* was cultured for 13 years with a total of 231 passages and considered attenuated from a loss of a gene encoding for the secretory proteins, ESAT-6 and CFP-10 (Ganguly, Siddiqui, & Sharma, 2008; WHO, 2013a). From 1924 to 1928, 114,000 infants were vaccinated without serious complications and thus BCG was deemed to be safe (Luca & Mihaescu, 2013). From then on the use of BCG was encouraged by WHO and by the United Nations Children's Fund (UNICEF) (Luca & Mihaescu, 2013). Since its use BCG has varied in the actual protection it offers from developing TB; it is unable to sterilize the lung after Mtb infection as shown by the large number of people diagnosed with LTBI who have received BCG (Moliva, Turner, & Torrelles, 2015). BCG does not protect adults from pulmonary disease and provides variable pulmonary protection in children. However, efficacy against meningeal and miliary TB in infants is 75% (WHO, 2009). It has been proposed that the variability in BCG protection emerged after worldwide laboratory manufacturing of BCG and lack of global standardization of procedures utilized in the production of BCG vaccine (Luca & Mihaescu, 2013). This has caused the development of 14 substrains of BCG, which have been used as vaccines in different parts of the world (WHO, 2013a). It is known that these substrains differ in genetic and phenotypic properties as well as immunogenicity (WHO, 2013a). The differences in BCG strains to induce a mycobacterial specific immune response have been noted in animal and human studies, however, there is insufficient data to recommend one strain over another (Luca & Mihaescu, 2013). In order to prevent more divergence from the original strain of BCG, WHO has kept lyophilized seed lots of vaccine strains since 1956 (WHO, 2004). The most common strains used worldwide are Danish 1331, Tokyo 172-1 and Russian BCG-I as

UNICEF only purchases vaccines through a prequalification process and distribute them in national immunization programs (WHO, 2013a).

There is no universal policy for the use of BCG, however WHO recommends vaccinating infants immediately after birth in high-burden countries (Luca & Mihaescu, 2013). WHO makes an exception for infants infected with HIV and does not recommend vaccination with BCG as it impairs the BCG-specific T cell response and increases the risk of disseminated BCG disease (WHO, 2009). BCG does not actually prevent infection with Mtb, rather offers protection against TB meningitis and disseminated TB disease in infants and young children (WHO, 2004). In addition, BCG does not prevent reactivation of LTBI which can be the main source for transmission in a community (WHO, 2004). The longevity of protection BCG offers is not well studied but is believed to gradually decrease after 10-20 years and booster vaccination has not shown additional protection (WHO, 2004).

Many mysteries remain about the immune response after vaccination with BCG. To date it is known BCG elicits a similar response as Mtb would by infecting phagocytes and stimulating T cell responses (Orme, Robinson, & Cooper, 2015). A study in the Cape Town region of South Africa used blood from 10 week old infants vaccinated with BCG to determine cytokine expression of T cells and the phenotype of IFN-γ and IL-2 expressing T cells (Andreia P. Soares et al., 2008). They concluded that vaccination with BCG induces a complex pattern of cytokine production and phenotypes from T cells. CD4+ T cells predominantly expressed IFN-γ, IL-2 and TNF-α while CD8+ T cells were less abundant and mostly produced IFN-γ and IL-2 with less TNF-α (Andreia P. Soares et al., 2008). IFN-γ has been proven to play a critical role in protection against TB as mutations of its receptor lead to severe mycobacterial disease, however, this study demonstrated that IFN-γ may not be

the only important cytokine in BCG or TB response. IGRA's used to determine LTBI or to describe immune responses to novel TB vaccines may underestimate the magnitude and complexity of induced immunity, it would be more beneficial if IL-2 and TNF-α were also considered (Andreia P. Soares et al., 2008). In addition, geography and population dynamics contribute to the protective efficacy of BCG. In the Native Alaskan Indians community, protection lasted over 50 years while other studies showed complete lack of protection in communities of India (Moliva et al., 2015). Global standardization of BCG vaccination may be able to minimize the variability in preventing TB disease, however without sufficient data to promote one BCG strain over another it is considered costly to standardize the global use of one BCG substrain (Moliva et al., 2015). While use of BCG is not optimal, it can provide a window of understanding to the cellular responses generated after vaccination in order to learn the components required for better protection against developing TB. Lastly, it is even more critical to understand immunity caused by mycobacteria and the possibility of using vaccines to prevent infection rather than TB disease.

Although BCG has variable efficacy in preventing pulmonary TB, it will most likely remain in use worldwide as its protection from disseminated TB in children is 75% (WHO, 2009) thus new vaccines must work in conjunction with it. BCG is administered in 80% of the countries considered endemic (Moliva et al., 2015). In 2014 there were 15 vaccine candidates in clinical trials some of which focus on preventing infection while others focus on preventing disease (WHO, 2015c). These candidates may prove effective against different conditions towards the development of TB disease such as in preventing initial infection, prophylactic use, assist chemotherapy, or prevent disease relapse (Orme, 2015).

One of the most promising subunit vaccine candidates was the modified vaccinia virus Ankara (MVA) expressing antigen 85A (MVA85A), the first to actually complete an efficacy trial since BCG (McShane et al., 2004). Early on MVA85A proved to be safe and cause high immunogenicity yet was unable to afford protection against TB infection or disease (Tameris et al., 2013). The pipeline of prospective vaccines has shifted from targeting disease prevention in infants towards prevention in adolescents and adults as infants are not considered to cause significant transmission of TB (Tameris et al., 2013). In addition, adolescents have been found to have more transmissible or active TB (Ellis et al., 2015). Recent modeling showed vaccinating this older group would decrease morbidity, mortality and would increase protection of infants rather than vaccinating infants alone with the same vaccine (WHO, 2015c). Nonetheless, much remains unknown about the immune system and its response to Mtb infection; continuing research in this relationship will aid in developing a more effective vaccine.

1.5d Treatment for TB disease

Between 2000 and 2014, treatment for TB saved an estimated 35 million lives in HIV-negative people and 8.4 million lives amongst HIV-positive people using antiretroviral therapy (ART) (WHO, 2015c). Treatment of TB aims to cure patients, prevent death, prevent relapse, reduce transmission and prevent drug resistance (WHO, 2010c). The standard treatment regimen for drug susceptible TB includes 6 months of isoniazid, rifampicin, pyrazinamide and ethambutol (WHO, 2015c). Rifampicin, considered the most effective first line anti-TB drug by WHO became available in the 1960's (WHO, 2015c). It works through intracellular activity to inhibit bacterial DNA-dependent RNA polymerase while isoniazid inhibits synthesis of mycolic acid during bacterial replication and prevents

formation of the mycobacterial cell wall (Munoz et al., 2015). Isoniazid also decreases the amount of bacilli in sputum during the first two weeks of treatment (Alliance, 2016). Pyrazinamide is weakly bactericidal against Mtb but has strong sterilizing activity in the acidic environment of macrophages and inflammatory areas (WHO, 2010c). Use of Isoniazid and Pyrazinamide together is powerful as it is believed there are two types of Mtb populations: one abundantly growing with rapid replication and another classified as persistent as it replicates slowly or may hibernate (Horsburgh, Barry, & Lange, 2015). Lastly, ethambutol is helpful in preventing emergence of resistant Mtb strains (WHO, 2010c). TB treatment can also be used to prevent LTBI from becoming active and therefore reduce the amount of TB incident cases worldwide. In this case individuals need continual clinical monitoring and may choose a treatment option such as six or nine month isoniazid, three month regimen of weekly rifapentine with isoniazid, 3-4 months isoniazid plus rifampicin or 3-4 months of only rifampicin (WHO, 2015d).

Although treatment success rates reported to WHO are 85% in new cases of drug susceptible Mtb the adverse effects, length of treatment and cost can deter many from seeking care. The standard treatment for drug sensitive Mtb is 6-9 months of antibiotics (Alliance, 2016). Adverse effects can include jaundice, abdominal pain, severe nausea, fever, hepatoxicity and clinical hepatitis (Getahun et al., 2015). Consequently, patients need to monitor liver enzymes along with liver function tests (Munoz et al., 2015). Treatments for MDR-TB and XDR-TB are even more costly, longer and toxic; these treatments can last over two years (Alliance, 2016). Side effects are severe and include hearing loss, depression, psychosis and kidney impairment (Alliance, 2016). In 2014, WHO approved two new drugs under interim policy guidelines, Bedaquiline and Delamanid, to be added in

the treatment regimen of MDR-TB for select individuals (WHO, 2015c). Directly observed short-therapy (DOTS) program has been instrumental in helping patients as well as countries adhere to treatment regimens (Fogel, 2015). The program aims to assure government entities build partnerships and commit to financing TB care, standardize diagnostic and treatment approaches, provide free treatment for all and continue improvement of evaluating the overall approach of treating TB (WHO, 2010b). There are many drugs in clinical trials as of 2015 with the desire to reduce the length of treatment, become more accessible and reduce the burden of TB disease globally.

1.6 Animal models in vaccine development for preventing TB

A major problem in vaccine development is identifying an animal model, which can portray the actual effects a vaccine can cause if given to a human. Animal models such as mice, guinea pigs, and non-human primates provide information of outcomes that can occur in humans but are not completely predictive. Developing a vaccine for Mtb is even more difficult as humans are the only natural host for the bacteria (Kaushal et al., 2012). MVA85A was the first vaccine candidate for TB disease protection to enter clinical trials in more than a decade (McShane & Williams, 2014). MVA85A, a modified *Vaccinia Ankara* virus expressing antigen 85A, was designed as a booster to improve the protective efficacy of BCG (Tameris et al., 2013). A total of nearly 3,000 newborns vaccinated with BCG in South Africa were enrolled to either receive MVA85A or a placebo (Tameris et al., 2013). Upon completing the study it was concluded MVA85A booster did not provide protection from TB disease as no differences were observed between the number of infants who developed TB disease and received only BCG with the placebo booster or BCG with the MVA85A booster (Arnold, 2013). Prior to clinical studies MVA85A showed variable efficacy

in mice, guinea pigs, cattle and non-human primates (NHP) (McShane & Williams, 2014). Mice vaccinated with BCG and boosted with MVA85A or BCG showed comparable levels of reduction in bacterial load (McShane & Williams, 2014). However, the MVA85A boosted mice generated higher levels of antigen specific CD4 and CD8 T cells (Moliva et al., 2015). Guinea pigs and NHP who were submitted to similar trials demonstrated no difference between BCG alone and BCG boosted with MVA85A (McShane & Williams, 2014). One study in NHP showed a positive trend towards improvement but overall was not statistically significant (Verreck et al., 2009). Cattle challenged with *M.bovis* revealed more disease-free animals in those vaccinated with BCG and boosted with MVA85A than those with BCG alone (McShane & Williams, 2014). These efforts in animal studies along with the clinical studies in infants highlighted information missing to develop a more effective TB vaccine strategy.

The variability in results shown in animal models boosted with MVA85A and the unsuccessful results of MVA85A in infants could be improved by establishing more consistent protocols in TB vaccine development. For starters, many studies of Mtb infection use laboratory strains rather than the more virulent clinical strains people are actually exposed to. In addition, studies also vary in the infectious dose used along with route of administration from what occurs in a natural infection (Ellis et al., 2015). It is presumed that exacerbating disease can appear after inhalation of very few organisms (1-10 bacilli) (Fennelly et al., 2012). However, studies in animal model challenges most commonly administer one single dose (between 50-1000 bacilli) of Mtb whereas infection in humans is likely to occur after multiple exposures of low doses of clinical Mtb strains (McShane & Williams, 2014). The definition of protection from animal models to human studies also needs a more relevant explanation. Efficacy in animal models are measured by

improvement of disease related parameters such as bacterial load, pathology, and length of disease until death. Vaccines are regarded as effective even with bacterial load and pathology present or if animals do not survive; it is considered protective as long as there is a relative reduction in organ colony forming units (CFU) or pathology (McShane & Williams, 2014). These studies cannot be replicated in humans, thus the method currently used to relate efficacy in animals and humans is to define the prevention of TB disease using clinical endpoints and classifying an individual as not protected if these endpoints are met (McShane & Williams, 2014). All of these factors put into question the efficacy of future TB chemotherapies and TB vaccines. There is no perfect animal model, as each will show different disease manifestation and immune response to infection or vaccination when compared to humans (McShane & Williams, 2014). Although MVA85A showed variable responses in animal models it was still important for it to move on through clinical trials as this is the only way to make progress towards achieving an effective TB vaccine strategy.

1.6a Mice

Many infectious disease studies involve the use of mice as they conveniently have a wide availability of immunological reagents, are low-cost and, with an array of inbred strains, can be genetically engineered to express a preferred genotype best fit for a study design. In TB research laboratories, the majority of mice strains studied have been found to be resistant to mycobacterial infection and show a strong immune response thus they have helped define the host protective immune response to the disease (D. J. Ordway & Orme, 2011). Studies in mice have provided insights to the relationships between T-cells, antigen presenting cells, and chemokines and highlighted the importance of how, when and where these interactions occur with Mtb infiltration (Robinson et al., 2015). The major caveat to

mouse models is the use of inbred strains. On one hand, this minimalizes the variability in responses and thus provides a baseline of the immunological response yet, on the other hand, the lack of variability cannot be related to the highly variable immune responses in humans and thus can hamper vaccine studies. Regardless, the mouse model has simplified the complexity of the immune system providing a manipulative living vertebrate lung, on a small scale, with the capacity for widespread experiments with definitive outcomes (Cooper, 2015). As for vaccine development, the mouse model is a useful tool in identifying the potential mechanisms of protection but not one to use to decide which vaccines move forward (Cooper, 2015).

1.6b Guinea Pigs

A major disadvantage in using mice to study TB disease is the inability to show morphological features in the pulmonary and extra-pulmonary pathology after challenge commonly seen in humans (D. J. Ordway & Orme, 2011). Most mouse strains do not show caseating granulomas, which are considered a main pathological trait of TB in humans (Ellis et al., 2015). Hence, guinea pigs are used to study the progressive pathology of TB as they demonstrate acute, subacute and chronic stages of infection (D. Ordway et al., 2008). In fact guinea pigs were used by Dr. Robert Koch to initially study TB and to develop his five postulates of infectious disease etiology (Padilla-Carlin, McMurray, & Hickey, 2008). Guinea pigs are highly sensitive to Mtb and can develop disease with a low dose aerosol exposure to laboratory strains and eventually require euthanasia after 100-150 days on the basis of humane grounds (Williams, Hall, & Orme, 2009). The susceptibility of guinea pigs may be from the inability of their macrophages to produce reactive nitrogen species, a factor needed for macrophages to destroy intracellular pathogens (Padilla-Carlin et al.,

2008). In addition, guinea pigs are considered the gold standard in vaccine testing (D. J. Ordway & Orme, 2011). Hormonally and immunologically guinea pigs are more similar to humans than rodents (Gupta & Katoch, 2005). Studies have shown a similarity in the innate immune response and complement system, however, a lack of immunological reagents for this species has prevented a full exploration of the immune response to infection and vaccination (Padilla-Carlin et al., 2008). Gene studies in guinea pigs have shown immunological similarities with humans in a homologous leukocyte antigen as well as homology with human 1 group CD1 proteins expressed on lymphoid and non-lymphoid tissues serving as antigen presenting molecules for non-peptide antigens to T cells during infections (Padilla-Carlin et al., 2008). The presence of CD1 in guinea pigs is important in vaccine studies as it is not part of mice immune systems (Clark, Hall, & Williams, 2015). The similarity in delayed type hypersensitivity between guinea pigs and humans have also made this model useful in evaluating reagents used for diagnostic skin tests (Clark et al., 2015). Although the guinea pig genome has been fully sequenced, gene knock-out, knock-in or transgene expression are not available as it is in mice (Institute, 2016; Padilla-Carlin et al., 2008). Further, guinea pigs rarely show liquefication and cavitation of pulmonary granulomas within infected tissue and do not demonstrate LTBI at all (Padilla-Carlin et al., 2008). Overall, until reagents are readily available the immune response to vaccines and efficacy testing will be limited in the guinea pig.

1.6c Non-human Primates

Being the closest relative to humans, NHPs are commonly used to model human disease through their similar genome, physiology and immunology (Kaushal et al., 2012). For the scope of TB, NHPs have the ability to demonstrate the spectrum of the disease

conditions such as LTBI, chronic infection and acute TB (Kaushal et al., 2012). More than half of infected macaques develop active disease while 40% become latently infected (Myllymäki, Niskanen, Oksanen, & Rämet, 2015). Human-like LTBI is shown by the lack of clinical signs with the presence of antigen-specific immunological response measured by TST or primate specific IGRA (Kaushal et al., 2012). The ability to study LTBI could aid in the development of antibiotic treatments and determine the role of granulomas in controlling Mtb (Fogel, 2015). Another major advantage of using NHPs is the potential to study TB/HIV co-infection; a problem affecting 12% of the 9.6 million new TB cases in 2014 (WHO, 2015c). Macaques have shown the cellular, molecular, immunologic mechanisms of TB reactivation with infection of Simian Immunodeficiency Virus (SIV) (Fogel, 2015). The obvious downfall of studies in NHPs is the cost involved in the care for these animals and how much it affects the statistical power of a study. In addition, the two subspecies of macaques used, rhesus and cynomolgus, have shown variable responses to vaccines and Mtb challenge (Orme, 2015). Different patterns of disease are observed depending on the method used to challenge the animals and studies have struggled to demonstrate protection by BCG (Orme, 2015). There is a general consensus of using NHPs as an endpoint model, however despite the similarity to humans the issues in variability of modeling aspects of Mtb disease need to be resolved before relying solely on this model.

1.6d Pigs

Domestic pigs have been compared to humans in a variety of studies with many similarities found between the two species. For starters, the anatomy, genetics, and physiology of pigs resembles that of humans (Meurens et al., 2012). There are functional similarities in organs as well, making pig-to-primate organ transplantation models

successful (Ibrahim et al., 2006). This has increased their use and progress in developing transgenic models to study human diseases such as Alzheimer's disease, cystic fibrosis and diabetes (Aigner et al., 2010). There are several advantages in using pigs for human studies such as the availability, size, and more ethical acceptance of their use as opposed to NHPs (Meurens et al., 2012). The size of pigs is of notable use as there are 541 different breeds available, outbred or inbred, and all allow extensive sampling as opposed to smaller animal models (Meurens et al., 2012). Of the different breeds, miniature pigs can be especially useful in human studies as they develop human sized organs and, due to genetic variability, can best demonstrate the full scope of responses to vaccines or therapeutics as would be seen in humans (Meurens et al., 2012). The use of pigs in TB research is not a novel idea. In 2010, Cardona and his group challenged miniature pigs with the clinical H37Rv Mtb strain and monitored the animals for a period of 20 weeks. In 2003, Molitor and colleagues monitored the γδ T cell response and IFN-γ production in pigs vaccinated with BCG (Lee, Choi, Olin, Cho, & Molitor, 2004). A more detailed review of these studies will be provided in chapters 2 and 3 respectively.

1.6di Pig Immunology

Aside from the primate and mouse immune system, the pig immune system is the third best characterized and offers a wide range of resources (Summerfield, 2009). The Swine Genome Sequencing Consortium (SGSC) was developed in 2003 in an effort to aid sequencing of the pig genome (Bambery, 2016). These efforts have led to further work in the pig immunome structure and function which has revealed greater similarities to humans compared to similarities of humans and mice (Dawson et al., 2013). About 50% of porcine immunology comes from infectious disease studies (Summerfield, 2009). The main

differences between the pig and human immune system are the inversion of lymph nodes, two types of Peyer's Patches, and passive immunity mechanism from a sow to her piglets (Meurens et al., 2012). The only way to transfer passive immunity to a piglet is from the sow's colostrum and milk; since the animals are precocial, piglets can be used to study interactions between pathogens and the immune system (Meurens et al., 2012; Salmon, Berri, Gerdts, & Meurens, 2009).

It is known that there are two subsets of CD3 associated T-cell receptors in pigs: αβ and γδ (M. Sinkora & Butler, 2009). These cells develop in the porcine thymus and resemble a similar process to maturity as in other species (M. Sinkora & Butler, 2016). Similar to humans, γδ T cells are the first T cells to develop followed by αβ (M. Sinkora & Butler, 2009). There are three main subsets of γδ T cells found in the periphery of pigs: CD2-CD8-, CD2+CD8-, and CD2+CD8α+ all of which are CD4- (M. Sinkora & Butler, 2009). γδ T cells fill diverse roles in the innate and adaptive immune response (Vantourout & Hayday, 2013). γδ T cells have been shown to recognize antigen without assistance from the major histocompatibility complex (MHC), function as antigen presenting cells (APC), and produce different types of cytokines (Stepanova, Samankova, Leva, Sinkora, & Faldyna, 2007). In comparison to humans and mice which have a majority of αβ T cells with 95%, pigs have a majority of γδ T cells that may account for 80% of T cells in the peripheral T cell pool (M. Sinkora et al., 1998). The variability of γδ T cells separates mammals into γδ "high" or "low," however whether that affects immune function is yet to be determined (M. Sinkora & Butler, 2016).

αβ T cells in the periphery of pigs are composed of cytotoxic T cells CD4-CD8αβ+ and two subsets of T helper cells: CD4+CD8- and CD4+CD8αα+ (M. Sinkora & Butler, 2016).

The helper T cells recognize foreign peptides presented by MHC molecules (M. Sinkora & Butler, 2016). CD4+CD8αα+ develop in the periphery as a result of activation and thus are considered effector/memory T cells (M. Sinkora & Butler, 2016). Six different CD4+ T cell subpopulations have been presented in humans and mice: Th1, Th2, Th17, regulatory T cells (Treg), follicular helper T cells (Tfh) and cytolytic T cells. Of these only regulatory T cells have been well documented in pigs while Th1, Th2, Th17 and cytolytic T cells have only been partially studied (Gerner et al., 2015). CD4+ T cells have shown to produce interferon IFN-γ, IL-17, TNF-α and IL-2 individually and in combination in the lung, lymph node and less prominently in peripheral blood mononuclear cells (PBMC) (Gerner et al., 2015). Cytotoxic T cells producing the same cytokines were found to be enriched in the lungs (Gerner et al., 2015).

A notable difference in the pig's immune system is that a double positive CD4+CD8+ T cell population remains high ranging from 8-60% in the blood and varies with age and immunological history of the animal (Saalmuller, Werner, & Fachinger, 2002). These double positive cells are thought to contribute to a higher population of γδ T cells (Holderness, Hedges, Ramstead, & Jutila, 2013). In addition, these cells have been found to proliferate more than CD4+CD8α- cells in response to different antigens such as Staphylococcus enterotoxin B, pseudorabiesvirus and classical swine fever virus (Gerner et al., 2015). Another peculiarity of pig lymphocytes is the ratio of CD4+ to CD8+ T cells of an average of 0.6, while in humans, this is only seen under pathological conditions (Lunney & Pescovitz, 1987). The normal range for humans is 1.5-2 (Lunney & Pescovitz, 1987).

