THESIS

THE EVALUATION OF CLINICAL ISOLATES OF MYCOBACTERIUM TUBERCULOSIS IN THE MURINE AND GUINEA PIG INFECTION MODELS

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2011

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ABSTRACT

THE EVALUATION OF CLINICAL ISOLATES OF MYCOBACTERIUM TUBERCULOSIS IN THE MURINE AND GUINEA PIG INFECTION MODELS

Globally the tuberculosis epidemic continues unabated, affecting over nine million people a year, with more than half a million of these cases being resistant to multiple drugs. Multiple drug resistant tuberculosis (MDR-TB) is becoming a growing problem to the world's population. Despite this growing problem, very little research is being focused on MDR-TB. One basic question not yet addressed is how drug resistance affects virulence levels.

A hypothesis, originating from classical studies of Mitchison, is that drug resistance results in a lower virulence level. Using the murine and guinea pig models of infection, I studied the ability of multiple isogenic pairs of *Mycobacterium tuberculosis* to grow in these particular animal models, in order to determine if acquired drug resistance increased or decreased the virulence of the drug resistant strain. In the murine model there was no discernable relationship between the drug resistance of a given strain and its virulence. Instead, isogenic drug resistant strains exhibited a range of virulence. Interestingly, the opposite was seen in the guinea pig infection model. In this model, it was observed that the drug resistant strain of the isogenic pair caused less severe disease and pathology.

Drug resistance is not the only cause for concern in the ever continuing tuberculosis epidemic. Many strains that are associated with outbreaks around the world are being classified

as either high or low transmission strains. High transmission strains are thought to be associated with increased rates of infections and higher virulence, the latter driving the former. Low transmission strains are the opposite; while they have been known to cause disease the numbers of cases where these strains have been identified appear to be fewer. I examined the virulence and pathogenicity of two strains selected for apparent high versus low transmission patterns, recently seen in a tuberculosis outbreak within the Chinese community of San Francisco, CA, USA that were typed as being W – Beijing strains. My studies did not support the hypothesis that high transmission strains have a higher virulence level.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Ian Orme for granting me the incredible opportunity to be a graduate student in his laboratory and the many wonderful opportunities this gave me through my time in his laboratory. I would also like to thank my other committee members, Dr. Anne Lenaerts and Dr. Robert Callan, for their willingness to serve on my committee and their many contributions to my training as a student.

I am appreciative of the help and guidance given to me by so many individuals on different aspects of this project. I would like to extend my appreciation to the members of the Lenearts' laboratory, especially Veronica Gruppo, and Lisa Woolhiser for your guidance and patience with the many murine model questions that I had. I could not have done any of the work for this project without the help of members of the Orme laboratory, including the student work studies and Jenny Harding. I would really like to thank Crystal Shanley for all her expertise and help throughout my work. I thank Dr. Colleen Duncan for helping me interpret murine histology. I need to also give my thanks to Dr.K. Midori and Dr.Burgoss for the use of their clinical strains of Mycobacterium tuberculosis.

I would like to thank the staff at the Fort Collins Museum and Discovery Science Center for allowing me the opportunity to participate in enriching the public's knowledge of science and the enjoyment to be had while learning something new.

I also thank my loved ones, including my family, friends, and pets for their patience, love and support throughout the years which has made all my past and future endeavors possible.

This research was supported by NIH grant U01 AI070456

DEDICATION

I would like to dedicate my thesis to my crazy yellow dog Daisy for all the times you reduced my stress levels and kept me sane. I would also like to dedicate my work to my family and friends for all your love and support during the past years of research, writing, and frustrations. I would not have gotten through this process without all of you behind me. Many thanks.

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Chapter 1 - Literature Review

Mycobacterium tuberculosis and Drug Resistance

Mycobacterium tuberculosis is the leading cause of death in the world from a bacterial infectious disease. There are over nine million new cases each year, with approximately one third of the world's population harboring latent tuberculosis (TB). TB has a high mortality rate compared to non-bacterial infections worldwide, with AIDS being the only other disease every year that kills more people than TB. According to the Center for Disease Control and Prevention (CDC) in developed countries, such as the United States, there is a decline in the number of reported cases of tuberculosis. In 2009, there were 11,545 cases of tuberculosis reported in the United States, which was a decline to 3.8 cases per 100,000 of the population, which is the lowest rate since 1953, when recording the disease rate originated (1). As indicated by the World Health Organization (WHO) global incidence rates of tuberculosis infection are mixed; some regions have rates that are either falling or staying stable, then there are the world's regions, such as South Africa, where the rates are increasing. The spread of tuberculosis globally is facilitated by human immunodeficiency virus infections (HIV) (2).