Porcine lymphocytes also express activation markers-for the purpose of our study we have focused on CD45RA, swine leukocyte antigen-DQ (SLA-DQ) and chemokine

receptor 7 (CCR7) on T cells and monocytes. CD45 has been found in four different isoforms based off molecular weight analysis, however only CD45RA and CD45RC have been identified with monoclonal antibodies (mAbs) in pigs (Schnitzlein & Zuckermann, 1998). The CD45RA isoform marks naïve and terminally differentiated cells, when these cells become active CD45RA is replaced by other CD45 isoforms (Bullido, Gomez del Moral, et al., 1997; Donovan & Koretzky, 1993). The MHC II is also found in pigs as swine leukocyte antigens. These glycoproteins are involved in foreign antigen presentation to the immune system by binding peptide fragments and displaying them for CD4+ T cells to recognize (Bullido, Domenech, et al., 1997). Three families of MHC II have been found in humans known as human leukocyte antigen (HLA) -DR, -DQ and -DP while in pigs only SLA-DR and –DQ have been characterized (Bullido, Domenech, et al., 1997). In pigs MHC II is expressed on B cells, natural killer cells, most CD8+ T cells and one-third of CD4+ T cells (Bullido, Domenech, et al., 1997; Hlavova, Stepanova, & Faldyna, 2014). Monocytes and macrophages in pigs have the same role as in humans in presenting antigen and performing phagocytosis (McCullough, Schaffner, Natale, Kim, & Summerfield, 1997). Therefore, modulating the expression of SLA-DQ provides insight of how well these cells are able to present antigen to initiate an immune response. CCR7 is expressed on pig naïve CD4+ T cells and on both naïve and memory CD8+ T cells (Moreno et al., 2013). It is important in directing lymphocytes to secondary lymphoid organs and its loss occurs after antigenic stimulation and differentiation (Campbell et al., 2001; Moreno et al., 2013). In blood it is expressed on 70% of TCR αβ T cells in naïve CD4+ cells and CD8αα+ cells

(Moreno et al., 2013). Many more cell types and markers of the immune response in pigs and humans can be found, however, as a pilot study only a few can be monitored for our scope.

1.7 Comparative review of human to pig ontogeny

Determining the ontogeny of the immune system development in infants is difficult to ascertain due to ethical concerns and factors contributing to the heterogeneous human population. Current studies have relied heavily on use of mice as well as human umbilical cord blood (Holt & Jones, 2000). Newborn mice differ in cellular differentiation and anatomical structure and have an underdeveloped immune response compared to infants (Hodgins & Shewen, 2012). In addition, the species' short gestation and distance in phylogeny from humans leaves development of new vaccines short-handed (J. Sinkora, Rehakova, Sinkora, Cukrowska, & Tlaskalova-Hogenova, 2002). Nonetheless, most of our knowledge of T-cell development in the thymus comes from mice studies (Murphy, 2011). Human umbilical cord blood is limited by the fact that cells in circulation at the time of collection are unlikely to be representatives of cells that will circulate within days after, when corticosteroids and maternal cytokines have decreased (Hodgins & Shewen, 2012). More studies in this area could aid in the discovery of a neonate's mechanisms established to protect from microbial invasion, pathogen resistance and ultimately improve development in more effective vaccines.

Pigs have a longer gestational period of 114 days allowing for more time in characterizing the immune system (J. Sinkora et al., 2002). In addition, since pigs are precocial they can be maintained isolated and under sterile conditions to determine the mechanisms of the interactions between a naïve immune system and microbes to

determine the immunological structural and functional changes developed during this time (J. Sinkora et al., 2002). The study of the naïve immune system in pigs is further strengthened by the fact there is no transplacental transfer of maternal antibodies (M. Sinkora & Butler, 2016). Using pigs to model the immune system could fill the gap of knowledge missing in understanding the response elicited from BCG vaccination in infants and improve vaccine efficacy outcomes.

Pigs contain the same T cell populations as other jawed vertebrates: αβ T cells can focus on peptides presented by MHC molecules while γδ T cells can recognize unprocessed antigens (M. Sinkora & Butler, 2016). At the end of the first trimester, a fetal pig is missing CD3+CD4+, and CD8+ cells in the thymus (J. Sinkora et al., 2002). However, at the start of the second trimester, CD3+ε is detected with expression of γδ receptor (J. Sinkora et al., 2002). Similarly, in human fetuses CD3+ T cells are detected in circulation at the beginning of the second trimester, 15-16 weeks of gestation (Holt & Jones, 2000). In pigs, mature αβ single positive thymocytes appear shortly after preceded by CD3+ε double positive pre-T precursors (J. Sinkora et al., 2002). Eventually, these immature CD3+ε double positive cells become mature single positive, CD4+CD8- or CD4-CD8+, expressing high levels of CD3 (M. Sinkora & Butler, 2009). In the periphery, γδ T cells dominate until the beginning of the second half of gestation when αβ T cells expand (J. Sinkora et al., 2002). From then on αβ T cells are predominant in the thymus and periphery until the end of gestation (M. Sinkora & Butler, 2009). Minimal to no double positive T cells are observed before birth and CD4+ T cells are the predominant phenotype in the spleen, mesenteric lymph nodes, and umbilical blood (J. Sinkora et al., 2002). CD8+ T cells are less frequent comparatively, but increase with age (J. Sinkora et al., 2002). Natural killer (NK) cells, CD3-CD8+CD2+, were detected at

a frequency below 5% in the spleen, mesenteric lymph nodes, and umbilical blood in the second trimester(J. Sinkora et al., 2002). In adult pigs, NK represent 15% of the lymphocyte population (M. Sinkora & Butler, 2009). Comparatively, human infants produce higher levels of NK cells early on and gradually decrease to adult levels at 5 years of age (Goenka & Kollmann, 2015). At the end of the second trimester, the innate response in the fetal pig is developed and can respond to some antigens (Butler et al., 2009). From mice studies, the ontogeny of human T cell development in the thymus is believed to be similar; CD3-CD4-CD8- thymocytes to double positive expression and finally single positive expression of either CD4 or CD8 (Murphy, 2011). At birth, thymocyte population in pigs resemble humans and mice with the exception of a larger $\gamma\delta$ population (J. Sinkora et al., 2002). At this time, the adaptive immune response of piglets is considered naïve and lymphocytes expand from colonization of the gastrointestinal tract and exposure to pathogens (Butler et al., 2009; M. Sinkora & Butler, 2009). Similarly, human infants are also considered naïve in their adaptive response and rely on their innate responses (PrabhuDas et al., 2011).

It is currently believed that neonates are able to elicit a Th_1 and Th_2 primary response, however, the secondary response is believed to be biased toward Th_2 (Zaghouani, Hoeman, & Adkins, 2009). Since a Th_1 response elicits inflammation and expansion of lymphocytes to counter microbes, it is important to understand this lack of Th_1 in the secondary response for the development of pediatric vaccines (Zaghouani et al., 2009). Much of this work has been done in mice, in which the animals showed that upon antigen stimulation in vitro and in vivo Th_2 respond but Th_1 undergo apoptosis (Zaghouani et al., 2009). Since mice are quite different than humans, specifically a delayed ontogeny of

the immune system, this information may not be translated in the human neonatal immune response (Hodgins & Shewen, 2012).

Overall, it is recognized that the immune responses of human infants are distinct and cannot be predicted from those of human adults or adult animal models (Sanchez-Schmitz & Levy, 2011). The few studies done in neonatal cells show different and contradictory results; TNF-α levels in neonatal cells have been reported as significantly less, equally, or even more than levels in adult cells (Kollmann et al., 2009). Current knowledge is largely reflective of mice studies and the use of other neonatal animal models could shed light on key components in the infant immune system and aid in improving vaccines. The neonatal piglet, with similarities in human physiology, anatomy, the ability of extensive sampling and the ease of study manipulation could provide this information (Butler et al., 2009; Meurens et al., 2012).

1.8 Rationale for study

Although the animal models listed above have provided great insight into the progression or resistance to TB disease, Mtb still remains the world's most successful pathogen. Despite many efforts, progress towards developing improved diagnostics, easier treatment regimens or a vaccine to prevent disease is lagging compared to studies with other infectious pathogens such as HIV. There may be no perfect animal model and in fact we may need to continue using multiple models to decipher Mtb completely as the course of TB in humans develops with wide variability. In this study, we suggest using mini-pigs as a neonatal model for TB vaccine development as there is a lack of immunological information in human neonates. Small animal models may not be the best models for neonatal response to TB in relation to humans due to aging and susceptibility differences. A

study in 2013 successfully established an aerosol challenge of Mtb in neonatal macaques demonstrating similarities in clinical and bacteriological characteristics as seen in human infants (Cepeda et al., 2013). Aside from that study there appears to be no other research with neonatology and Mtb. As mentioned before, NHPs may provide the best translational results to humans compared to all other animal models. However, use of pigs as an alternative large animal model to the mouse or guinea pig model may provide crucial information missing from our knowledge of the immune response as, they are remarkably similar to humans in terms of anatomy, genetics and physiology (Gerner et al., 2015). The following chapters highlight benefits of using mini-pigs in establishing Mtb infection with the highly virulent clinical strain HN878, along with results to a longitudinal immune response to BCG. The objective was to follow components of the immune response in vaccinated and unvaccinated neonatal piglets to BCG and relate the response to human infants in hopes of improving vaccine development against TB and ultimately provide more stable results for vaccine efficacy trials before progressing to human clinical trials.

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Chapter 2: Mtb HN878 challenge in mini-pigs

2.1 Introduction

Mini-pigs may provide the missing information in the study of human TB disease, caused by a pathogen, which continues to outsmart us even as research technology improves. Unlike small animal models or macaques, pig lungs resemble humans' extensive interlobular and intralobular connective tissue joining the major vessels and bronchi to the pleural space (Gil et al., 2010; Plopper & Harkema, 2005). In addition, the upper respiratory tract including the Waldeyer's ring of pigs and humans are similar (Horter, Yoon, & Zimmerman, 2003). One major difference between human and pig lungs are the amount of lobes present: pigs have two lobes on the left side and four on the right while humans have two on the left and three on the right (Meurens et al., 2012). In order to study the pathological effects Mtb has on pigs we chose the Sinclair mini-pig model as it can develop adult human-sized organs starting at 6 months of age while maintaining a manageable size for housing and handling purposes (Meurens et al., 2012).

Notably, Mtb in pigs has been reported from contaminated garbage and contact with sputum or body excretions (Thoen, Lobue, Enarson, Kaneene, & de Kantor, 2009). One group has already characterized Mtb granuloma development in mini-pigs, presenting for the first time the evolution of Mtb infection in mini-pigs (Gil et al., 2010). Our approach here differs by use of the clinical HN878 virulent strain as opposed to the laboratory H37Rv strain used by Gil and colleagues. Mice challenged with HN878 have shown a more rapid mortality in comparison to other clinical isolates (Manca et al., 2001). In addition, we challenged the animals via aerosol delivery versus a transthoracic method used in Gil's group to more accurately present Mtb exposure as it occurs in humans. Our aim was to

follow the progression of disease in adult mini-pigs for the duration of 36 weeks and in 2 month old mini-pigs for 8 weeks. We were particularly interested in disease progression in the younger mini-pigs in the hopes of using these animals as a model for TB disease in children. Studies in this age group are few even though children account for more than 20% of TB cases in high burden countries (Moyo et al., 2010).

Our objectives were to (1) monitor clinical symptoms induced by Mtb in challenged animals, (2) test occurrence of transmission by housing unchallenged animals under the same conditions as challenged animals, (3) evaluate tissue pathology and level of colonization resulting from Mtb exposure in all animals.

2.2 Materials and Methods

2.2a Animals

Ten adult pigs of six months of age (group 1) and 10 two month old (group 2) Sinclair Miniature Swine gilts (Sinclair Bio-Resources, MO) were used to study Mtb infection and disease progression under protocols approved by the Institutional Animal Care and Use Committee of Colorado State University. Each group of animals was socially housed in an ABSL (animal biosafety level) III barrier under standard conditions (72 ± 6 degrees F, 30-70% humidity, 12:12 light cycle) for the duration of the experiment and were allowed to acclimate for two weeks prior to Mtb challenge. Animals had ad libitum water access and were fed a locally sourced complete feed (Panepinto Show Feed, CO). Temperatures were taken daily from group 1 along with weekly weights. Temperatures and weights were taken monthly from group 2 gilts. CSU veterinary residents monitored animals daily for any clinical symptoms associated with TB disease. Two animals from each group remained unchallenged and were housed with the challenged animals for the

duration of the study. After challenging the eight adult pigs, the two unchallenged pigs remained in a separate building during the aerosol challenge and were not housed with the challenged animals until three months post challenge. The unchallenged pigs in group 2 were separated from the challenged pigs during the aerosol challenge and were reunited two days post challenge.

2.2b HN878 *Mycobacterium tuberculosis* stock and inoculum

Mtb HN878 strain was obtained from Colorado State University Mycobacteria repository bank as a frozen suspension at $1.07x10^7$ colony-forming units (CFU)/ml. A fresh stock was thawed and serially diluted in sterile deionized water for a final concentration of $1x10^3$ CFU/ml for high dose challenge and $1x10^2$ CFU/ml for low dose challenge at the time of challenge for each pig group. Each inoculum was confirmed by plating an aliquot onto nutrient Middlebrook 7H11 agar plates and calculating bacterial numbers. The actual CFU count for the high dose was 338 CFU and for the low dose 25 CFU.

2.2c Aerosol exposure to Mtb HN878

An inoculum of Mtb HN878 was prepared as described above at a high and low dose (HD and LD respectively). As a pilot study, it was important to use different inoculum doses in order to determine which dose is effective in this particular animal model. The low and high doses used were proven effective previously (Gonzalez-Juarrero et al., 2013). The inoculum aerosol was created using a ViosH air compressor (PARI Respiratory Equipment, Inc. Midlothian, VA, USA) and LC SprintH nebulizer (mass mean diameter 3.5mm) as reported previously in the *M.bovis* model of infection in goats (Gonzalez-Juarrero et al., 2013). The nebulizer was modified in order for all exhaled air to pass through a HEPA filter. The modified nebulizer was then connected to a cuffed endotracheal tube for intratracheal

delivery of the aerosol into the mini-pigs. Mini-pigs received 5 ml of inoculum while under general anesthesia using xylazine hydrochloride 1.5mg/kg and ketamine hydrochloride 15mg/kg through intramuscular injection. Mini-pigs were monitored after challenge for signs of distress from the procedure.

2.2d PPD

Tuberculin Purified Protein Derivative (Mantoux) Tubersol® (Sanofi Pasteur Limited, Toronto, Ontario, Canada) was administered intradermally in all mini-pigs at a dose of 5TU per 0.1ml as recommended for human use. PPD was administered at 24 weeks post challenge in group 1. In group 2, PPD was administered two days before euthanasia and read during necropsy, 11 weeks post challenge.

2.2e Dexamethasone Treatment

At seven months post challenge, oral Dexamethasone (DEX), 0.5mg/kg/day, was mixed into a soft baked breakfast bar and given to 5 pigs for 60 consecutive days until sacrificed for group 1.

2.2f Post- mortem examination

Animals were euthanized with an intracardiac overdose of pentobarbital 80-100mg/kg after general anesthesia described above; 2 adults at week 16 post challenge, remaining 8 adults at week 36 post challenge and younger pigs at 11 weeks post challenge. As a pilot study, end points were determined due to limited housing space and financial resources. Necropsies of all animals were performed under the expertise of Dr. Richard Bowen, an infectious disease veterinarian. Right and left lung lobes, spleen, and lymph nodes (mediastinal and submandibular) were collected. Gross lesions from tissues were collected through observation and palpation. Samples of tissue with no apparent gross

lesions were also collected. All samples collected were isolated and weighed to approximately 0.5g prior to use for bacterial load determination as described below. Samples were used for bacterial load determination or histology.

2.2g Bacterial load determination

Bacterial loads from lung and lymph node were determined by plating serial dilutions of the organ homogenates on nutrient Middlebrook 7H11 agar plates. A total of 15 samples from lung and lymph node tissue were plated for each animal. The number of CFU that appeared on the agar plates after incubation at 37 ^{o}C during 3-5 weeks determined the bacterial load per gram of tissue sample. Pigs are known to host other mycobacteria species such as NTMs (Thoen et al., 2009) thus CFU appearing on agar plates were collected and processed by PCR using specific primers for Mtb as described below.

2.2h Speciation of Mtb by PCR

Colonies counted as CFU were further verified through DNA extraction Trizol® reagent (Thermo Fisher), following manufacturer's instructions. Extracted DNA was used in PCR with specific primers for rpoB (210bp) for Mtb and (372bp) for NTM (Helb et al., 2010). The forward primer for this gene was 5'CGTGGAGGCGATCACACCGCAG3' and the reverse primer was 5'AGCTCCAGCCCGGCACGCTCAC3'. PCR products confirmed with 2% agarose gel, a band around 200 bp was considered positive. The rpoB primers were as follows: A PCR for NTM specific primers of rpoB (372bp) were also used to assure presence of Mtb (Wang et al., 2015). The forward primer sequence was

5'CCTGTTCTTCAAGGAGAAGCGCTACGACCTGG3'

Reverse primer sequence was 5'GGACGGATGTTGATCAGGGTCTGCGG3'.

2.2i Histology

Tissue samples collected from each animal were fixed in 4% paraformaldehyde. Sections were stained with hematoxylin and eosin along with acid-fast Auramine-Rhodamine. Hematoxylin and eosin (H & E) and acid-fast stained samples were observed under an IX70 Olympus microscope. Auramine Rhodamine stained samples were examined under a Zeiss LSM 510 confocal microscope.

2.3 Results

2.3a Group 1: Adult mini-pigs

Four miniature pigs were challenged with a high dose aerosol of Mtb HN878 and four miniature pigs were challenged with a low dose of Mtb HN878. Two unchallenged pigs were united with the challenged animals shortly after Mtb HN878 aerosol exposure. The pigs were monitored for clinical symptoms for a total of nine months. A main reason pigs were chosen to model TB in our study is due to their ability to cough and sneeze, common symptoms associated with TB. Weight loss and fevers are also common symptoms of TB. Pig temperatures were monitored daily and temperatures taken weekly with no changes indicating illness. At seven months post challenge, mini-pigs in group 1 adult pigs did not show clinical symptoms. Thus in order to assess whether mini-pigs could develop clinical symptoms 5 animals in group 1 were subject to oral Dexamethasone (DEX) at 0.5mg/kg/day. DEX is known to cause immunosuppression and change T-cell development and function (van Mierlo et al., 2013)

Gross pathological examination at necropsy revealed TB-compatible lesions in all pigs, as summarized in **Table 2.1**. The decision to euthanize 2 pigs (43 and 76), 16 weeks post challenge was due to the lack of clinical symptoms in all of the animals and a desire to

evaluate the status of infection. Further, treatment with DEX did not induce clinical symptoms in any of the animals. These animals demonstrated small lesions in the lungs, lymph nodes, and spleen. The remaining pigs were continuously monitored for weight loss, fever, cough and sneezing until euthanasia 36 weeks post challenge. All challenged animals showed calcified lesions varying in size as well as softer lesions with variable size mostly found in the apical lobes and near the bronchial tree of the lungs; similar lesions were found in mediastinal lymph nodes. The heterogeneity of lung lesions is represented in **Figure 2.1 A-G** with **Figure 2.1A** representing disseminated lesions throughout the lungs, **Figure 2.1 B, D** and **G** showing calcified lesions, **Figure 2.1C**and **F** demonstrating soft lesions, and **Figure 2.1 E** representing a soft calcified lesion. Enlargement was noted in thoracic and submandibular lymph nodes of some animals, however these organs were not measured for comparison in healthy animals. No abnormalities were seen in other organs. Two pigs, 58 and 59, were found to have severe pathological changes in comparison to the rest of the animals. Both of these pigs had disseminated lesions throughout both lung lobes and apical lobes hardened by calcified lesions represented in **Figure 2.2 A** calcification of apical lobe, **Figure 2.2B** calcified mediastinal lymph node, **2.2C** disseminated lesions throughout the lungs, **Figure 2.2D** enlarged mediastinal lymph node, **Figure 2.2E** lesions near bronchial tree, a cross-section of a calcified lesion is shown in **Figure 2.2 F**.

None of the animals showed clinical symptoms indicative of active TB, even after DEX treatment, coinciding with results from a previous study (Gil et al., 2010). However, the two unchallenged animals, 68 and 74, housed under the same conditions as the challenged animals demonstrated lesions upon gross pathological examination, as summarized in **Table 2.2**. Lesions in the lung were small and none were calcified. Both

unchallenged pigs showed enlarged lymph nodes, though were not measured to compare to healthy animals. The submandibular lymph node of pig 74 contained small lesions while no lesions were observed in this lymph node of pig 68. All other organs appeared healthy in these unchallenged animals. Colony counts of the plated homogenized tissue samples revealed there was no statistical difference between the high or low inoculum doses used, **Figure 2.3**. Further, this testing demonstrated that not all lesions produced bacilli growth on 7H11 agar. PPD was administered in the neck of all challenged pigs, 24 weeks post challenge, and all of the animals developed a large induration indicative of a positive PPD result, however the size of indurations were not measured.

Histopathological analysis confirmed the TB-compatible lesions observed through gross pathology examination. Through H & E stains, inflammation of the parenchyma was noted **Figure 2.4E** along with necrotic granulomas containing calcified centers surrounded by fibrosis in the lymph node **Figure 2.4B** and lung tissue **Figure 2.4 C-D.** Most sections contained healthy lung tissue, **Figure 2.4A**, demonstrating the heterogeneity of lesions caused by Mtb. Lesions were infiltrated with cells, that were most likely lymphocytes, starting a classical granuloma, **Figure 2.4F**. The presence of lymphocytes could be further confirmed by immunohistochemistry in future studies. Histopathology of lymph nodes revealed those with necrotic and calcified granulomas with fibrosis of the lymph node, **Figure 2.5**. All lesions appeared similar to granulomas previously reported in guinea pigs challenged with Mtb (Orme & Basaraba, 2014).