A serious aspect of the TB epidemic is the prevalence of the W-Beijing strains of tuberculosis. The W - Beijing strain family is globally distributed, highly virulent in multiple animal models, associated with HIV infection, associated with drug resistance, and appears to be an emerging strain family (3). These strains compose a separate clade than other TB strains, based on defined evolutionary markers (4). In studies done globally, it has been found that the incidence of W - Beijing infections has been increasing exponentially over time. In East Asia these strains represent about 50% of infections, while they represent at least 13% of the strains

worldwide (5). The globally observed increased infection rate of W - Beijing strains has many hypothesizing that this particular family of tuberculosis has a selective advantage over other strains. The wide geographic distribution of the W - Beijing genotype family, and its genetic homogeneity has led to the possibility that the W - Beijing strains have a selective advantage (3, 5).

The emergence of multiple drug resistant tuberculosis (MDR-TB), along with HIV is further facilitating the spread of tuberculosis worldwide. Multi-drug resistant tuberculosis is defined as any strain that is resistant to two or more primary anti-tubercular drugs, such as isoniazid (INH) and rifampicin (RIF), which are used in the treatment of the disease. Bacterial resistance to anti-tubercular drugs can occur due to many reasons such as naturally occurring mutations, poor adherence to therapeutic regimens, improper prescription by clinicians, and drug interactions or malabsorption. One of the reasons that patient compliance is so low in MDR-TB is that many of the second line drugs used in treatment are toxic. Once acquired resistance is developed, treatment of the disease is compromised, thus allowing further resistance to evolve and allowing resistant tuberculosis organisms to be transmitted to others that will also fail to respond to standard therapy. Since it is highly unlikely that a single bacilli will spontaneously mutate to be resistant to more than one drug, therapy including multiple effective drugs is recommended, which is why the standard treatment of *Mycobacterium tuberculosis* includes a minimum of three drugs. Delayed recognition of drug resistance, which can result in the initiation of effective therapy being deferred, is one of the major factors that has driven the worldwide MDR-TB outbreak. Drug resistance of a strain is determined from bacterial cultures from an infected person. Many undeveloped, and even developing countries, do not have the

technology to do bacterial cultures, so determining resistance to anti-tubercular drugs is slow to nonexistent in these countries. It can be fatal when people are not put on the most effective/correct therapy. This is the case with MDR-TB, and especially with extensively drug resistant (XDR)-TB.

Treatment of MDR-TB is lengthy, less effective, costly, and poorly tolerated. MDR-TB therapy involves a second line of drugs that are primarily used in the therapy of MDR or XDR-TB, which is defined as the resistance to the front line drugs isoniazid, rifampicin, and second line drugs that includes any fluoroquinolone and at least one injectable agent. Estimates are that 6-74% of patients with tuberculosis worldwide have multi-drug resistant tuberculosis, with more than 40% of these patients having been previously treated for the disease. Eastern Europe has the highest rate of MDR-TB, with the disease being found in about 10% and 40% of new and previously treated cases, respectively (6). Unfortunately the very success of drug treatment of tuberculosis has been the catalyst for the emergence of a new wave of drug resistance.

Genetic and molecular analysis of drug resistance in tuberculosis suggests that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation or by titration of the drug through over-production of the target. MDR-TB results primarily from accumulation of mutations in individual drug target genes. The probability of resistance is very high for less effective anti-tubercular drugs such as ethionamide (EMB), capreomycin (CPM), and cycloserine (10^{-3}); intermediate for drugs such as isoniazid, streptomycin (STM), ethamtubol, and kanamycin (10^{-6}); and lowest for rifampicin (10^{-8}). Consequently, the probability of a mutation is directly proportional to the bacterial load. A bacillary load of 10^{9} will contain several mutants resistant to any one anti-tubercular drug. Because the mutations

conferring drug resistance are chromosomal, the likelihood of a mutant being simultaneously resistant to two or more drugs is the product of individual probabilities; thus the probability of MDR is multiplicative. Resistance to a drug does not confer any selective advantage to the bacterium unless it is exposed to that drug. Under such circumstances, the sensitive strains are killed and the drug resistant mutants flourish. When the patient is exposed to a second course of drug therapy with yet another drug, mutants resistant to the new drug are selected and the patient may eventually have bacilli resistant to two or more drugs. Serial selection of drug resistance thus is the predominant mechanism for the development of MDR strains; the patients with MDR strains constitute a pool of chronic infections which propagate primary MDR resistance. In addition to accumulation of mutations in the individual drug target genes, the permeability barrier imposed by the *Mycobacterium tuberculosis* cell wall can also contribute to the development of low-level drug resistance (7).