Acid fast staining with auramine rhodamine, revealed bacilli present in different lung structures, including necrotic granulomas, **Figure 2.6A,B**. Presence of Mtb bacilli inside a bronchiole of a challenged pig and in a salivary gland from an unchallenged pig

with positive staining for Mtb bacilli indicative of Mtb transmission, **Figure 2.6C,D.** A representative lung sample of pig 58 stained with auramine rhodamine, demonstrates a granuloma with a necrotic center, **Figure 2.7**. Mtb bacilli surround the necrotic center along with macrophages further surrounded by a lymphocytic ring and fibrosis. **Figure 2.8** are isolated areas of **Figure 2.7** to highlight areas where Mtb bacilli were most prominent.

Table 2.1 Summary of gross pathological lesions observed in HN878 challenged adult pigs at time of necropsy

Pig ID	Inoculum Dose	Lungs	Lymph Nodes(LN)	Other organs
43	HD	Small lesions	Small lesions	Extra-pulmonary lesions in spleen
59	HD	Calcified lesions near bronchial tree Apical lobe hardened with lesions	Small lesions	No abnormalities noted
46	HD	Small lesions near bronchial tree	No abnormalities noted	No abnormalities noted
80	HD	Small lesions	Calcified lesion in mediastinal LN	No abnormalities noted
42	LD	One major calcified lesion Several small lesions disseminated	Calcified lesion in mediastinal LN	No abnormalities noted
67	LD	Several small lesions	Calcified lesions	No abnormalities noted
58	LD	Disseminated numerous lesions Calcified lesions near bronchial tree Apical lobe hardened with lesions	Calcified lesions in submandibular LN	No abnormalities noted
76	LD	Small lesions	Small lesions	Extra-pulmonary lesions in spleen

Table 2.2 Summary of gross pathological lesions observed in unchallenged adult pigs co-housed with HN878 challenged pigs at time of necropsy

Pig ID	Inoculum Dose	Lungs	Lymph Nodes (LN)	Other organs
68	none	Small lesions	No abnormalities noted	No abnormalities noted
74	none	Small lesions	Small lesions in submandibular LN	No abnormalities noted

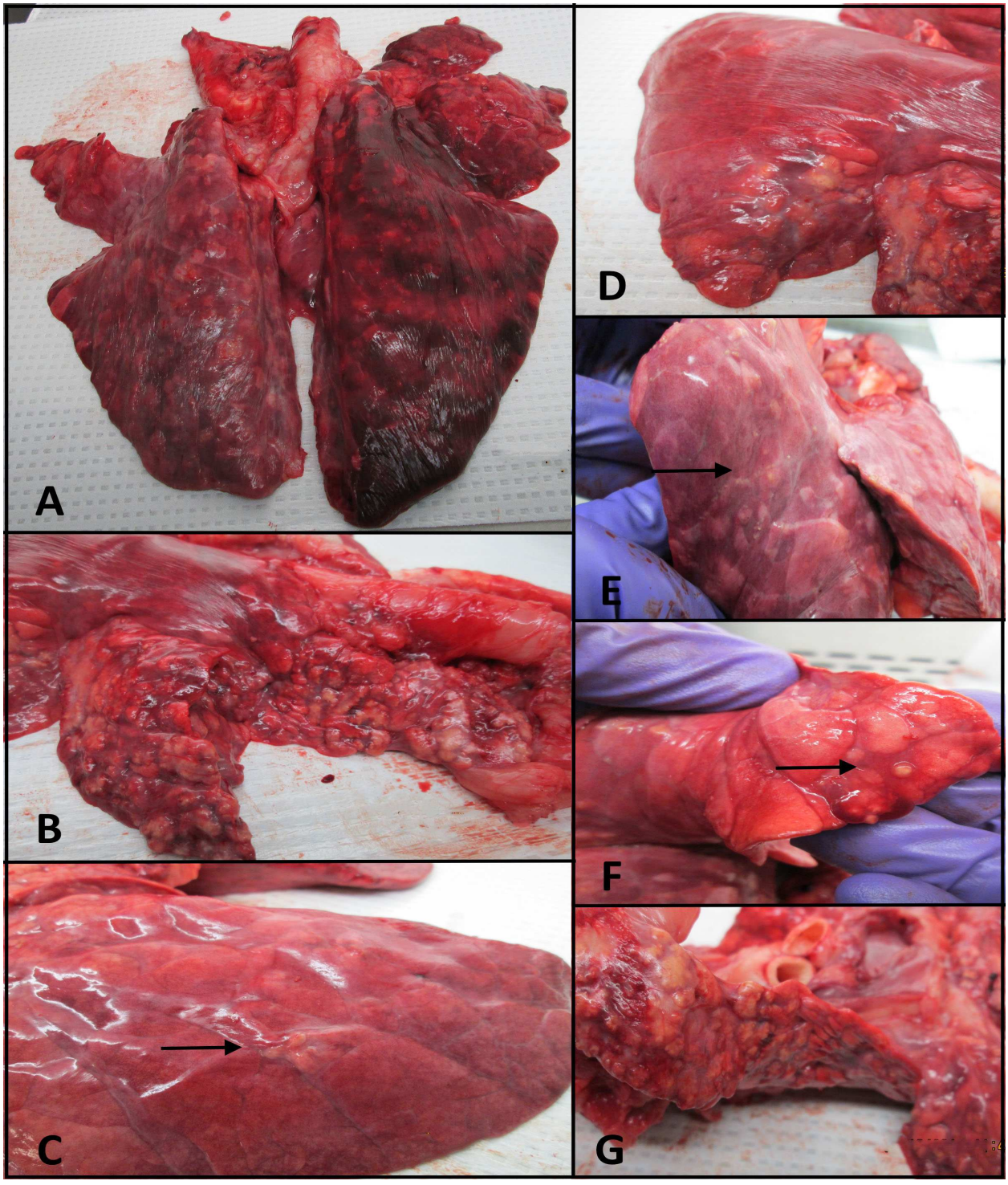

Figure 2.1 Representative tissue of animals demonstrating heterogeneity of TB-compatible lesions observed in pigs 36 weeks post challenge with HN878 Mtb. (A) Disseminated lesions (B, D, G) calcified lesions, (C and F) soft lesions, (E) soft calcified lesion.

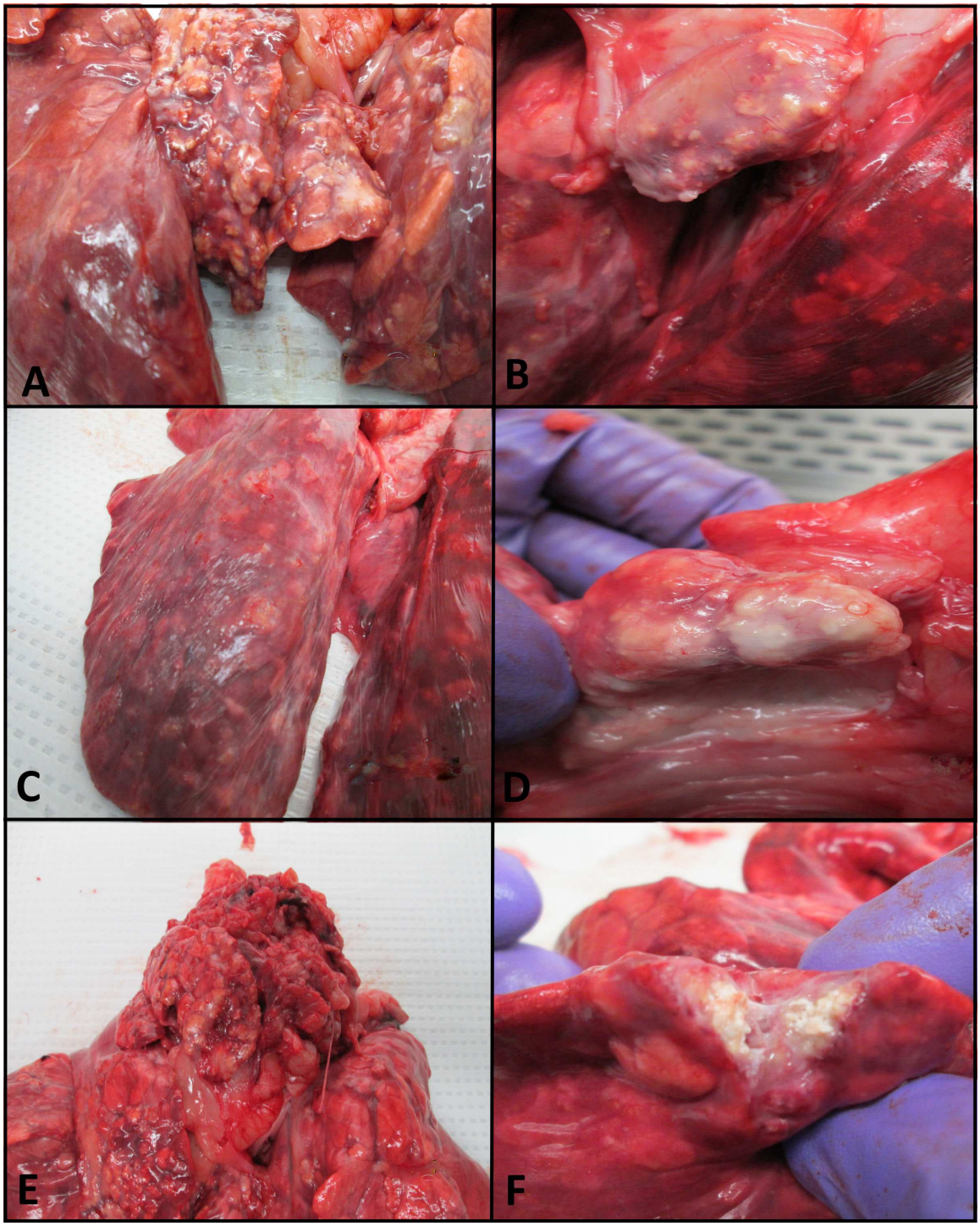

Figure 2.2 Severe pathology observed in two pigs 36 weeks post challenge with HN878 Mtb. (A) numerous lesions in apical lung lobe. (B) calcified mediastinal lymph node. (C) disseminated lesions in the lungs. (D) enlarged mediastinal lymph node. (E) lesions near bronchial tree (F) cross-section of calcified lesion.

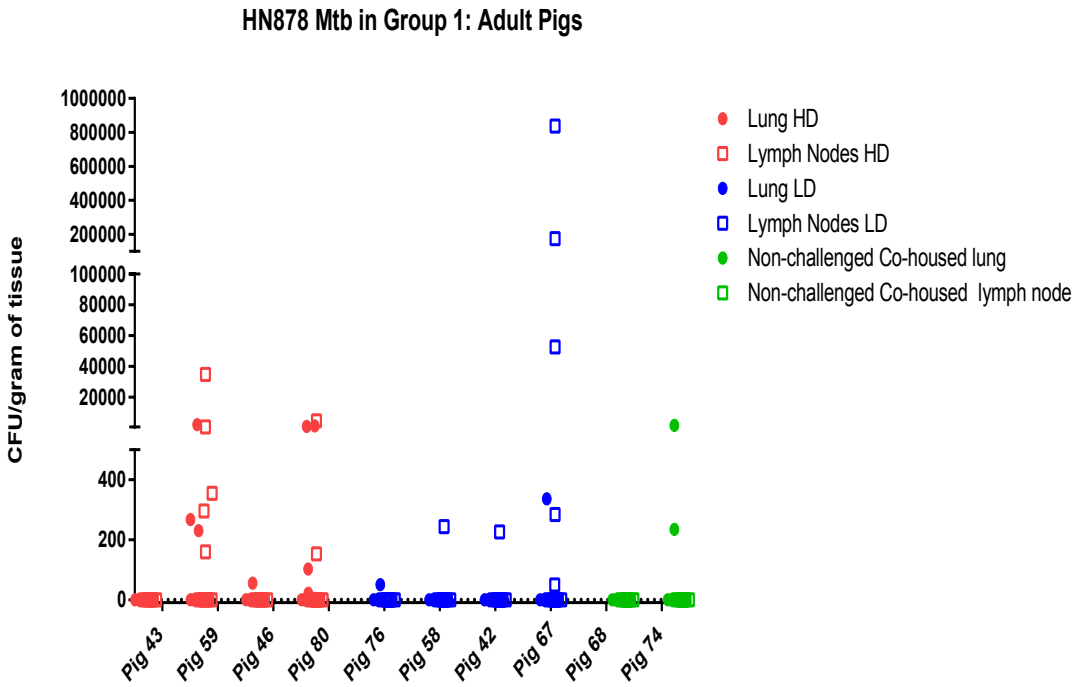

Figure 2.3 Bacterial load in samples collected from lesions of lung and lymph node tissue in pigs challenged with a high dose (HD) and low dose (LD) aerosol of Mtb HN878 and from pigs left unchallenged but co-housed with the challenged animals.

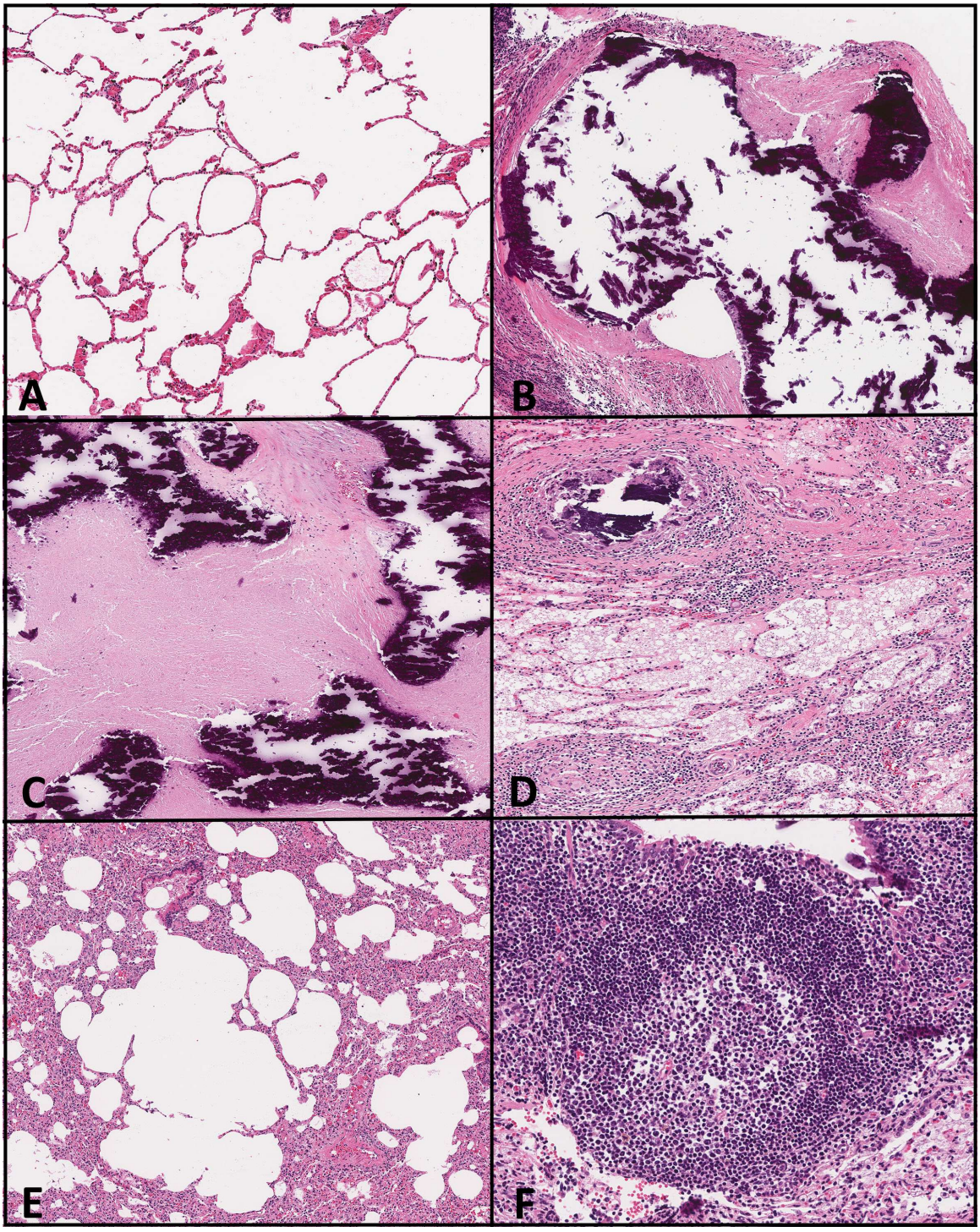

Figure 2.4 Representative H&E stained tissue of pigs 36 weeks post challenge of aerosol HN878 Mtb. (A) Healthy lung tissue. (B) Necrotic granuloma with calcified center and surrounding fibrosis in the mediastinal lymph node and lung tissue (C-D). (E) Inflammation of the lung parenchyma. (F) Infiltration of lymphocytes starting a classical granuloma.

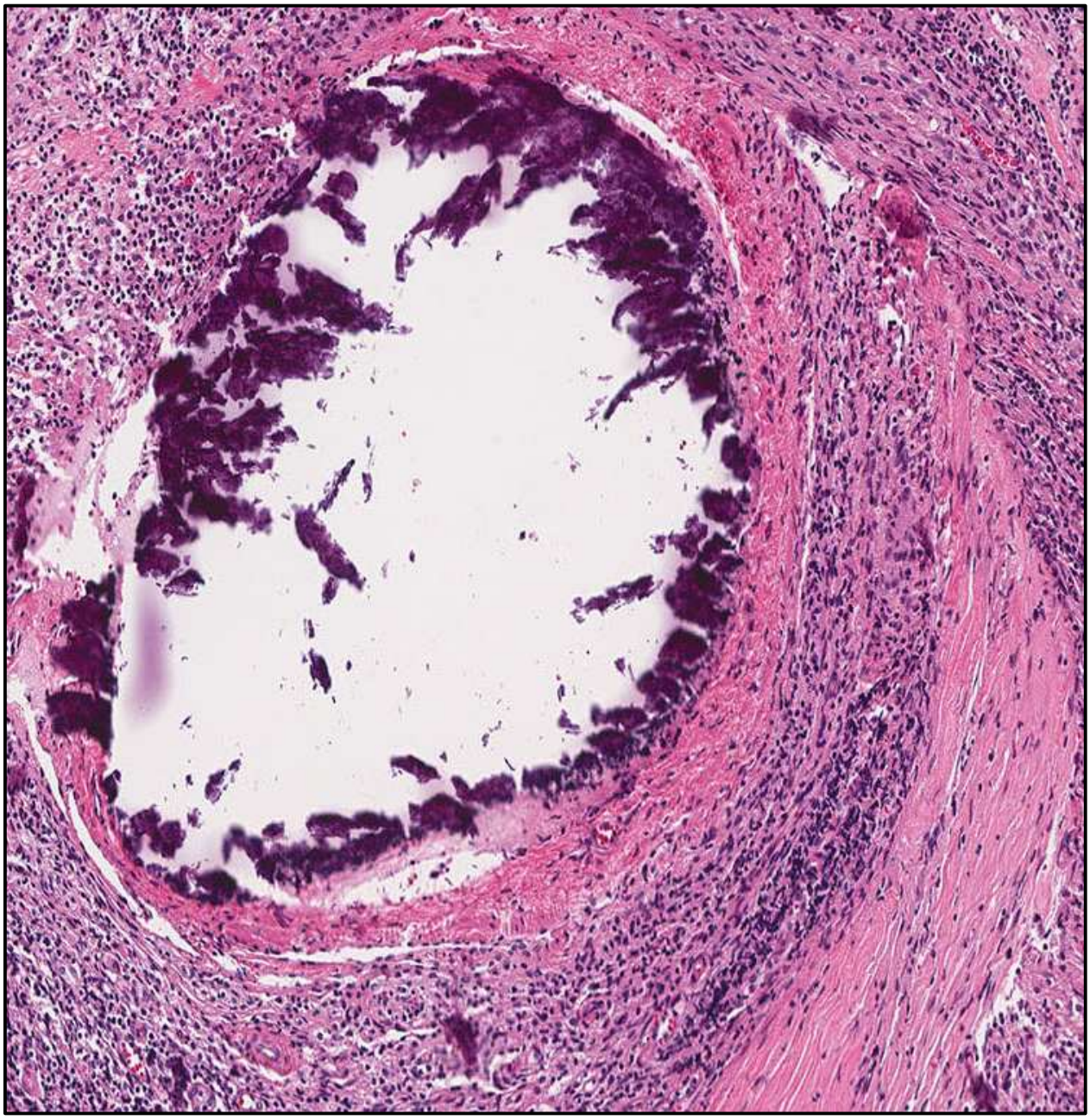

Figure 2.5 Representative H&E stained tissue of pigs 36 weeks post challenge of aerosol HN878 Mtb. A necrotic and calcified granuloma surrounded by fibrosis and lymphocytic ring.