Treatment for all forms of tuberculosis – susceptible, multi-drug resistant, and extensively resistant strains is lengthy. One of the main reasons for the lengthy therapy time is that tuberculosis bacilli have a long generation time, and in turn take a long time to die and thus are able to persist within the host. Currently available drugs are less effective against persisting bacilli (8). Chemotherapy for tuberculosis originated in the 1950s with the introduction of streptomycin and para-aminosalycilic acid (PAS). Streptomycin was originally used by itself but cases of relapse were occurring so the practice of multi-drug therapy was started when PAS was added to the use of streptomycin, with the emergence of drug resistant bacteria prevented (9).

Innate Immunity to *Mycobacterium tuberculosis*

Infection with *Mycobacterium tuberculosis* takes place when aerosolized droplet nuclei of bacteria are inhaled deep into the alveoli of the lungs. It is thought that only a small number of bacilli are required to cause disease, as few as one to three (2). Once in the alveoli, bacteria are phagocytosed by alveolar macrophages. This results in the death or inhibition of the vast majority of bacilli (10). Those that are not destroyed multiply intracellarly, leading to the destruction of the macrophage and the release of bacteria which are then phagocytosed by other macrophages and dendritic cells. This leads to the activation of the adaptive immune response, resulting in the recruitment of lymphocytes, monocytes, and other inflammatory cells to the foci of infection (11).

Phagocytic cells play a key role in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens and expression of co-stimulatory signals and cytokines. In addition, innate defense mechanisms of phagocytic cells are important, as highlighted in Lurie's fundamental studies with resistant and susceptible inbred rabbits (12). Seven days after primary infection through inhalation of tubercle bacilli, the lungs of mycobacterium-susceptible rabbits contained 20- to 30-fold more viable mycobacteria than did the lungs of mycobacterium-resistant rabbits (13).

Different receptors on phagocytic cells are involved in the endocytosis of *Mycobacterium tuberculosis*. These receptors either bind to non-opsonized *M. tuberculosis* or recognize opsonins on the surface of *M. tuberculosis*. An example of the latter mechanism, mycobacteria can invade host macrophages after opsonization with complement factor C3, which is followed by binding

and uptake through complement receptor 1 (CR1), complement receptor 3 (CR3), and complement receptor 4 (CR4) (14,15,16). For opsonization with C3 to occur the complement system must become activated, which generates the split product C3b. Part of the classical pathway of complement activation is utilized by *M. tuberculosis* by direct binding to C2a, even in the absence of C4b; in this way the C3b required for binding to CR1 is formed (17). Mycobacterial uptake in environments low in opsonins, such as the lungs, is expedited by this mechanism. Nevertheless, non-opsonized *Mycobacterium tuberculosis* can bind directly to CR3 and CR4 (18). However, the best characterized receptor for non-opsonin-mediated phagocytosis of *M. tuberculosi*s is the macrophage mannose receptor (MR), which recognizes terminal mannose residues on mycobacteria (16). Macrophages may also internalize *M. tuberculosis* through the type A scavenger receptor, when uptake by complement receptors and mannose receptor is blocked (19).

A risk factor for developing clinical tuberculosis is enhanced binding of *Mycobacterium tuberculosis* to epithelial cells or alveolar macrophages. Collectins, which are a structurally related group of proteins that includes surfactant proteins, mannose-binding lectins (MBLs), and C1q appear to be important in the binding of tuberculosis to either epithelial cells of alveolar macrophages. Surfactant protein A (Sp-A) facilitates the uptake of *M. tuberculosis* (20), through binding to macrophages (21), type II pneumocytes (22), or neutrophils (23). The uptake of pathogenic strains of tuberculosis in macrophages has been found to be blocked by surfactant protein D (Sp-D) (24). Accordingly it can be hypothesized that the relative concentrations of different surfactant proteins correlate with the risk of infection.