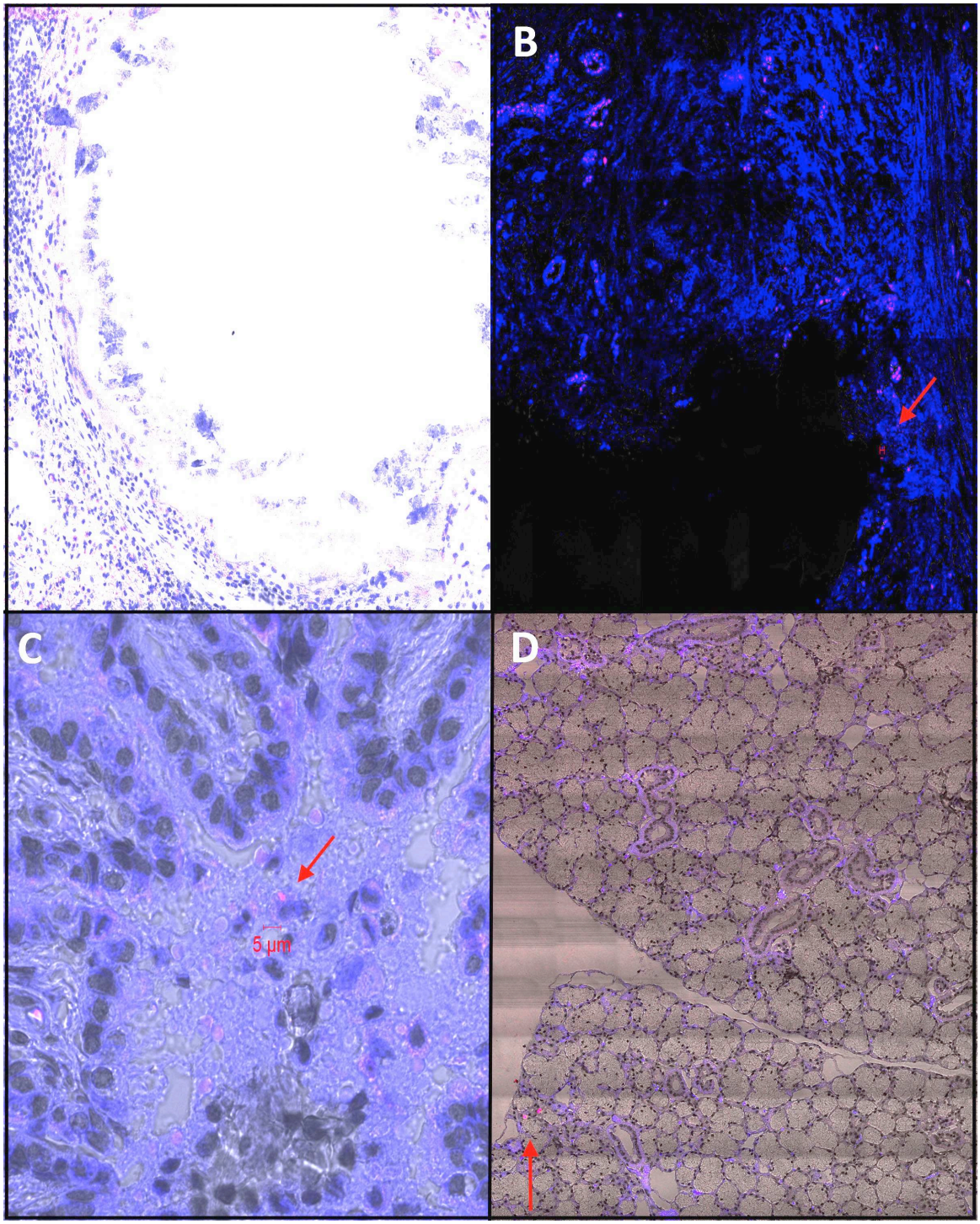

Figure 2.6 Representative acid fast stained bacilli found in tissue of pigs 36 weeks post challenge of aerosol HN878 Mtb. (A) Granuloma with necrotic center in the lung. (B) Acid fast stained bacilli in necrotic rim of granuloma in the lung. (C) Bronchioles with acid fast stained bacilli (D) Salivary gland of an unchallenged pig co-housed with Mtb challenged animals demonstrating acid fast bacilli.

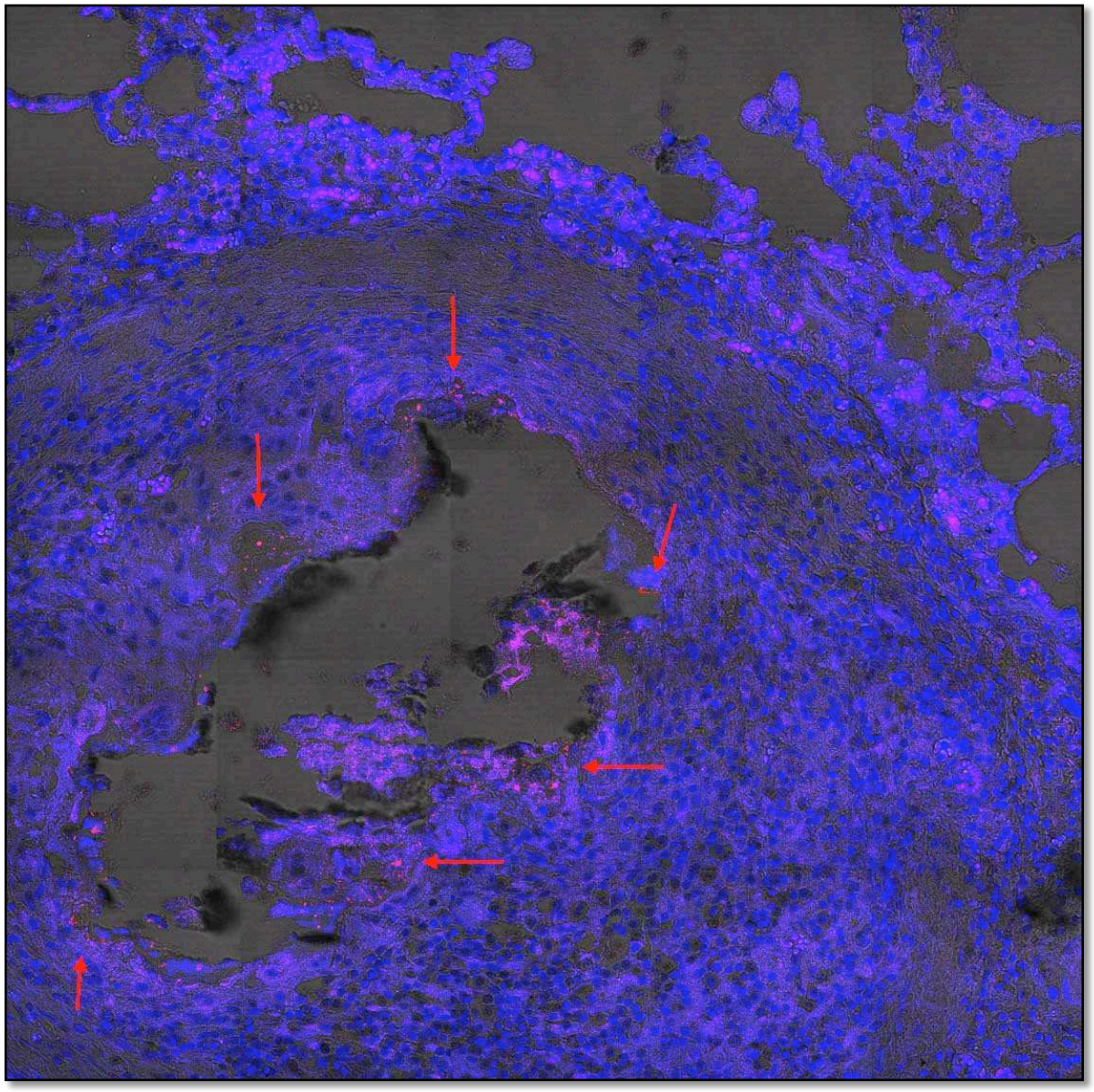

Figure 2.7 Lung tissue of a representative pig 36 weeks post challenge of aerosol HN878 Mtb demonstrating a necrotic granuloma surrounded by lymphocytes and fibrosis with numerous Mtb bacilli present.

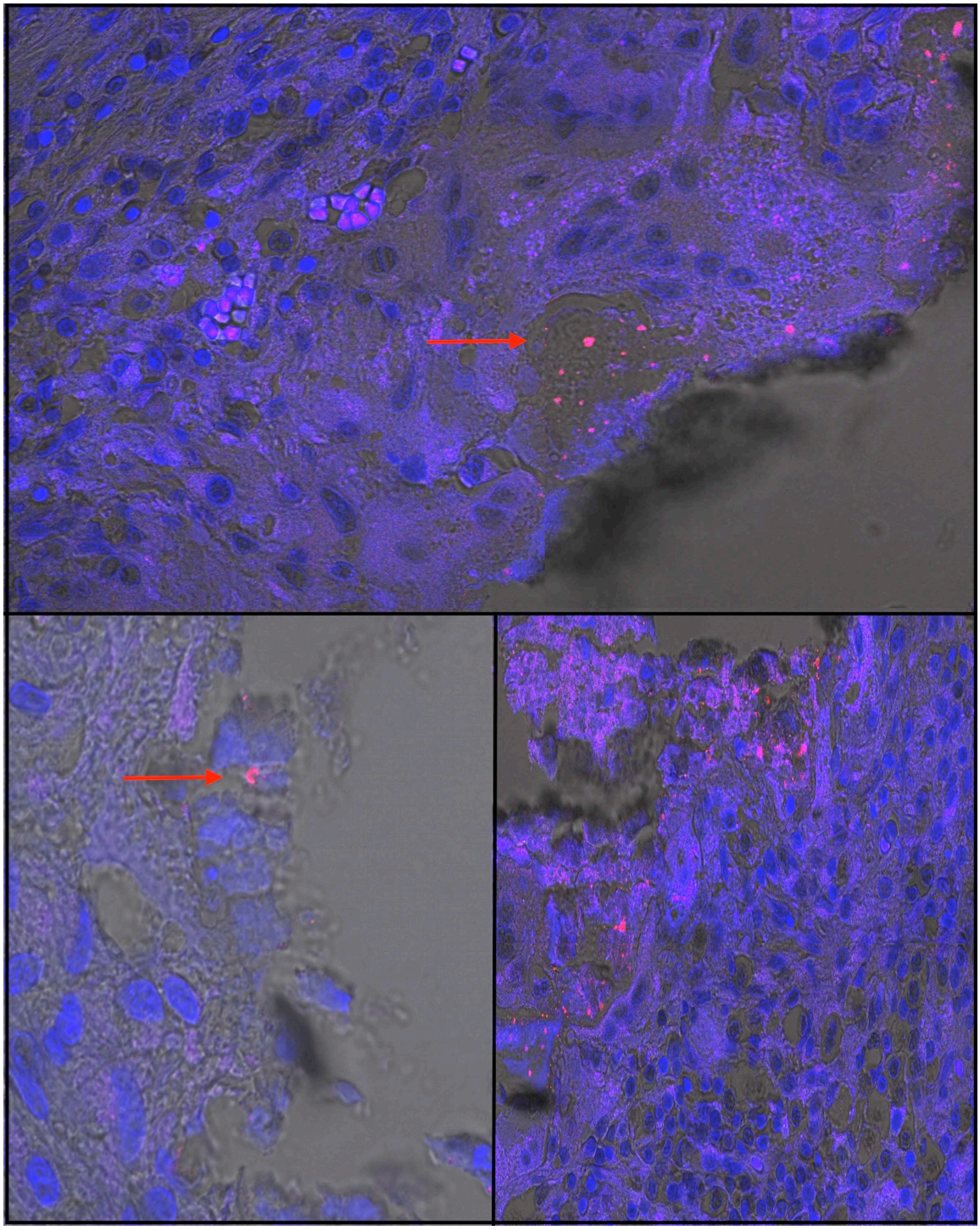

Figure 2.8 Isolated areas of **Figure 2.7**, highlighting presence of Mtb bacilli.

2.3b Group 2: Two months old piglets

All pigs in this group were euthanized 11 weeks post challenge. Gross pathological examination of the lungs, lymph nodes, and spleen did not reveal as many lesions as in group 1. Small and few lesions were observed in the lungs and lymph nodes of all animals, summarized in **Table 2.3.** None of the animals showed overt clinical symptoms throughout the study yet the two unchallenged animals showed TB-compatible lesions, **Table 2.4**. Representative gross pathology images are shown in **Figure 2.9** with most cases appearing healthy or with small lesions indicated by arrows; only pig 94 showed a difference in pathology with an active caseous lesion, **Figure 2.9E**, and pig 88 showed small disseminated lesions through its lung lobes, **Figure 2.9B**. Comparative analysis of colony counts of the plated homogenized tissue samples revealed there was no statistical difference between the high or low inoculum doses used, **Figure 2.10**. Further, it demonstrated that not all lesions produced viable bacilli when plated onto 7H11 agar.

In comparison to the adult animals, the H & E stained tissue in these piglets revealed few granulomas **Figure 2.11A**, inflammation of the parenchyma is seen **Figure 2.11C** with **Figure 2.11B** as healthy lung alveoli. Auramine rhodamine staining did not demonstrate acid-fast bacilli as shown in the adult animals, **Figure 2.12**. All of the pigs in this group were inoculated with PPD two days before euthanasia. The site of inoculum was read at necropsy with only pig 88, noted to have disseminated lesions in its lungs, showing a positive induration reaction to PPD.

Table 2.3 Summary of gross pathological lesions observed in HN878 challenged young pigs at time of necropsy

Pig ID	Inoculum Dose	Lungs	Lymph Nodes	Other organs
68	LD	Small lesions	Small lesions	No abnormalities noted
92	LD	Small lesions	Small lesions	No abnormalities noted
93	LD	Small lesions	Small lesions	No abnormalities noted
94	LD	Small lesions 1 caseous lesion	Small lesions	No abnormalities noted
72	HD	Small lesions	Small lesions	No abnormalities noted
78	HD	Small lesions	Small lesions	No abnormalities noted
79	HD	Small lesions	Small lesions	No abnormalities noted
88	HD	Several small lesions disseminated	Small lesions	No abnormalities noted

Table 2.4 Summary of gross pathological lesions observed in unchallenged young pigs co-housed with HN878 challenged pigs at time of necropsy

Pig ID	Inoculum Dose	Lungs	Lymph Nodes	Other organs
73	none	Small lesions	No abnormalities noted	No abnormalities noted
80	none	Small lesions	Small lesions	No abnormalities noted

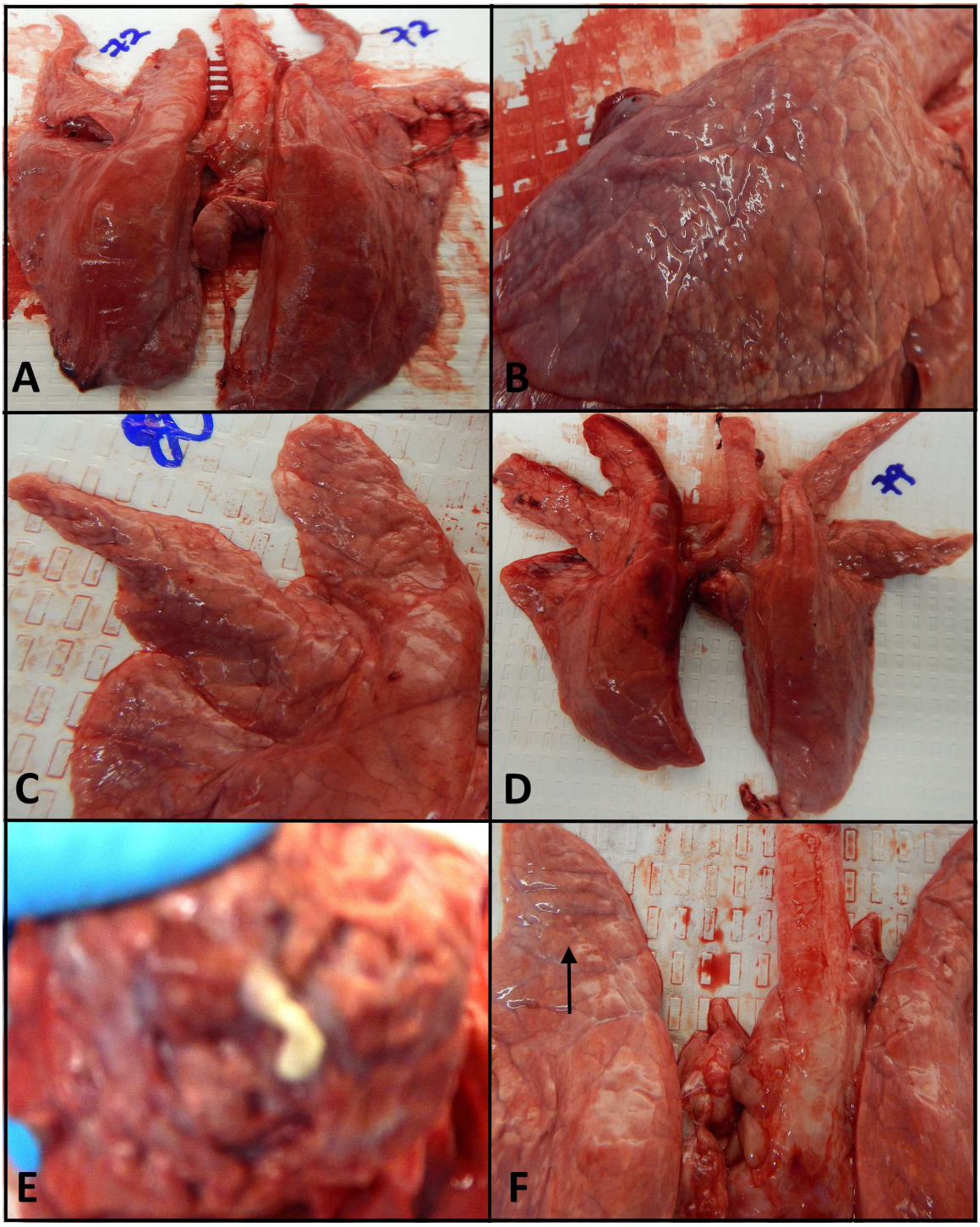

Figure 2.9 Gross pathological tissue examination 11 weeks post challenge of aerosol HN878 Mtb in pigs (A, C,D, F) Healthy appearing tissue with minimal to no small lesions present. (B) Disseminated lesions through the lungs. (E) Caseous lesion, image out of focus but only one available.

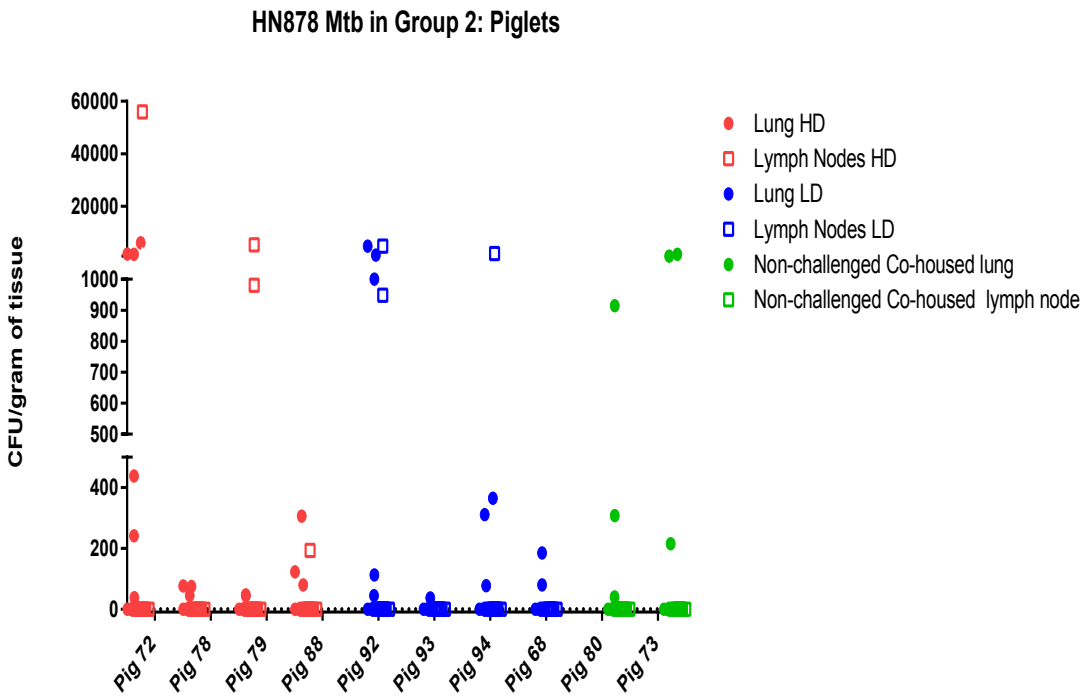

Figure 2.10 Bacterial load in samples collected from lesions of lung and lymph node tissue in two month old pigs challenged with a high dose (HD) and low dose (LD) aerosol of Mtb HN878 and from two month old piglets left unchallenged but co-housed with the challenged animals.

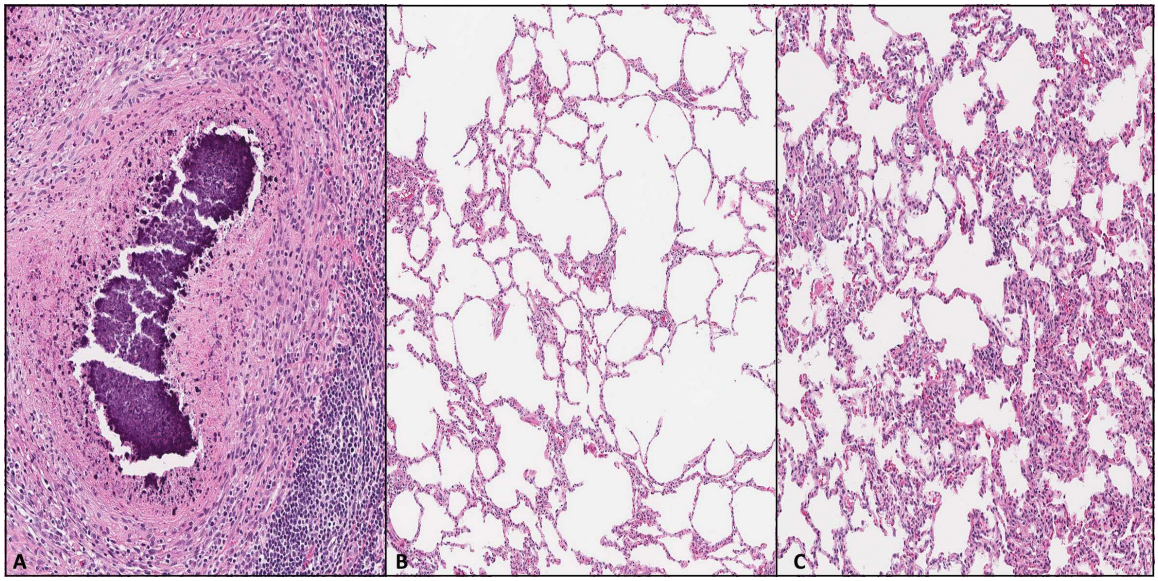

Figure 2.11 Representative H & E stained tissue 11 weeks post challenge of aerosol HN878 Mtb in pigs. (A) Calcified lung granuloma. (B) Healthy lung alveoli. (C) Inflammation of lung parenchyma.

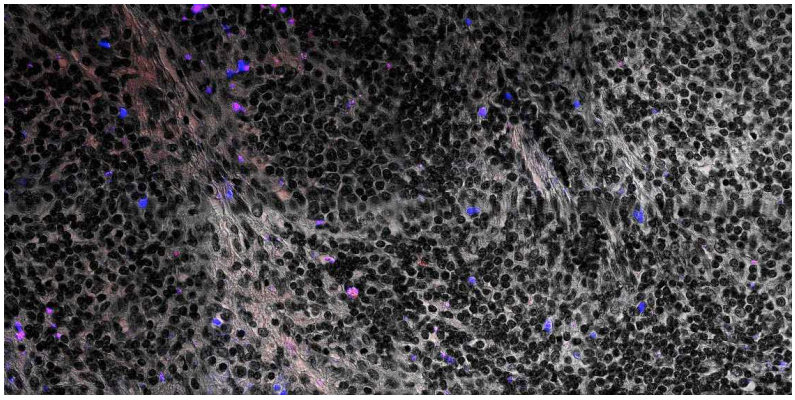

Figure 2.12 Acid fast staining did not reveal bacilli in tissues of two month old pigs challenged with HN878 Mtb.

2.4 Conclusion

The pathogenesis of Mtb HN878 from an aerosol challenge in adult and two month old piglets is reported in the present study. Post-mortem examination revealed both groups of pigs demonstrated TB-compatible lesions in the lungs and lymph nodes with differing heterogeneity. The pigs in the adult group showed more severe pathology with lesions varying in size, dispersed throughout lungs and lymph nodes, and with calcification

present in many lesions. The younger pigs, group 2, had lesions smaller in size with little dispersion and no calcification present; the lesions in this group were more homogeneous overall. Histopathology revealed acid fast positive Mtb bacilli within granulomatous lesions in adult pigs. Granulomas were found to have necrotic centers with pronounced fibrosis, a common feature found in human TB but not reproduced in some TB animal models (Driver et al., 2012; Russell, Cardona, Kim, Allain, & Altare, 2009). The presence of fibrosis indicates a favorable environment for Mtb to survive, preventing penetration of chemotherapeutics (Gonzalez-Juarrero et al., 2013).

Neither pig group demonstrated overt clinical symptoms of TB such as weight loss, temperature fluctuations, cough, sneezing, or sputum production. Lack of clinical symptoms suggest LTBI as seen in a previous study with Mtb H37Rv transthoracic challenge in mini-pigs (Gil et al., 2010). However, in both groups of pigs the unchallenged animals revealed small TB-compatible lesions at time of necropsy with some viability for CFU growth. This finding suggests that the animals challenged with Mtb HN878 were able to possibly transmit viable bacilli through aerosol droplets under the same housing. Furthermore, in group 1 of the adult animals, two of the challenged pigs, 58 and 59, showed severe pathology in comparison to the other unchallenged animals. Most important, these animals demonstrated severe pathology in the apical lung, an observation frequently documented in human TB (Hunter, 2011). As mentioned previously none of the animals demonstrated any clinical symptoms. However, on one occasion approximately 32 weeks after challenge, pig 59 produced a sneeze with mucus. This mucus was collected and plated on 7H11 agar but did not produce viable CFU. Although this was a chance incident, it is likely this animal may have sneezed or coughed at other times when researchers were not

present; 2 researchers reported coughing in this same pig once before this incident. From the severe pathology observed at necropsy, **Figure 2.2,** it could be inferred this animal could have progressed to a more prominent active stage of disease if maintained in the study longer. This could have also been the case with pig 58 who showed similar pathology. These animals could have also been the hosts responsible for transmission in the unchallenged animals. Furthermore, **Figure 2.6D**, shows presence of Mtb bacilli after acid fast staining in the salivary gland of one of the unchallenged animals; supporting the incidence of transmission.

While a previous study suggested the mini-pig as a model for LTBI due to the pathological classifications found at 20 weeks post challenge with H37Rv (Gil et al., 2010), our model with an HN878 inoculum suggests mini-pigs could be used to model childhood TB as no symptoms were shown by any animals yet transmission seemed to have occurred between the challenged and unchallenged animals. Children are often undiagnosed because of their inability to show symptoms such as sputum production with cough. Sputum smear microscopy is the most common diagnostic method used for adults as they commonly produce cough with sputum. In addition, diagnostic methods such as bacteriological culture or WHO endorsed Xpert MTB/RIF are often unavailable in the majority of locations where TB often occurs (WHO, 2016). Further, Xpert MTB/RIF lacks sensitivity in detecting TB in children in comparison to culture confirmation or clinical diagnosis (WHO, 2016). Reasons for the lack of sensitivity of Xpert MTB/RIF in children are currently unknown due to insufficient studies in this age group (WHO, 2016)

Research in improved diagnostics in children is needed but has been underdeveloped as children have been perceived to carry a lower bacillary load than adults

and therefore thought to be less infectious (Seddon & Shingadia, 2014). WHO has recommended development of diagnostic methods to be affordable and used in low-resource settings as a priority for researchers and policy makers (WHO, 2016). While children may not always produce overt symptoms of active TB, if continued to be ignored, they will contribute to the TB epidemic as they are the reservoir out of which future cases will develop (Seddon & Shingadia, 2014). Most cases of TB in children occur in TB endemic countries, but due to diagnostic limitations the actual burden of childhood TB is unknown; in 2014 one million incident cases were believed to be in children (WHO, 2015c, 2016). If the mini-pig can transmit Mtb bacilli without visible symptoms, it has potential to be used as a new model for diagnostic development in children and further therapeutic and vaccine development. However, this model with an HN878 challenge should be further classified pathologically as done in Gil and colleagues study to determine whether there were differences in pathology caused by the laboratory strain H37Rv and the hyper virulent clnical strain HN878. Further, an optimal dose of Mtb inoculum or time frame should be developed in order to elucidate a more prominent active TB disease in these animals.

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Chapter 3: Neonatal piglet response to BCG

3.1 Introduction

In development of TB vaccines, a gap exists in the current knowledge of the T cell response caused by BCG, the only available vaccine offering protection against disseminated TB (A. P. Soares et al., 2013). Further, recent clinical trials of new vaccines to protect against pulmonary TB have proven ineffective (Tameris et al., 2013). Without information on the kinetics of T cells induced by BCG, in particular when this response peaks, improved progress in preventing TB will continue to be impeded. It has been suggested that the best time for boosting is after the peak effector phase, when effector T cells have established memory (A. P. Soares et al., 2013). Vaccination during the primary effector phase can lead to T-cell exhaustion or activation induced cell death (A. P. Soares et al., 2013). Another potential indicator to the lack of efficacy found in infants to new vaccines is the use of adult animal models in pre-clinical trials even though it is known there are immunological discrepancies between adults and infants (Marchant & Goldman, 2005). Studies in neonatal immunology and TB are few. Neonatal piglets with resemblance to infants may provide insight into the BCG induced response and further developed to test other vaccine candidates.

In this study, longitudinal response in T cells and monocytes was followed in neonatal piglets vaccinated at birth with BCG SSI and monitored from 4 weeks of age to 24 weeks through flow cytometry for phenotype and cytokine production; an ELISA was done for production of IL-2. A total of 10 piglets were used, with 5 vaccinated with BCG Statens Serum Institute (SSI) and 5 left unvaccinated. This experimental design purposefully

resembles the longitudinal study done by the South African Tuberculosis Vaccine Initiative (SATVI) on healthy infants vaccinated with BCG SSI 48 hours after birth in Cape Town, South Africa (A. P. Soares et al., 2013). 73 infants were enrolled and peripheral blood mononuclear cells (PBMCs) were collected for flow cytometry of the following surface phenotype and cytokine production: CD3, CD4, CD8, Ki67, Bcl-2, CD38, HLA-DR, CD45RA, CCR7, granulysin, granzyme B, perforin, TNF-α, IFNγ, IL-2, IL-17 (A. P. Soares et al., 2013). Our study is limited in the amount of phenotypes and cytokines we could measure, we chose markers indicative of the main activation of T cell and monocyte response. The results will be compared to the SATVI study mentioned above as well as other BCG studies in infants, in an effort to demonstrate the similarities between neonatal pigs and infants, and further develop pigs as a neonatal model for TB vaccine efficacy testing.

3.2 Materials and Methods

3.2a Animals

Two pregnant Sinclair Miniature Swine sows (Sinclair Bio-Resources, MO) were delivered to CSU at 2 and 3 weeks before expected birth delivery dates and kept under standard housing conditions (72 ± 6 degrees F, 30-70% humidity, 12:12 light cycle) in separate runs but with visual and tactile contact with each other. Sows had *ad libitum* water access and were fed 1000g/day of a locally sourced complete feed (Panepinto Show Feed, CO). Feed was increased to 1500g/day during last week of gestation and was provided *ad libitum* during lactation. Sows were monitored once daily up to the last week of gestation then were monitored 5 times a day for labor/farrowing. Neonates were examined, had umbilical cord stumps dipped in iodine, and were provided with a partitioned area containing supplemental heat sources and free choice milk replacer.

Needle teeth were trimmed at 1-2 days old, a dose of iron was administered at 1-2 days old and 10-12 days old, males were neutered under general anesthesia at 10-12 days old, and all piglets were weaned at 3-4 weeks old. Ten healthy neonatal piglets were used for the study; 5 piglets were vaccinated 48 hours after birth by intradermal injection with BCG Statens Serum Institute (SSI), provided by Dr. Angelo Izzo, with 0.05ml as recommended for children below 1 year of age, the other 5 served as non-vaccinated controls. Piglets were not vaccinated for any other diseases and were kept under barrier conditions to prevent exposure to natural swine infectious diseases. Piglets were bled at various time points as described below. At 4 months of age, the piglets were moved into an ABSL3 facility and challenged with Mtb HN878 as described in Chapter 2 at 5 months of age. Piglets were euthanized as described in Chapter 2 at 7 months of age.

3.2b Immunological study

Isolation, culture and antigenic stimulation of PBMC

Direct jugular venipuncture was used to collect 2 ml of blood into sodium heparin tubes (BD) from all animals at week 4, 6, 8, 10, 12, 20, 21 and 24 after birth. Blood samples were also collected 1 week and 4 weeks after aerosol challenge with Mtb HN878. Monitoring for adverse symptoms and positive reinforcement were provided to animals immediately after blood collection. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by density-gradient centrifugation with Lympholyte® Mammal Cell Separation Media (Cedarlane) according to manufacturer's instructions. Viability was assessed with Trypan blue (BD) and counted with Cellometer-mini (Nexcelcom). PBMC were cultivated in round-bottom 96-well plates in RPMI 1640 (ThermoScientific) medium supplemented with 10% fetal bovine serum, 1 M-HEPES buffer (Sigma), and 100mM

sodium pyruvate (Sigma) was used. PBMC from each animal was divided equally into aliquots (10^6 cells/ml) for 18 hour stimulation in the presence of complete RPMI media as negative control, 5ug/ml phytohemagglutinin (PHA) as positive control, and 10^6 CFU/ml BCG SSI in preparation for phenotypical and intracellular staining detailed below. All PBMC were incubated at 37 ^oC in 5% CO overnight. Supernatant from PBMC was collected, stored at -80 ^0C and used for IL-2 ELISA.

Flow Cytometry Panels

By use of eight color flow cytometry, four different panels were arranged to study surface markers and intracellular cytokine production. These panels were used for all time points and summarized in **Table 3.1**. All antibodies used were monoclonal. Unless specified all antibodies used were anti-pig and were found by referencing US Veterinary Immune Reagents Network Swine Reagents Update: Reagent Source, those which are anti-human have been tested for cross-reactivity (Gerner et al., 2015). The following three antibodies and three isotypes were used for all four panels: Alexa Fluor 700-conjugated anti-CD3 (IgG2a, clone BB23-8E6-8C8, custom-conjugation by BD Biosciences, San Diego, CA, USA), V450-conjugated anti-CD8α (IgG2a, clone 76-2-11, custom-conjugation by BD Biosciences), PerCP-Cy 5.5-conjugated anti-CD4 (IgG2b, clone 74-12-4, BD Biosciences), Alexa Fluor 700-conjugated mouse IgG2a isotype control for CD3 (clone MOPC-173, BD Biosciences), V450-conjugated mouse IgG2a isotype control for CD8 (clone MOPC-173, BD Biosciences), PerCP-Cy 5.5-conjugated mouse IgG2b isotype control for CD4 (clone 27-35, BD Biosciences).

For panel 1 the following three additional antibodies were used to measure cellular activation and determine T cell memory phenotype: PE-Cy7-conjugated anti-human CCR7

(IgG2a, clone 3D12, BD Biosciences), PE-conjugated anti-CD45RA (IgG1, clone MIL13, Thermo Fisher, Rockford, IL, USA), Allophycocyanin(APC)-conjugated SLA-DQ (IgG2a, clone TH16, generously provided by Dr. Joan Lunney, USDA). SLA-DQ was custom conjugated in-house according to manufacturer's instructions with Lightning-Link® APC conjugation kit (Innova Biosciences, Babraham, Cambridge, UK). Panel 1 also identified monocytes using FITC-conjugated anti-CD172 (IgG2b, clone 74-22-15A, BD Biosciences).

Panel 1 isotype controls contained the following: PE-Cy7-conjugated rat IgG2a isotype (clone R35-95, BD Biosciences) for CCR7, PE-conjugated mouse IgG1 isotype (Thermo Fisher) for CD45RA, APC-conjugated mouse IgG2a isotype (clone G155-178, BD Biosciences) for SLA-DQ and FITC-conjugated mouse IgG2b isotype (cloneMPC-11, BD Biosciences) for CD172.

Panel 2 used the following antibodies for intracellular cytokine detection: FITC-conjugated anti-human TNF-α (IgG1, clone Mab11, BD Biosciences), PE-conjugated IFN-γ (IgG1, clone P2G10, BD Biosciences), and Alexa Fluor 647-conjugated anti-human IL-17α (IgG1, clone SCPL1362, BD Biosciences).

Panel 2 isotype controls contained the following: FITC-conjugated mouse IgG1 isotype (clone MOPC-21, BD Biosciences) for TNF-α, PE-conjugated mouse IgG1 isotype (clone MOPC-21, BD Biosciences) for IFNγ, and Alexa Fluor 647-conjugated IgG1 isotype (clone MOPC-21, BD Biosciences) for IL-17 α.

Table 3.1 Monoclonal antibodies used to identify surface and intracellular markers

Filters in BD FACS CANTO II	**510/50 -502LP**	**450/50**	**530/30 -502LP**	**780/60-735LP**	**585/42-556LP**	**670LP-655LP**	**660/20**	**720/40-685 LP**
Panel 1	V510 Viability Dye	CD8-V450	CD172-FITC	CCR7-PECY7	CD45RA-PE	CD4-PerCP Cy5.5	SLADQ-APC	CD3-ALEXA700
Panel 1 isotype controls	V510 Viability Dye	CD8-V450	IgG2bκ--FITC	IgG2a κ-PECY7	IgG2-PE	CD4-PerCP Cy5.5	IgG1κ-APC	CD3-Alexa 700
Panel 2	V510 Viability Dye	CD8-V450	TNF-α-FITC		IFNγ-PE	CD4-PerCP Cy5.5	IL-17-ALEXA647	CD3-Alexa 700
Panel 2 isotype controls	V510 Viability Dye	CD8-V450	IgG2bκ--FITC		IgG2-PE	CD4-PerCP Cy5.5	IgG1κ-ALEXA647	CD3-Alexa 700

Surface staining

After 12 hour stimulation, PBMC needed for intracellular cytokine staining, panel 2 and isotype controls, were further stimulated with Brefeldin A (1ug/ml) for an additional 6 hours. PBMC needed for surface staining, panels 1 and 2 above, remained under stimulation during this time as well. Thereafter, PBMC were harvested and stained first with V510 Fixable Viability Dye (BD Biosciences) followed by incubation for 30 minutes at 4 ^{0}C with surface antibodies listed in each panel above. Finally, cells were washed in Stain Buffer (BD Biosciences) and fixed in 4% paraformaldehyde. Thereafter, cells were stained for intracellular cytokines and immediately read for analysis.

Intracellular cytokine staining

Following surface staining detailed above, cells designated for panels 3 and 4 were permeabilized with Perm/Wash (BD Biosciences) at 4 ^{0}C for 15 minutes. Thereafter, incubated with anti-cytokine antibodies and corresponding isotypes for 30 minutes at 4 ^{0}C.

Flow cytometry analysis

Samples were read using a FACSCANTO II flow cytometer (BD Biosciences) with data acquisition and analysis performed by FACSDiva (BD Biosciences) and Flowjo software (Flowjo, LLC, Ashland, OR, USA), respectively. Compensation was accomplished using single-stained samples. For each sample, 100,000 events were acquired. Gating strategies were used uniformly through all panels, representative gates are shown in **Figure 3.1**.

IL-2 ELISA

Supernatants from PBMC used in flow cytometry analysis were collected upon harvest. A sandwich ELISA was prepared to detect swine IL-2 in PBMC supernatant using Swine IL-2 Cytoset™ and antibody pair buffer kit (Thermo Fisher), following manufacturer's protocol. A positive IL-2 response was defined as 62 pg/ml.

Statistical analysis

Graph Pad Prism version 6 was used for data presentation. For flow cytometry statistical analysis, frequencies or median fluorescence intensity (MFI) of each phenotype from the BCG stimulated samples were used for a repeated measures ANOVA to compare the mean response of unvaccinated versus vaccinated animals. Statistical analysis of the mean differences was computed with R, version 3.2.3 using a repeated measures ANOVA model to account for multiple testing of each animal throughout the study. This analysis allowed for testing for significant differences between the vaccinated and unvaccinated animals at each time point. P values less than 0.05 were considered statistically significant. A similar approach was used for the IL-2 ELISA.

One pig from the vaccinated group was excluded in the statistical analysis of any phenotype with CD4+ T cells. This pig continuously demonstrated lack of production of CD4+ T cells according to the flow cytometry gates used and with PHA stimulation throughout all time points in comparison to the other 4 vaccinated pigs **Figure 3.2**. However, this pig remained healthy throughout the course of the study and even after Mtb challenge suggesting this pig had a genetic loss of the CD4 monoclonal antibody epitope not a loss of CD4+ T cells. A polymorphism in CD4 has been identified in pigs by failure of the monoclonal antibody clone 74-12-4 to react while pigs show no signs of clinical immunodeficiency (Sundt et al., 1992).

3.2c Mtb HN878 challenge

At 5 months of age, all miniature pigs were subjected to aerosol challenge with Mtb HN878 at a target dose of $1x10^3$ CFU/ml. Protocols for inoculum preparation, aerosol challenge, PPD test, post-mortem examination, bacterial load, Mtb PCR colony confirmation and histology were followed as described in chapter 2. Inoculum dose used was confirmed by plating an aliquot onto nutrient Middlebrook 7H11 agar plates and calculating bacterial numbers. The actual CFU count was 438. PPD was administered 60 days post challenge.

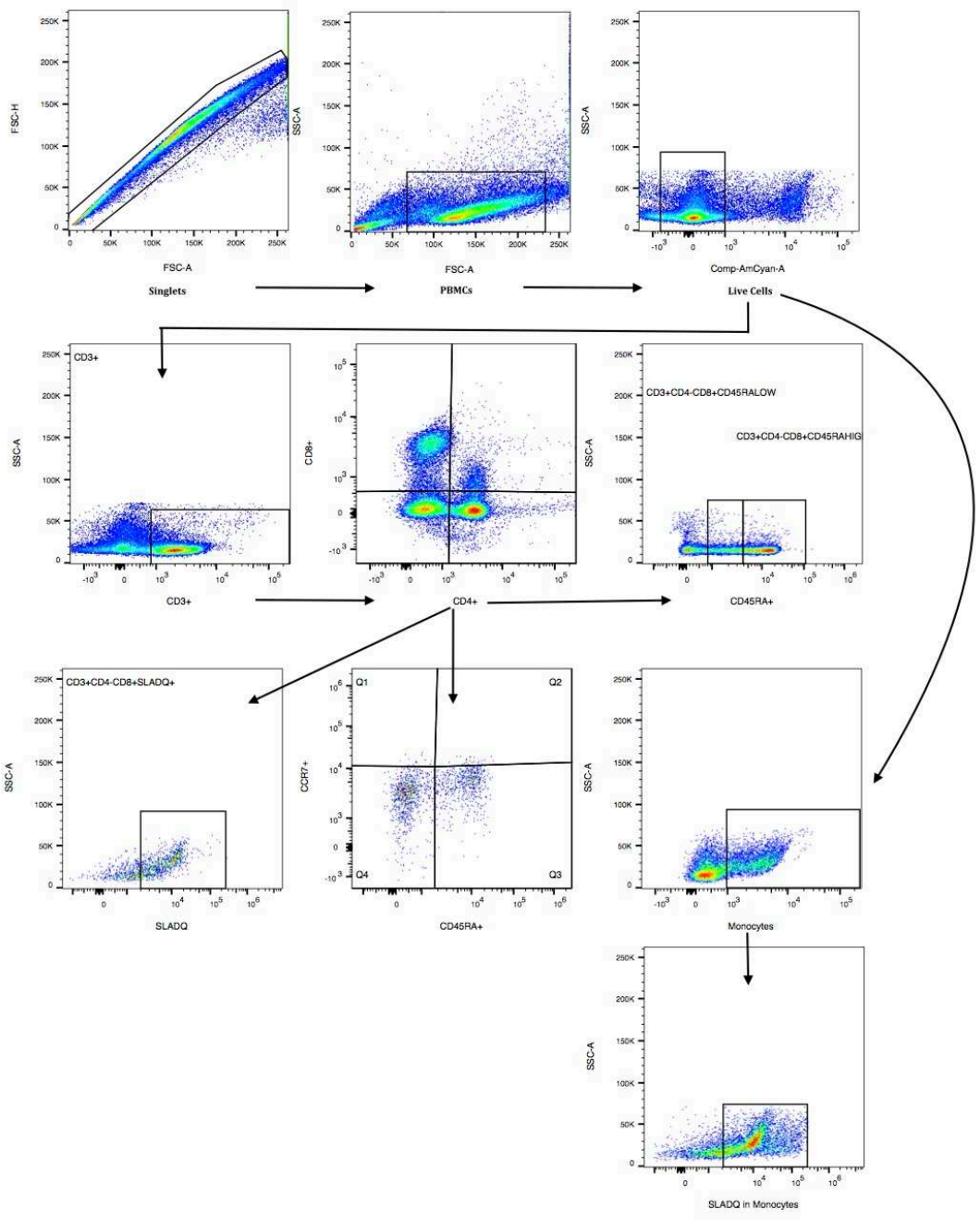

Figure 3.1 Gating strategy for the analysis of surface markers in panel 1, identifying T cell phenotypes and monocytes.