Plasma factor Mannan-binding lectin (MBL), another member of the collectin family, may also be involved in the uptake of mycobacteria by phagocytic cells. MBL recognizes carbohydrate configurations on a wide variety of pathogens (25) and induces phagocytosis directly through a yet-undefined receptor or indirectly by activation of the complement system (25).

Toll-like receptors (TLRs) are essential for microbial recognition on macrophages and dendritic cells (26,27). Members of the TLR family are transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system. The cytoplasmic domain of TLR is homologous to the signaling domain of IL-1 receptor (IL-1R) and links to IRAK (IL-1R-associated kinase), a serine kinase that activates transcription factors like NF-κB to signal the production of cytokines (28).

To this date 13 TLRs have been identified in humans; TLR2 is the most studied; this TLR, along with all identified TLRs recognizes PAMPS (Pathogen-Associated Molecular Patterns) from gram-positive bacteria, including lipoproteins, peptidoglycans, and lipoteichoic acid, but also liparabinomannan from mycobacteria (29). It is unclear how a single receptor can recognize such a broad diversity of stimuli. A possible explanation is the association between TLR1 and TLR6 which were demonstrated to form heterodimeric complex with TLR2 (30,31). TLR6 association with TLR2 induced recognition by diacylated lipopetide (31) but TLR1-TLR2 heterodimer binds preferentially triacylated lipopetides (30).

TLR4 importance in the innate immune response was initially demonstrated in C3H/HeJ and C57BL/lOScCr mice strains that are resistant to endotoxic shock, due to their possession of

a mutation in the *TLR4* gene. TLR4 is essential for LPS detection but, like TLR2, TLR4 recognizes other molecules from different origins. Activation of TLR4 by endogenous ligands, such as heat shock protein 60 (HSP60) (found in *M. tuberculosis*), HSP70, fibronectin, hyaluronic acid, fibrinogen, and heparan sulfate. The formation of a protein complex containing accessory molecules is needed for LPS recognition by TLR4. LPS is generally bound to LPS-binding protein (LPB), which is present in the serum. This complex is recognized first by the CD14 receptor, which is strongly expressed in peripheral blood monocytes and macrophages (32). Once it is bound to CD14, LPS comes in close proximity with TLR4; however the expression of the secreted protein MD-2 is required for a potent triggering of an inflammatory response (32).

The activation of mammalian cells via a TLR3 dependent manner has been shown through several mycobacterial products. One such product is arabinose-capped lipoarabinomannan (AraLAM), purified from rapidly growing mycobacteria (33). Like LPS of gram-negative bacteria, TLR2 dependent activation of cells by AraLAM was also found to depend on CD14. Subsequent studies have demonstrated that *M. tuberculosis* does not contain significant amounts of AraLAM, but it does possess its own TLR2-agonists. LprG (Rv1411) was found to be a TLR2 agonist that upon prolonged stimulation through TLR2, inhibited major histocompatibility complex-class II (MHC-II) antigen processing (34). The 19 kDa lipoprotein, a cell wall-associated protein from *Mycobacterium tuberculosis*, was found to activate cells in a TLR2-dependent manner (35). *M. tuberculosis* has been shown to have another factor along with the cell wall associated 19kDa lipoprotein that activates cells in a TLR2 dependent manner (36). Investigators saw that culture filtrates of *M. tuberculosis* contained a heat-stable and protease

resistant factor (STF). The fraction of STF that had TLR2 stimulating activity has a mass of 6kDa and contained material that co-migrated by SDS-PAGE with a small mycobacterial glycolipid, phospatidylinositol dimmoniside (PIM). PIM was found to activate cells in a TLR2-dependent manner, similar to STF (37). These findings indicate that *M. tuberculosis* is fully capable of expressing distinct factors that can activate or deactivate cells via TLR2.

The high output expression of nitric oxide (NO) in response to cytokines or to pathogen-derived molecules is an important component in the host defense against intracellular organisms, such as *M. tuberculosis* (38,39,40). NO is formed when the guanidine nitrogen of L-arginine is oxidized by a family of iso-enzymes known as NO synthases (NOSs). Exposure to NO at low concentrations, e.g. <100 ppm, was shown to kill more than 99% of *Mycobacterium tuberculosis* in culture (41). Bacterial DNA, protein, and lipids at the microbial surface and intracellularly can be modified by NO and Reactive Nitrogen Intermediates (RNI). Other destructive activities by NO include deamination, direct damage to bacterial DNA, the interaction with accessory protein targets such as iron-sulfur groups, heme groups, thiols, aromatic or phenol residues, tyrosyl radicals, and amines, resulting in enzymatic inactivation or other protein malfunctions (42).