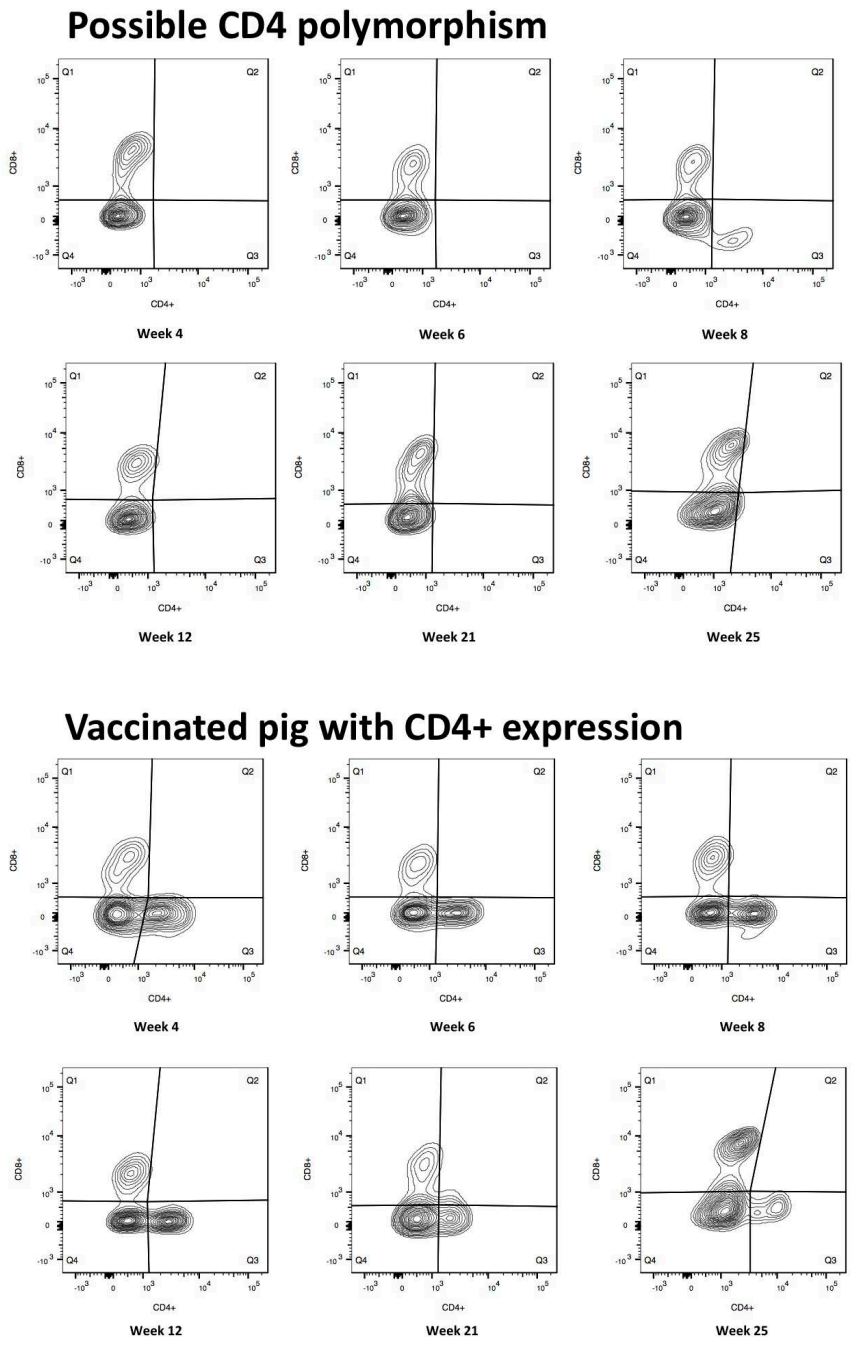

Figure 3.2 CD4+ vs CD8+ longitudinal comparison of vaccinated piglet 5 in comparison to a representative vaccinated piglet in response to PHA stimulation. Piglet 5 continuously demonstrated lack of CD4+ presence in flow analysis yet maintained a healthy state suggesting a polymorphism in the CD4 marker inhibiting the monoclonal antibody from recognizing it.

3.3 Results

3.3a Flow Cytometry

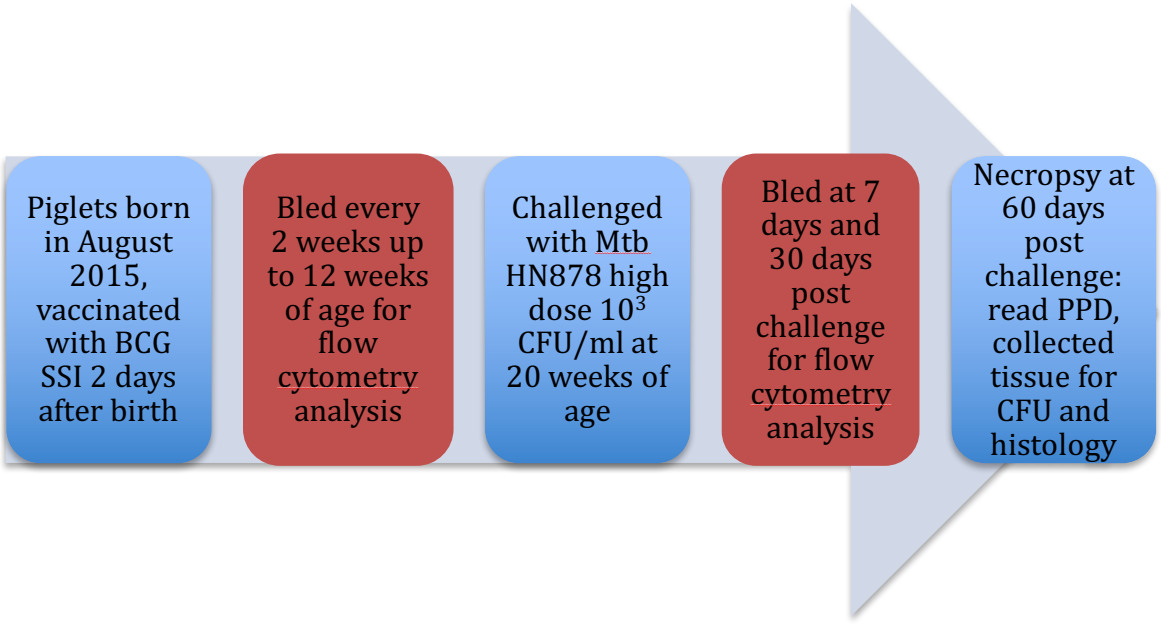

Figure 3.3 Study Timeline

Cell phenotypes and T cell kinetics

Longitudinal changes in the abundance of T cell subsets and monocyte cell populations of PBMC in response to BCG stimulation were measured first by analyzing changes in the percentage of positive cells for each subset **Figure 3.4**. As explained in materials and methods all PBMC cultures were normalized to 1x10 ^{6} ml. Thus, through flow cytometry analysis we were able to quantify the frequency of CD3+ T cells in response to BCG stimulation (CD3+CD4-CD8-) referred to as double negative cells, CD8+ T cells (CD3+CD4-CD8α+), CD4+ T Cells (CD3+CD4+CD8α-) and double positive T cells or (CD3+CD4+CD8α+) as well as monocytic cells defined by CD172 +, **Figure 3.11**. Double negative cells were most abundant through the course of the study. CD8α+ T-cells remained at a constant level until they peaked at 4 weeks (24 weeks of age) post challenge

while CD4+ T cells were higher at weeks 6, 8 and 12 but returned to similar levels observed at week 4 post challenge. Double positive cells defined as a subset of T helper cells with antigen experience in pig studies and interestingly also identified in NHPs (Talker et al., 2013), were the least abundant and were the only T cell phenotype to show a statistical significant difference between the unvaccinated and BCG vaccinated pigs. The abundance of monocytes in PBMCs measured as the percentage of CD172 positive cells remained at a constant expression through weeks 4-12. However, there was a slight decrease 1 week post Mtb challenge (Week 21)-in both groups. Interestingly, the monocyte population more than triple in abundance after 4 weeks of Mtb challenge.

The expression of cytokines IFNγ, TNF-α and IL-17 were also quantified in the four T cell populations, representative gates and frequency are shown in **Figure 3.5**. IFN-γ showed a peculiar constitutively high response, near 100%, from week 4-8 in all T cell populations with a decrease at week 12 and increase 1 week post challenge (21 weeks). Even more peculiar was the drastic drop of IFNγ, near or below 25%, in all T cell populations 4 weeks post challenge (24 weeks). Levels of TNF-α expressing cells remained at a constant range between 25-50% through all time points and T cell populations. Frequency of IL-17 remained at a constant level for both CD4+ and double negative T cells through all time points and nearly identical between the vaccinated and unvaccinated animals. For CD8+ T cells expressing IL-17, a decrease was observed in weeks 6 and 8 yet for weeks 12, 21 and 24 levels increased similar to levels observed at week 4. There were no statistical significant differences between the vaccinated and unvaccinated animals, yet a lower expression was found 4 weeks post Mtb challenge (24 weeks) in the unvaccinated animals in comparison to the vaccinated animals, however, both groups expressed less

than 2%. The T helper cells (CD4+CD8+) expressing IL-17 had the opposite trend as CD8+ T cells, with a higher expression from weeks 4-6 and a decrease for the remainder of the study.

Memory Phenotype

Our aim for these studies was to use the neonatal mini-pig as an animal model for vaccine efficacy in infants. Thus we chose to define memory phenotype of T cells as previously reported by the SATVI studies. For T cell populations we were able to further differentiate the memory phenotype through co-expression of CCR7 and CD45RA (B. M. Kagina et al., 2009). Four different memory phenotypes have been classified as naïve cells (CD45RA+CCR7+) **Figure 3.6**, central memory cells (CD45RA-CCR7+) **Figure 3.7**, effector cells (CD45RA-CCR7-) **Figure 3.8**, and effector memory cells (CD45RA+CCR7-) **Figure 3.9**. A low frequency of naïve cells was observed in all T cell phenotypes with CD8+ and double negative T cells presenting similar frequencies. CD4+ T cells and double positive T cells followed a similar trend with a high expression of CD45RA+CCR7+ at 4 weeks of age, a decrease at week 6 with constitutive low expression through week 21. Notably, all four T cell populations started with a higher frequency of naïve cells expressing CD45RA+CCR7+, with a decrease through week 6-21 and a slight increase at week 24. A similar trend was observed in central memory CD45RA-CCR7+ cells with CD4+ and double positive T cells being more alike and CD8+ and double negative T cells expressing lower levels and similar trend as in naïve cells. Focusing further on the double positive and CD4+ T helper cells for weeks 4 and 24, the double positive cells express a near two-fold statistically significant difference of CD45RA+CCR7+ in comparison to CD4+ T cells, **Figure 3.10.**

The amount of effector cells expressing CD45RA-CCR7-rose continuously from week 6 to 21 in CD4+, double positive, and double negative T cells with a slight decrease at week 24. While CD8+ T cells had the fewest amount of effector cells and maintained a constant expression through all time points. However, the frequency of effector memory cells, CD45RA+CCR7-, was highest in CD8+ T cells and maintained through all time points with a decrease observed post challenge. The CD4+ and double positive T cells again behaved similarly, starting with a high expression of effector memory cells at week 4 and a decreased expression thereafter. The double negative cells expressing memory remained at a frequency range of 40-60% with higher levels in the unvaccinated group, though no significant differences were found. Both naïve and central memory cells had a lower frequency, less than 20%, in comparison to effector and memory cell frequency. No significant differences were found between the unvaccinated and vaccinated pigs except for week 4 in the double positive and double negative T cell population of the central memory cells.

Activation

With SLADQ, a homolog of HLADQ, we were able to determine the activation response in all T cell populations studied as well as monocytes through median fluorescence intensity (MFI). In monocytes, upregulation of SLADQ gradually increased from week 4-12 with a peak 1 week post challenge **Figure 3.12**. As for T cells a peak in SLADQ is observed at 4 weeks post Mtb challenge with double positive T cells expressing the most. Both CD4+ and double negative T cells upregulated SLADQ at low levels, CD4+ T cells expressed higher and similar levels at week 4 and 24 while double negative T cells started with low levels and peaked at week 24, **Figure 3.13**.

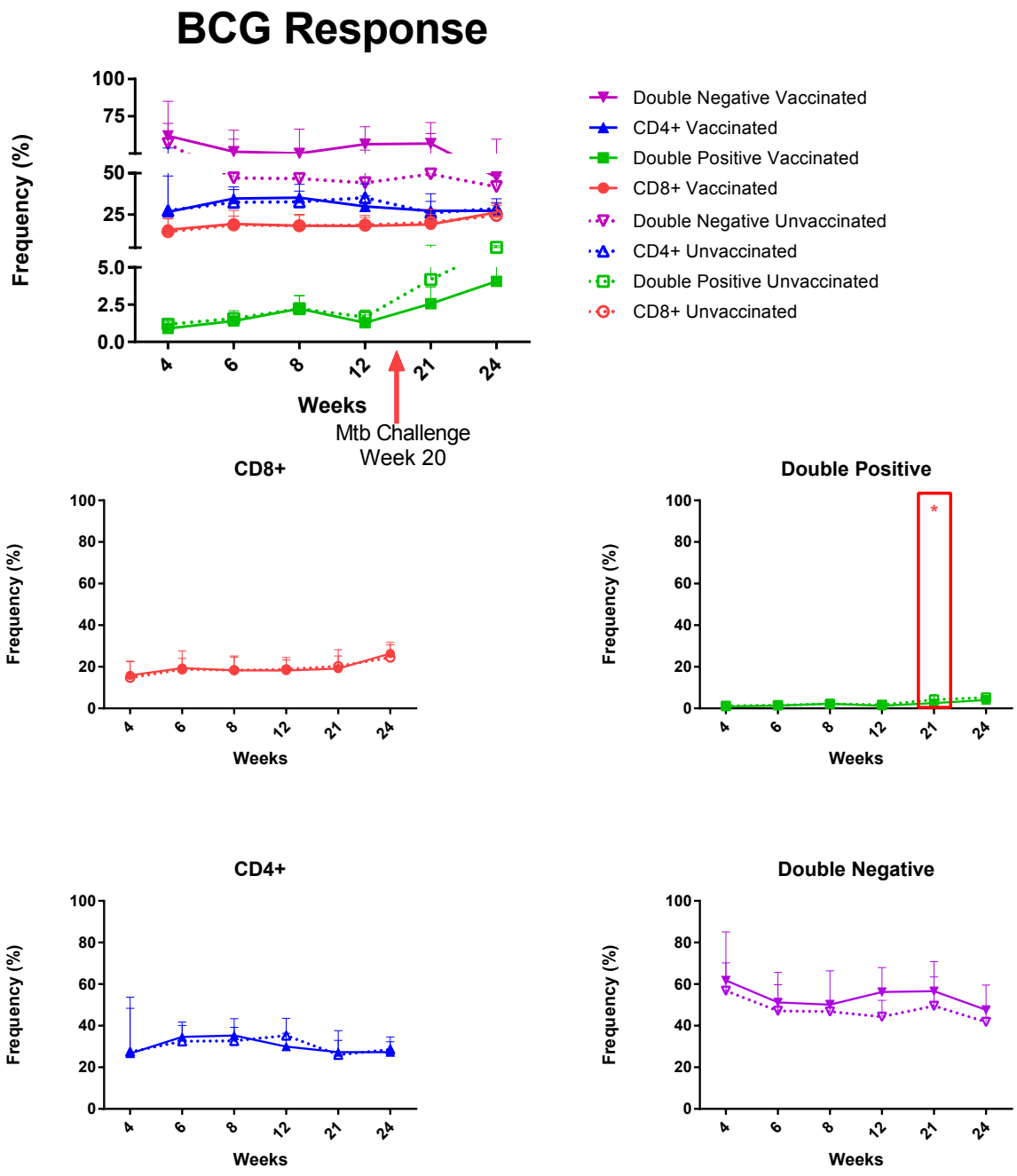

Figure 3.4 T cell phenotypes frequencies from parent population of CD3+ cells over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG.

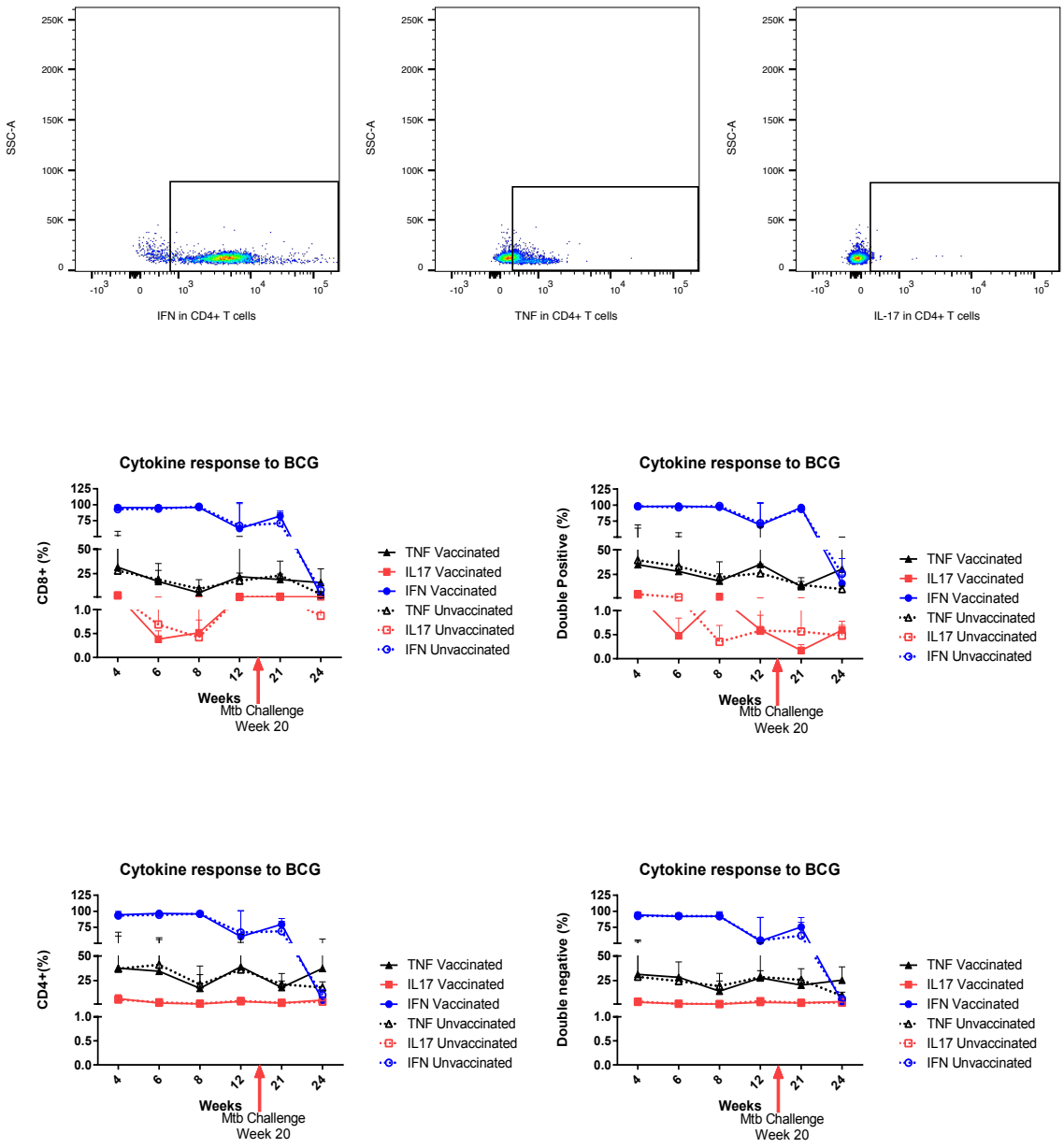

Figure 3.5 Frequencies of cytokines: IFNγ, TNF-α and IL-17 in the four T cell phenotypes cells over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG.

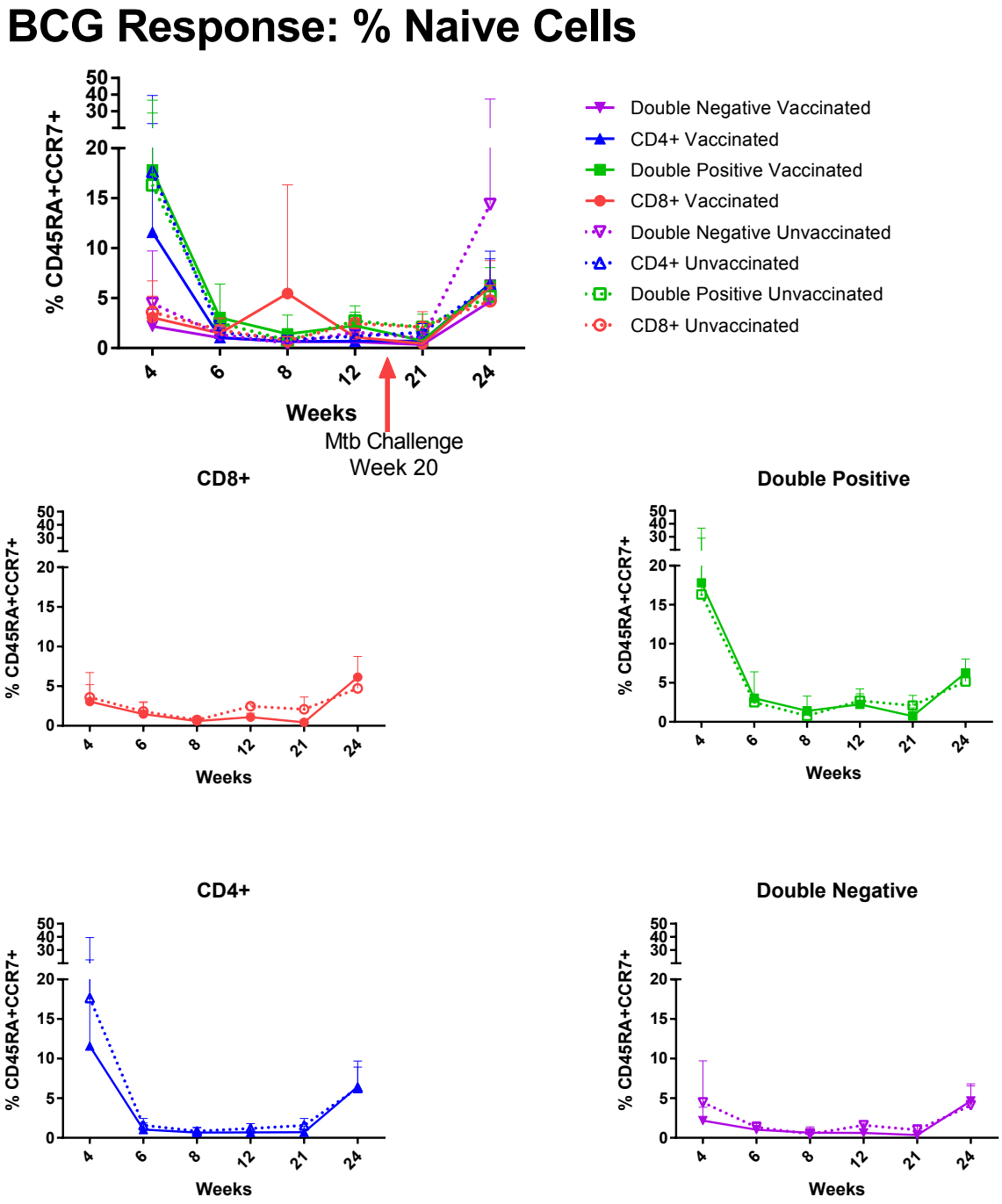

Figure 3.6 Frequency of naïve T cells over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG.

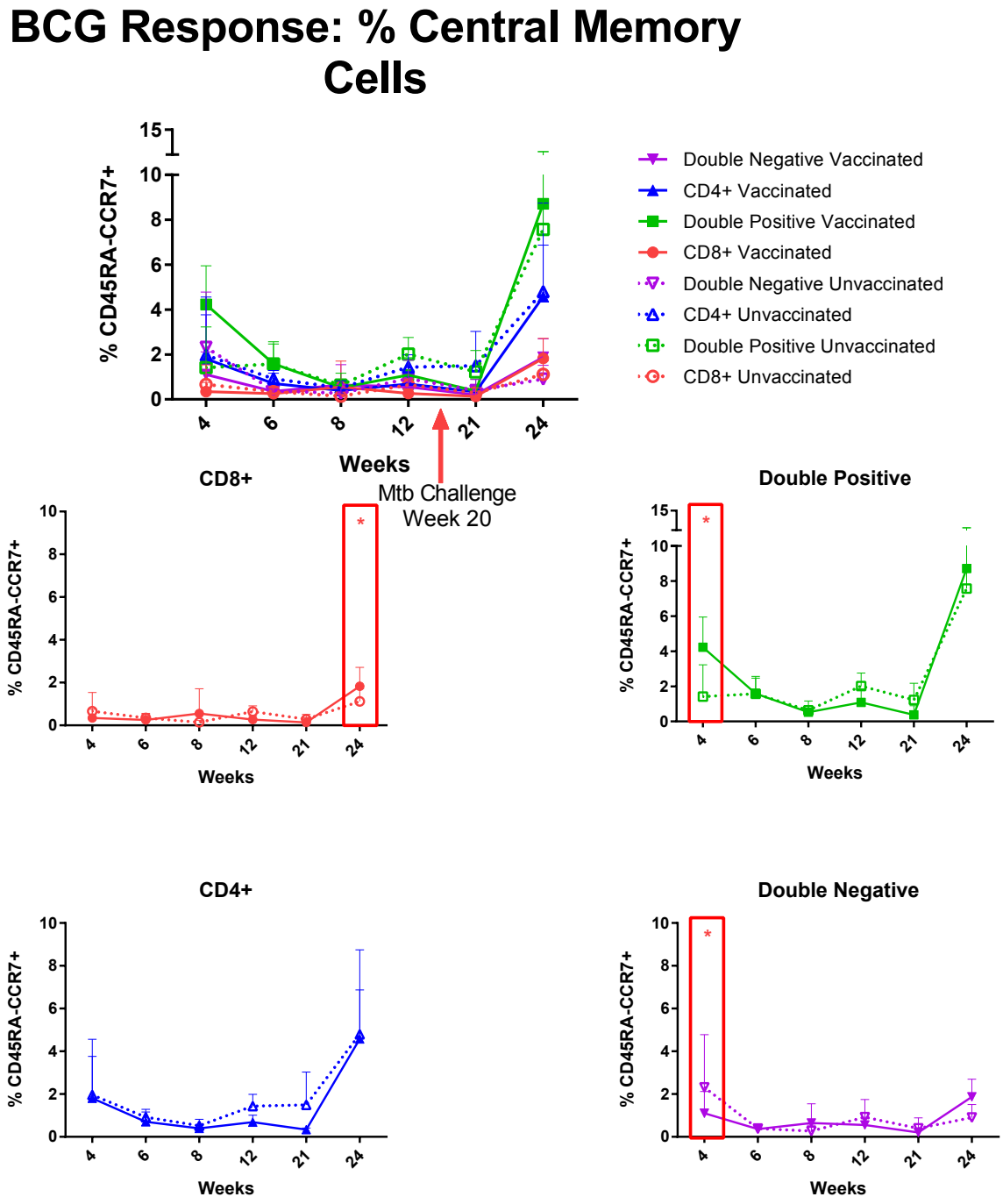

Figure 3.7 Frequency of central memory T cells over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG.

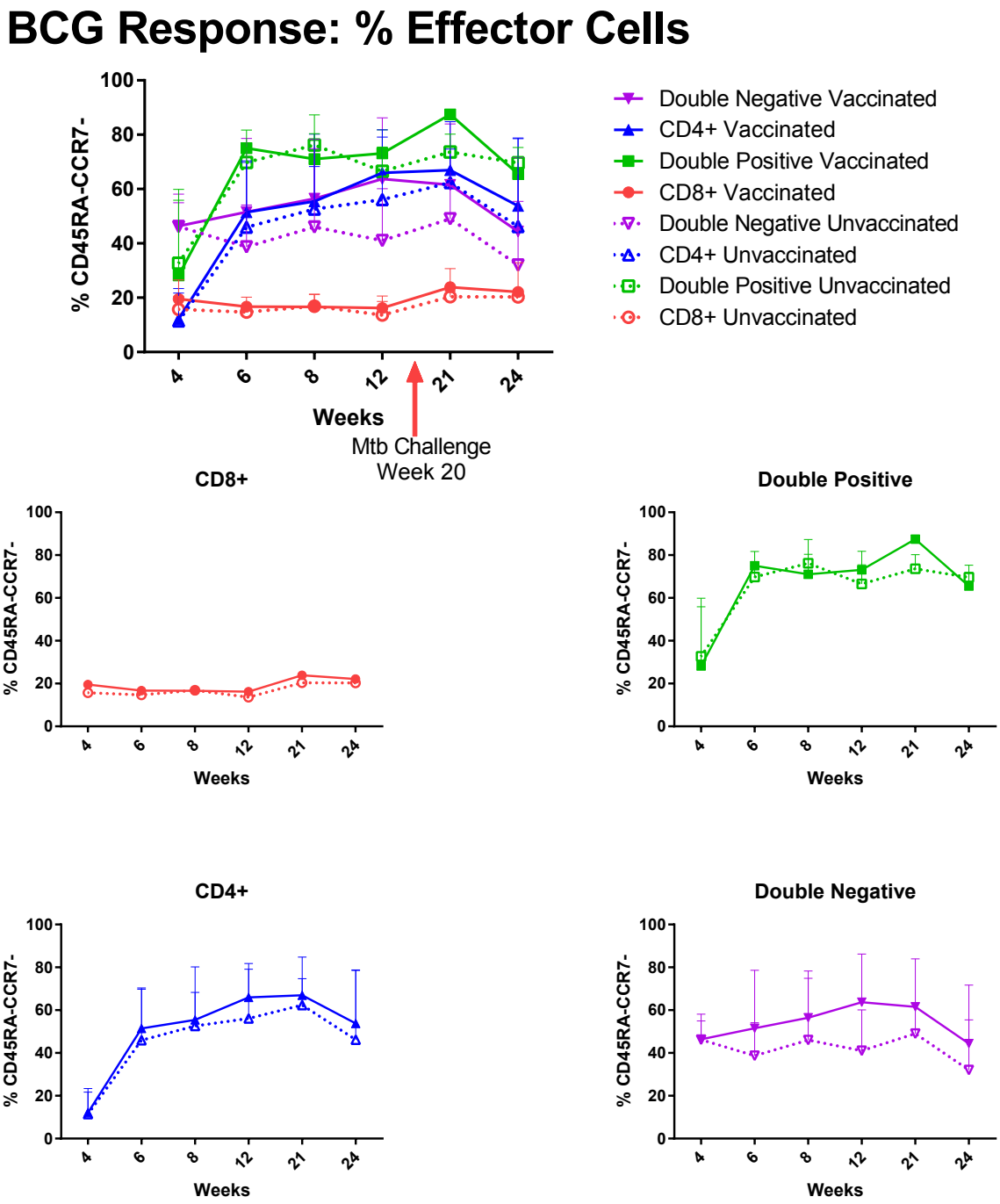

Figure 3.8 Frequency of effector T cells over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG.

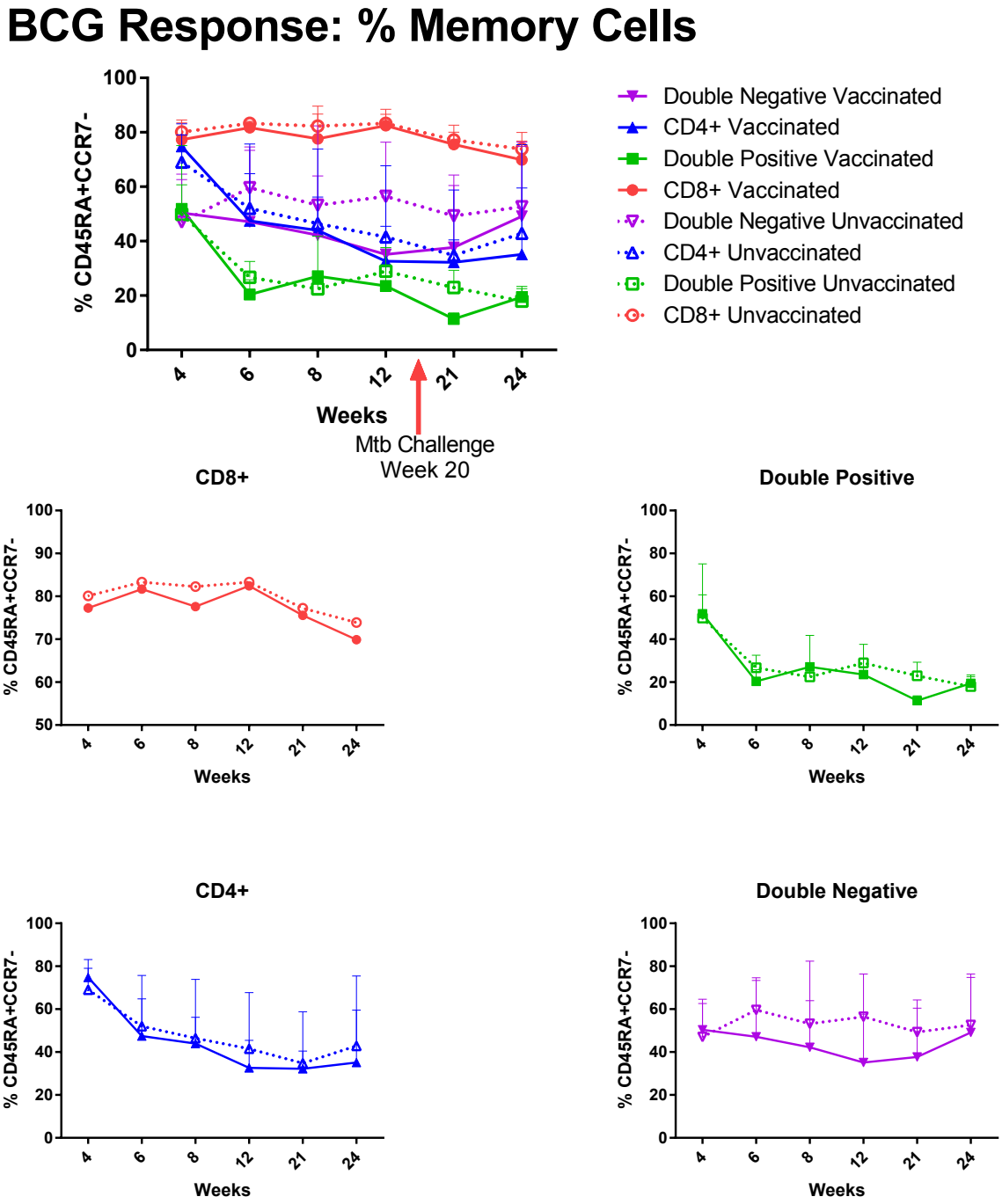

Figure 3.9 Frequency of memory T cells over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG.

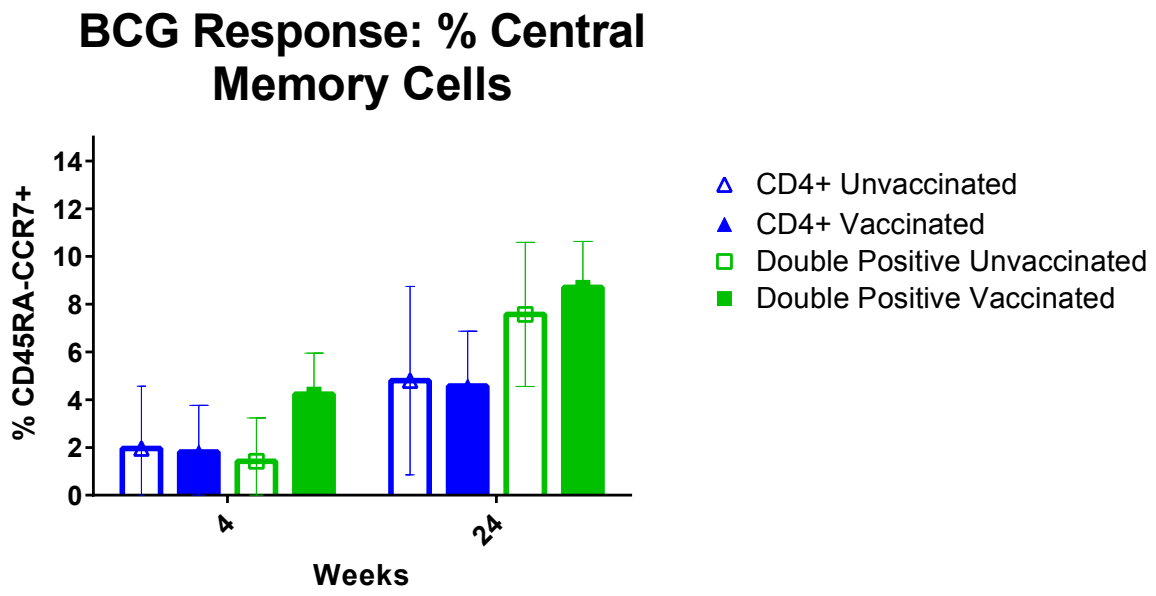

Figure 3.10 Comparison of CD4+ T cells to double positive T cells with a central memory phenotype 4 weeks after birth and 4 weeks after HN878 Mtb aerosol challenge.

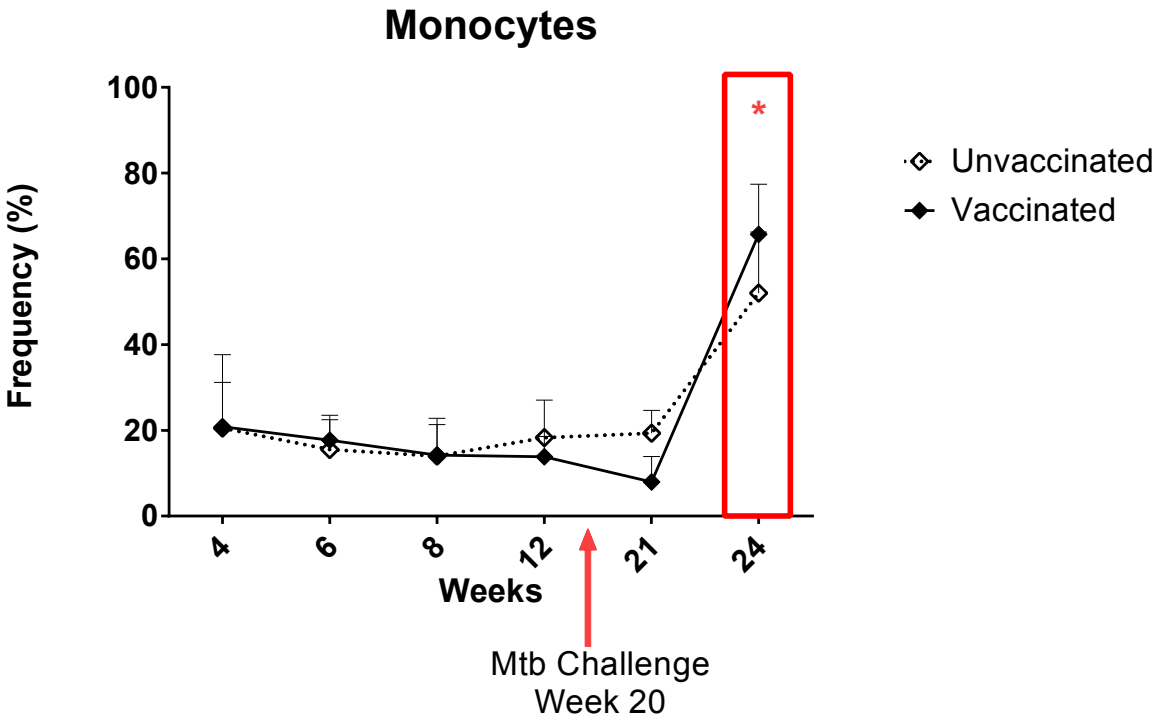

Figure 3.11 Frequency of monocytes over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG. A statistical significant difference was determined at week 24 in which vaccinated animals show a higher production of monocytes.

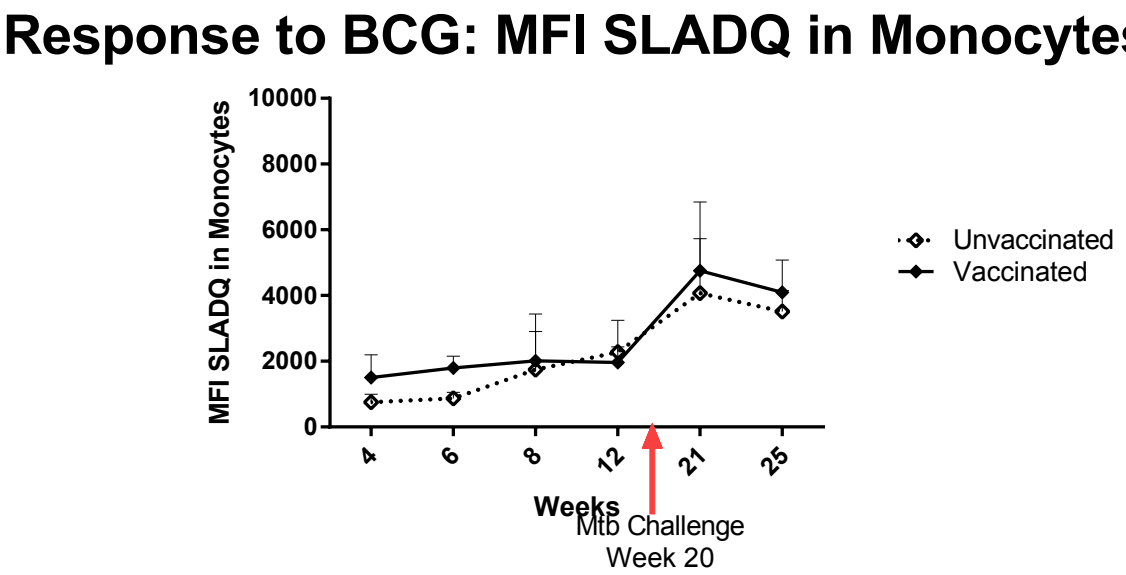

Figure 3.12 MFI of SLADQ upregulation in monocytes over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG.

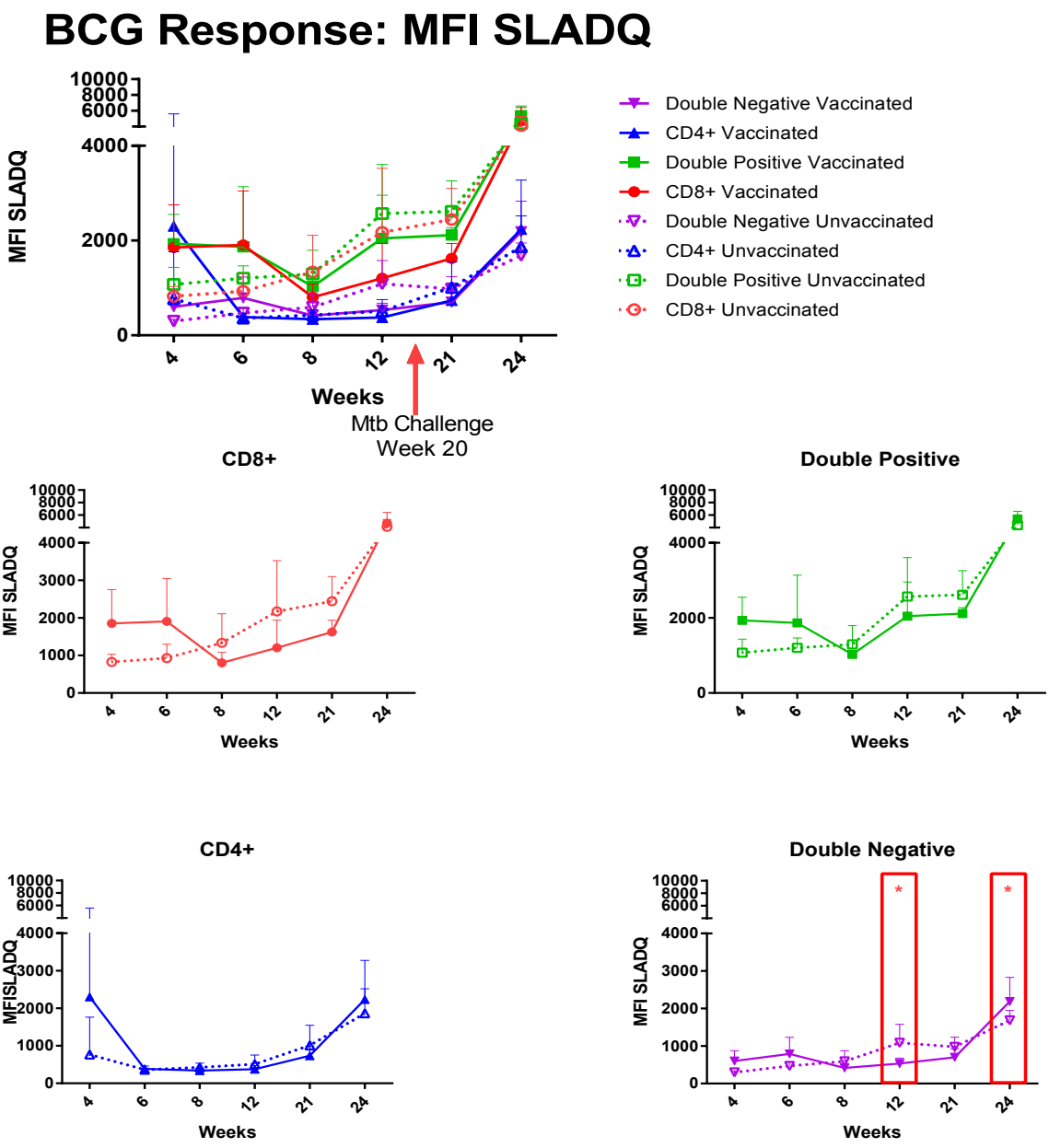

Figure 3.13 MFI of SLADQ upregulation in T cells over time in BCG vaccinated and unvaccinated piglets after in-vitro stimulation with BCG. Statistical significant differences were determined in double negative T cells at week 12 and 24.