Alveolar macrophages that reside within the lungs are considered to be the main cellular host for mycobacteria *in vivo* (43). This is due to the fact that *M. tuberculosis* persists in immature phagosomes by preventing their maturation into phagolysosomes and thus evade being killed by lysosomal enzymes. Mycobacteria are taken up via phagocytosis and the receptor type that mediates this event can inflluence the response generated within the macrophage. Mannose receptors that bind mannosylated molecules on the bacterial surface, Fc receptors binding opsonized cells, and complement receptors are receptor types/molecules that are associated with

the uptake of *Mycobacterium tuberculosis*. The use of CR3 by mycobacteria may be advantageous for the bacilli, since potentially cytotoxic Reactive Oxygen Intermediates (ROI) are not released when this receptor is triggered and then activated (44). Binding to the mannose receptor has also been suggested as a possible safe route of entry for mycobacteria that facilitates their intracellular survival (45).

Once the mycobacteria is phagocytosized and the phagolysosome fuses successfully the bacteria is destroyed and the bacterial antigens are processed and presented to T cells in the context of MHC molecules. The adaptive immune response is activated by the stimulation of T cells and thus induces IFN-γ release from $CD4^+$ and $CD8^+$ T cells that are the antimicrobial defense system. Bacteria persistence with the host occurs when mycobacteria evade the normal phagosome-lysosome fusion pathway. Mycobacteria appear to slow the maturation of the phagosome in such a way that they retain early endosomal markers and cannot fuse with the lysosome.

The interaction of mycobacteria and antigen presenting cells (APCs) has been reported to have an effect on cytokine synthesis and the expression of molecules found on the cell surface of macrophages. Macrophages infected with *Mycobacterium tuberculosis* preferentially secrete pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 (46). Infected macrophages are known also to secrete chemokines such as IL-8, RANTES, and MCP-1 (reviewed by (47)) which would aid the recruitment of lymphocytes to the lung and granuloma formation, thus leading to containment of the mycobacteria. *M. tuberculosis* infected macrophages also secrete IL-10, instead of IL-12, which could suppress Th1 responses in late infection (46,48). IL-10 may also inhibit export of MHC class II molecules to the cell surface, which would down regulate T cell

responses. A reduced ability of these cells to signal T lymphocyte activation combined with recruitment of cells to form granulomas may help mycobaterial persistence within the host. It is known that stimulation of macrophages by other components of the immune response, such as IFN-γ or TNF-α released by T cells, can enhance macrophage microbial activity and this is associated with reduced IL-10 secretion (46,48).

Alternatively, phagocytosis of particulate antigens (secreted by the mycobacteria) and apoptotic cells by dendritic cells and macrophages lead to MHC-I antigen processing and presentation through a process termed cross-presentation. *Mycobacterium tuberculosis* lipids and lipoproteins, actively trafficking in phagosomally derived vesicles (49), are cross-presented by bystander APC in a TAP independent and proteosome-independent manner (50,51). This mechanism, of antigen processing, explains why nearly all of the known mycobacterial antigens recognized by CD8 T cells are found in bacterial culture supernatant, many of them known to be actively secreted by the bacterium (52,53,54).

Autophagy is another macrophage function. Cells undergo autophagy by sequestering their own cytoplasm into an autophagosome that is then delivered to the lysosome (55). Autophagy has been described as a defense mechanism inhibiting bacilli Calmette-Guérin (BCG) and *Mycobacterium tuberculosis* survival in infected macrophages. IFN-γ induces autophagy and the inhibition of this process increases the viability of intracellular mycobacteria in mice and humans (56).

Dendritic cells (DCs) are cells that are specialized for the presentation of antigen to T cells. These cells are the most potent of the antigen presenting cells and are central to the

initiation of immune responses in naive animals (57,58). DCs originate in the bone marrow but there is evidence that they may also be derived from both myeloid and lymphoid precursors.