3.3b IL-2 ELISA

An ELISA measuring IL-2 produced by PBMC supernatant after BCG stimulation was performed at each time point in accordance to the flow cytometry assay. **Figure 3.1**4 shows positive results above 62 pg/ml in both pig groups. The vaccinated pigs show a higher response yet both groups show a similar trend starting with high expression at week 4, a slight decrease at week 8 and a more drastic decrease 1 week post Mtb challenge. IL-2 levels appear to rise at 4 weeks post challenge but are still lower than the initial levels observed at week 4. No statistically significant differences were found between the pig groups.

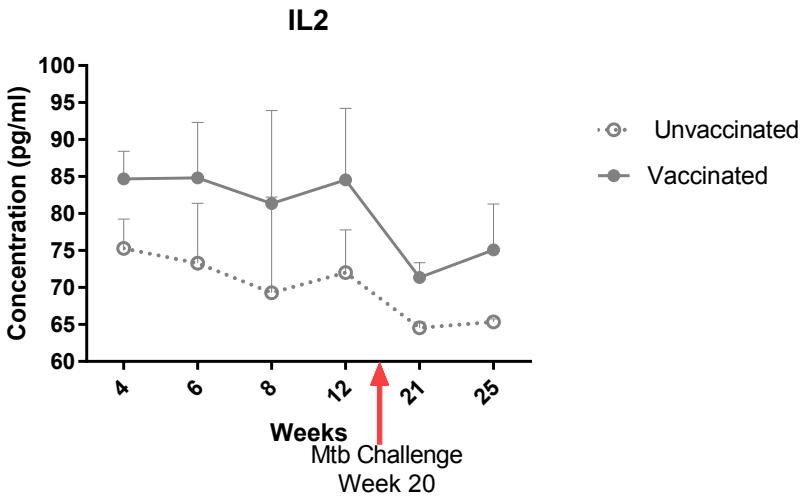

Figure 3.14 Longitudinal changes of IL-2 production from PBMCs of BCG vaccinated and unvaccinated piglets stimulated with BCG in vitro.

3.3c Mtb challenge

At 5 months of age (20 weeks) all 10 pigs were challenged with a dose, 10^3 CFU/ml, of Mtb HN878. Pigs were monitored for clinical symptoms 8 weeks after challenge and bled at 2 time points for PBMCs, week 21 and 24 for flow cytometry and ELISA assays. Similarly, to the previously challenged adult pigs and 2 month old unvaccinated pigs, this third group of pigs did not show any clinical symptoms. Further upon necropsy, gross pathology showed small and few lesions in all animals, **Table 3.2**, however none of these lesions produced viable bacilli on 7H11 agar. In addition, histology, revealed little indication of disease with inflammation of the parenchyma observed as the most severe pathological classification, **Figure 3.15B**. Furthermore, no differences were found between the unvaccinated and vaccinated pigs.

Table 3.2 Summary of gross pathological lesions observed in HN878 challenged adult pigs at time of necropsy comparing BCG vaccinated animals and unvaccinated

Pig ID (Vaccinated)	**Lung**	**Lymph Nodes(LN)**
1	Small lesions	No abnormalities noted
2	Small disseminated lesions in upper lobe	No abnormalities noted
3	1 small lesion in lower left lobe	1 lesion in mediastinal LN
4	No abnormalities noted	No abnormalities noted
5	Small disseminated lesions	No abnormalities noted
Pig ID (Unvaccinated)	**Lung**	**Lymph Nodes(LN)**
6	Small lesions in apical lobe only	No abnormalities noted
7	Small disseminated lesions	Hard lesions present in submandibular and mediastinal LN
8	1 lesion in top right lobe Lesions near bronchial tree	Hardened mediastinal and submandibular LN
9	Small lesions in apical lobe only	No abnormalities noted
10	Small lesions in apical lobe only	No abnormalities noted

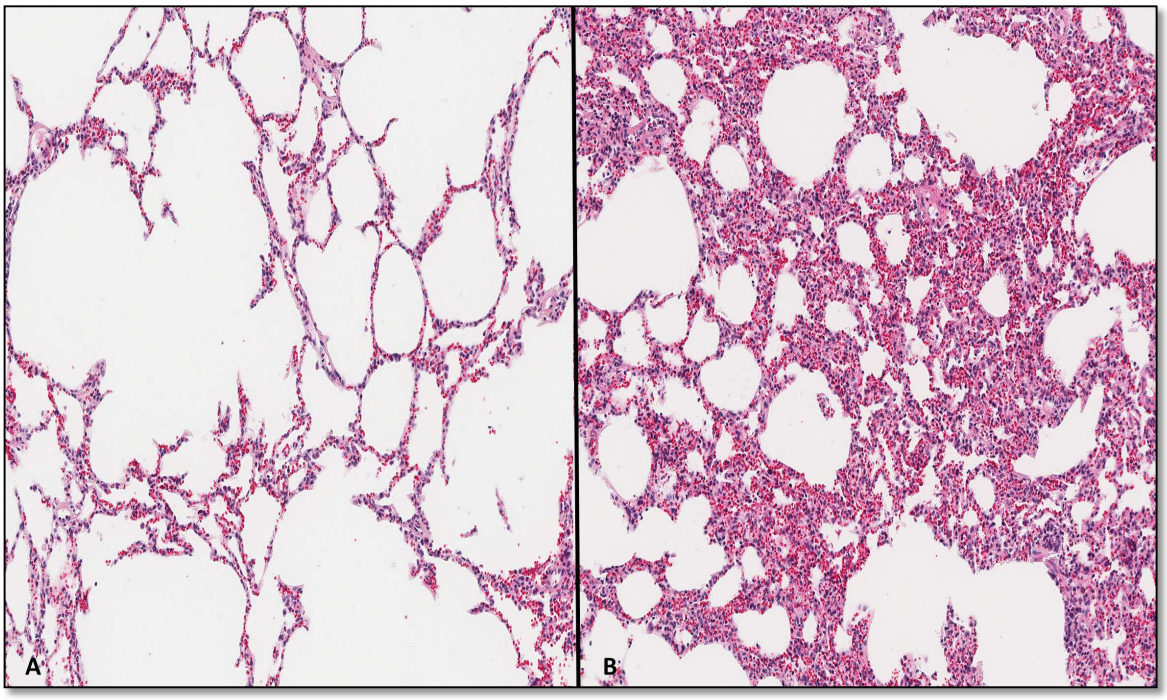

Figure 3.15 Representative H & E stained tissue 60 days post challenge of aerosol HN878 Mtb in pigs. (A) Healthy lung alveoli. (B) Inflammation of lung parenchyma.

3. 4 Conclusion

In this longitudinal study of neonatal piglets, we characterized T cells, cytokine profile, and monocytic activation in response to BCG stimulation from 4 weeks of age to 24 weeks. Our aim was to show neonatal piglets can have similar immunological responses to infants vaccinated with BCG whose immunological responses to in-vitro stimulation with BCG have been previously studied; comparisons of the piglets to infants are summarized in **Table 3.3.**

Notably, the predominant T-cell phenotypes were CD4+ effector T cells which included the T helper populations (CD3+ CD4+ and CD3+CD4+CD8α+). This finding is in agreement with three other studies measuring T cell kinetics to BCG in infants (B. M. Kagina et al., 2009; Andreia P. Soares et al., 2008; Tena-Coki et al., 2010). These 3 studies,

however, only looked at one time point and were found to be in disagreement to the longitudinal infant response to BCG study done in 2013 by Soares et al, which is the one we aimed to resemble in setting up our assays (A. P. Soares et al., 2013). In this longitudinal study, infants were found to have a peak response of CD4+ T cells 10 weeks after BCG vaccination with a predominant central memory cell phenotype with characteristics of effector memory cells from their cytokine expression(A. P. Soares et al., 2013). At the peak response these infant CD4+ T cells expressed high levels of IFNγ, TNF-α and IL-2 (A. P. Soares et al., 2013). From our flow cytometry assay, we can conclude that CD4+ T cells were predominantly effector cells and expressed high levels of IFN-γ with a decline 4 weeks post Mtb challenge and a steady level of TNF-α. The central memory cells found in the pigs were expressed at a low frequency, less than 10%, in comparison to effector cells ranging from 40-80% in the four T cell phenotypes. In the 2013 infant study, central memory cells are reported to range from 30-80% as well, however only two time points were compared in this study which may indicate why the results differ from previous BCG studies in infants (A. P. Soares et al., 2013)

A peculiarity observed in our study was the high constitutive expression of IFN-γ from weeks 4-12 and even one week post Mtb challenge but a sharp decline 4 weeks after challenge. It is expected for IFN-γ to be a marker of the magnitude of an inflammatory response (B. M. N. Kagina et al., 2010), therefore it is unusual to observe high levels before the pigs were challenged with Mtb and low levels after challenge. High expression of IFN-γ was also observed in 2013 longitudinal infant study of PBMCs at an early age with a peak at 10 weeks of age and decrease thereafter. In infants, it is possible to explain high levels of IFN-γ due to continuous exposure to Mtb found in TB endemic countries. However, our pigs

were not exposed to any pathological antigens and showed similar high frequencies to infants, therefore, there is reason to believe this high expression may not be due to environment alone. In addition, the piglets in our study demonstrated a drastic decline in the percentage of cells expressing IFN-γ 4 weeks post challenge while the infants showed a decline starting at 10 weeks of age (A. P. Soares et al., 2013). Further, the high levels of IFN-γ should have induced higher frequencies of monocytes and upregulation of SLADQ early on in the piglets. However, frequency of monocytes did not increase until four weeks post challenge **Figure 3.11** and SLADQ was not upregulated until one week post challenge **Figure 3.12.** Recent neonatal infant studies have proposed an impaired ability of monocytes to respond adequately from TLR stimulation(Kollmann et al., 2009). Human infants have a limited exposure to antigens in utero to stimulate adaptive immunity and therefore rely on their innate immune system to protect against infections (PrabhuDas et al., 2011). The immaturity of innate cells present in infants decreases the capacity of MHC class II antigen presentation and subsequent stimulation of antigen specific T-cell memory (Dowling & Levy, 2014). The same naïve response in piglets could account for the reason monocytes in the piglets studied here did not have high frequencies nor upregulated SLADQ in the presence of high IFN-γ producing cells at an early age (Butler et al., 2009; M. Sinkora & Butler, 2009).

It is important to remember that our study only measured expression of cytokines and immune cell phenotypes in the peripheral blood. We speculate the lower frequency of IFN-γ positive cells after challenge was due to a recruitment of these cells to the site of infection. Future pig studies could be expanded to monitor cytokine expression and immune cell phenotypes at the site of infection such as lungs and lymph nodes; this is an

added benefit in using neonatal pigs as tissue availability in infants is not possible. Further studies to assess IFN-γ response in PMBC at a neonatal age could provide information to the role this cytokine plays at this time. IFN-γ is the current indicator used for vaccine efficacy though it has been suggested that measuring this cytokine alone could be misleading due to the complexity of memory response, the short time periods tested and lack of studies in different T cell subsets (Henao-Tamayo et al., 2010; B. M. Kagina et al., 2009).

For expression of TNF-α in infants, the longitudinal study appeared to have steady states ranging from 50-60% in CD4+ T cells from week 6-40 with a decrease at 1 year of age (A. P. Soares et al., 2013). IL-17 was less frequent in both infants and piglets. The longitudinal infant study was able to incorporate IL-2 into their flow cytometry assay and when compared to IFNγ, it was observed to have the opposite expression with IFN-γ being highly expressed; this observation made the authors conclude a low expression of IL-2 was not typical of effector cells (A. P. Soares et al., 2013). Our study was limited and therefore IL-2 was measured from PBMC supernatant and found to maintain a steady expression through most time points. Without further knowledge of which cells were producing IL-2, we can only conclude that general expression of IL-2 stimulated T-cell proliferation. Comparing the upregulation of SLADQ in CD4+ T cells, the 2013 infant study only measured two time points and showed a decrease while the six time points measured in piglets show a higher upregulation overall.

There were three main benefits to our study not possible to observe in the longitudinal infant study. For one, we were able to study CD8+, double positive T helper, and double negative T cells longitudinally. Second, we were able to compare vaccinated

animals to unvaccinated control animals. Third, we were able to challenge all pigs with a highly virulent clinical strain of Mtb and determine whether vaccination with BCG made a difference in disease outcome. The second and third points would be unethical to study in infants, nonetheless, in TB endemic countries.

Data for the BCG CD8+ T cell response exists but is limited in infants. Most studies report undetectable or low frequency of IFNγ, TNF-α and IL-2 cytokine production by these cells (B. M. Kagina et al., 2009; B. M. N. Kagina et al., 2010; Andreia P. Soares et al., 2008). However, two studies report CD8+ T cells with predominant effector and memory phenotype supportive of the results observed in pigs (Andreia P. Soares et al., 2008; Tena-Coki et al., 2010). The use of the CD8α monoclonal antibody was able to detect both αβ and γδ T cells in our study, thus further characterization of CD8+ phenotype is necessary. Lee and colleagues have already demonstrated the enhanced ability of γδ T cells to produce IFN-γ in BCG vaccinated pigs (Lee et al., 2004). Generally, αβ T cells are considered the most important T cell subtype in response to TB, however, with Lee's results and our observations of double negative T cells which contain γδ T cells it is clear this phenotype plays an important role in mediating an immune response (Lee et al., 2004).

From an extensive search, no studies appear on double negative T cells or double positive T helper cells in infant response to BCG. Inclusion of the CD8α monoclonal antibody along with CD4 in our flow cytometry panels allowed the study of the double positive T helper cell population not included in any of the BCG infant studies referenced here. Our study suggests this population may have a role in establishment of central memory T cells with BCG antigen early on after vaccination and the T helper cell population with the highest upregulation 4 weeks post challenge, **Figure 3.7**. In addition, these were

the most prominent T cells demonstrating an effector phenotype, **Figure 3.8**. It is generally believed that co-expression of CD4 and CD8 only occurs transiently in the thymus before negative selection in which T cells are committed to single expression of CD4 or CD8 (Koch & Radtke, 2011). However, both pigs and NHPs have shown large populations of these double positive T cells in peripheral lymphoid tissues and blood. This population of T cells was first described in humans in 1986(Blue, Daley, Levine, Craig, & Schlossman, 1986). Double positive T cells have the capability of expressing memory markers and maintain co-expression of CD4 and CD8 for one year in culture (Overgaard, Jung, Steptoe, & Wells, 2015). Further, these cells are proven to be thymically derived as they express CD8$\alpha\beta$ (Overgaard et al., 2015). They have been found to increase with age in both humans and NHPs yet their function and development is largely unknown and conflicting studies exist (Overgaard et al., 2015). In pigs, these double positive cells have also been described and defined as T helper cells(Gerner et al., 2015; Saalmuller, Reddehase, Buhring, Jonjic, & Koszinowski, 1987). Further, ontogenic studies have shown this T cell population continuously expands in the blood from birth to adulthood and proliferate with antigenic stimulation (Gerner et al., 2015).

These different T cell populations may be important in determining whether frequencies of specific cells, patterns of cytokine expression and memory phenotype may be important for long-term protection against TB (B. M. N. Kagina et al., 2010). The few statistical differences found between the BCG vaccinated and unvaccinated pigs support the theory that BCG does not confer reliable protection against Mtb and additional factors play a role in its variable efficacy. Furthermore, the two pig groups were more similar than different and could be used in the future to study general neonatal immunology not just to

BCG response. These similarities were observed again after Mtb challenge, in which TB-compatible lesions were found to be homogeneous through all 10 pigs, none of the animals produced viable bacilli or demonstrated clinical TB symptoms. A difference may have been observed if the animals were kept with the infection for a longer period of time such as for 9 months as the adult pigs were.

Overall, our results support previous infant BCG studies with the observation of CD4+ and CD8+ effector T cells. Additionally, it provides the study of populations, which may play a role in the BCG response and currently ignored. Importantly, it demonstrates pigs are capable of producing similar immune cell phenotypes to infants and thus should be considered as an important animal model in future TB vaccine studies.

Table 3.3 Comparison of results from previous studies in infants vaccinated with BCG and mini-pig results

T cell phenotypes and cytokine profiles in response to BCG in-vitro stimulation	**Mini-pigs**	**Infants**	**Reference**
Predominant CD4+ T cell memory phenotype	effector T cells	effector T cells	(B. M. Kagina et al., 2009; Andreia P. Soares et al., 2008; Tena-Coki et al., 2010)
Predominant cytokines expressed by CD4+ T cells	IFNγ, TNF-α	IFNγ, TNF-α	(A. P. Soares et al., 2013)
Frequency of central memory cells	10%	30-80%	(A. P. Soares et al., 2013)
Upregulation of SLADQ in CD4+ T cells	Increased over 6 time points measured	Decreased over 2 time points measured	(A. P. Soares et al., 2013)
Predominant CD8+ T cell memory phenotype	Memory and effector T cells	Memory and effector T cells	Andreia P. Soares et al., 2008; Tena-Coki et al., 2010)

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Chapter 4: Concluding Remarks

The overall goal of this project was to demonstrate the capabilities of pigs as a model for TB vaccine development with a focus on neonatal immunology. In order to demonstrate its potential for vaccine development we first established Mtb infection in adult pigs and later in two groups of younger pigs. As reported, in the adult pigs TB-compatible lesions were abundant and of heterogeneous nature similar to human TB lesions. The two month old pigs and the pigs used to monitor T cell and monocyte kinetics in response to BCG demonstrated fewer TB-compatible lesions with more homogeneity but were also only monitored for two months post challenge as opposed to nine months in the adults. In all three groups of pigs, none of the animals showed clinical symptoms indicating active TB except for the one time reported in chapter 2. However, the TB-compatible lesions seen in the unchallenged animals as well as some culturable CFUs suggest transmission from the challenged animals. These results support the continuous use of pigs for future studies in the TB field. Since these animals did not demonstrate any symptoms yet showed transmission of Mtb bacilli they can be further studied as a model for TB disease in children. Further, new diagnostic techniques for children could be studied in pigs, since children do not typically show prominent TB symptoms as adults do thus making it difficult to diagnose them with the current available techniques. In addition, establishing Mtb infection with a highly virulent clinical strain in pigs was an important accomplishment as later vaccine efficacy or new therapeutics could be tested against a similar Mtb challenge.

More importantly, a gap in the knowledge of the immunological kinetics induced by BCG continues to exist almost 100 years since the first doses of BCG were administered. Pigs have a full range of innate and adaptive immune effectors and with the wide availability of pig specific reagents along with more than an 80% similarity in immunity to humans makes for a model with great potential to study the immunological response induced by BCG (Meurens et al., 2012). Similar to infants, piglets are immunocompetent at birth but immunologically immature (Meurens et al., 2012). By use of flow cytometry, we were able to characterize the neonatal immune response induced by BCG longitudinally in piglets and compare it to recent studies in infants living in TB endemic countries. Our findings were similar to BCG vaccinated infant studies with a predominance of effector cells, supporting the use of neonatal piglets for further use in TB vaccine development. In addition, we were able to monitor unvaccinated pigs and compare them to BCG vaccinated pigs. Although, few significant differences between the groups existed, this could be further studied to determine the lack of efficacy BCG has on pulmonary TB as well as lack of protection from the recent phase IIb MVA85A clinical trial or the neonatal immune response overall.

It is important to highlight the benefits of neonatal piglets for potential TB studies in the future. Lee and colleagues have already confirmed pigs are an appropriate animal model to study cell-mediated immune responses to BCG setting up a platform for our more in-depth longitudinal study (Lee et al., 2004). In order to better understand the neonatal adaptive immune response, it is valuable to separate environmental factors from intrinsic (Butler et al., 2009). The piglet has better potential in separating these concerns in comparison to rodents or NHPs as neither the environmental nor maternal factors, such as

transmission of maternal immunoregulators in utero or rearing after birth without their mothers, can be controlled in these animals (Butler et al., 2009). Pigs develop precosial offspring and only acquire protective antibodies from colostrum, thus can be reared after birth in various ways depending on a study's interest (Butler et al., 2009). In germ-free (GF) isolators, studies can control the piglets exposure to food, commensal organisms, pathogens, maternal antibodies and other maternal regulatory factors (M. Sinkora & Butler, 2016). In addition, the 114 day gestation period of pigs provides ample time to study the lymphoid system development of fetal pigs without interference from passive maternal antibodies and other maternal regulatory factors (M. Sinkora & Butler, 2016). For the purpose of our study, we did not investigate fetal development and allowed the neonatal piglets to nurse from their mothers' until 3-4 weeks of age. For improved establishment of pigs as an animal model in TB it would be important to find an optimal dose of Mtb causing more prominent active TB symptoms. It would also be beneficial to study the immunological response sooner than 4 weeks of age as done here. The current regimen of BCG vaccine administration is done 48 hours after birth in most countries, studies have suggested delaying vaccination after 10 weeks of age would be more optimal (B. M. Kagina et al., 2009; A. P. Soares et al., 2013), however little to no data exists on the immunological state in newborns 48 hours after birth; neonatal pigs could provide insight into this early response without the ethical concern of studying infants at such a young age. Since taking extensive samples from human subjects is logistically and ethically challenging, the pig offers comparative similarities with infants and can be used as subjects allowing extensive sampling for long periods of time (Lee et al., 2004). The current use of murine models do not translate directly to humans and thus mandate use of intermediate animals before

human clinical trials; pigs have been proven to be useful in modeling the human immune system and thus have the potential to skip intermediate animal models (Boeker, Pabst, & Rothkotter, 1999). Overall, with the establishment of Mtb infection, wide availability of pig specific reagents, ease of animal handling and capability of study manipulation, the piglet is an important model for TB and needs to be incorporated in future vaccine efficacy studies, diagnostics and improved therapeutics.

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