Different properties have been established that are critical to the functions of DCs, as a critical antigen presenting cell population. These properties include the ability to effectively take up antigen by a number of routes, which may include endocytosis by clathrin-coated caveolae, as well as, macropinocytosis or phagocytosis depending on the maturation stage of the cell. The efficiency of DCs as APCs are due to high levels of MHC-II expression and a number of co-stimulatory molecules that include CD80, CD86, and CD40 (59). DCs serve as sentinels monitoring the exposure of the body surfaces to antigen in the periphery. At these sites, DCs are considered to be immature cells that express low to moderate levels of MHC-II and co-stimulatory molecules and have a high capacity to phagocytose microorganisms and other particulate antigens (57). Immature DCs are efficient in antigen uptake, but are poor simulators of T lymphocyte responses. Immature DCs are stimulated to migrate away from the body surface by tissue injury, inflammation, or infection (58). This process of migration from periphery, via the afferent lymphatic vessels to the draining lymph node, which is associated with functional and phenotypic maturation that upon arrival in the lymph node, the DCs have acquired the capacity to effectively stimulate naive T lymphocytes.

Antigen presentation is not the only functions of DCs; these cells also act as modulators of the immune response and link innate and adaptive immune responses. The innate response of DCs to microbial antigens is thought to polarize the T cell response towards Th1 or Th2 phenotypes. This is linked to the capacity of DCs to produce the cytokines IL-12 and IFN-γ

(60,61) that can up-regulate the secretion of IFN-γ by T lymphocytes and NK cells thus driving a Th1 biased response.

The involvement of DCs in the transport of the bacteria to the lymph node is essential to the initiation of the immune response. Cell maturation and activation that is characterized by changes in cell surface phenotype and cytokine profiles results from the interaction of DCs with BCG or *M. tuberculosis (*46,48). The uptake of mycobacteria by DCs triggers their expression of surface molecules that are involved in the interaction with T cells, notably MHC-II and the co-stimulatory molecules CD40 and CD80.

The expression of IL-12, TNF-α, IL-1, and IL-6 is increased when DCs become infected with either BCG or *M. tuberculosis.* These cytokines play major roles in protective anti-mycobacterial immune responses. As previously noted, IL-12 secreted by DCs can enhance IFN-γ and TNF-α secretion by T cells and in turn may serve to enhance the anti-microbial activity of macrophages to destroy invading bacilli. Mycobacterial infection of DCs is also associated with the secretion of IL-10, which may inhibit the cellular response to mycobacteria through the down regulation of IL-12 secretion (46,48). This may serve to limit the extent of DC and macrophage activation and thus regulate the potentially damaging immune response that occurs in tissues in vivo.

DCs, like non-activated macrophages are reported to provide an environment where mycobacteria can survive and replicate, although to a lower extent (62,63). It appears that the replication of mycobacteria within DCs is not enough to kill the host cell and the outcome of slow replication is reflected by the constant availability of antigens for presentation to T cells

that will therefore augment the immune response. Following macrophage activation by IFN-γ or TNF-α, almost all of the mycobacteria residing within the phagosome may be killed. The killing of mycobacteria does not occur in activated DCs following stimulation with IFN-γ DCs are able to control the replication of mycobacteria but are not able to kill the bacilli. Instead the bacilli appear to reside in vacuoles separated from the normal recycling pathway (62,63). Since DCs are unable to kill the mycobacteria, these cells become a reservoir, especially in the lymph nodes where these cells migrated to after the initial infection by the bacteria.

In an infection, neutrophils are the first phagocytes to arrive from the circulation and attempt to eliminate invading pathogens via oxygen-dependent and oxygen-independent mechanisms. The oxygen dependent mechanism results from the generation of reactive oxygen species (64), whereas the oxygen independent mechanism reflects the capacity of neutrophils to degranulate and release preformed oxidants and proteolytic enzymes from granules (65). Neutrophil degranulation is meant to target the pathogen; but other cells and tissues can become damaged or destroyed (66). A strict regulation of neutrophil influx and their infected tissues is essential for minimizing tissue damage.

Neutrophils have been implicated in the control of mycobacterial infections (67,68) but it is not known whether these cells have any direct protective functions, due to their short life span. Initial *in vitro* studies suggested that human neutrophils are able to kill virulent *M. tuberculosis* (69,70). The recruitment of neutrophils to the lungs has been described for acute TB (71) and in experimental animals infected with mycobacteria (72). The role of neutrophils may lie in the transmission from innate to adaptive immune responses by producing critical cytokines and chemokines, in both humans and animals, instead of in the clearance of mycobacteria (73,74,75).

Natural Killer (NK) cells are also effector cells of innate immunity. These cells are distinct from T and B lymphocytes in that they do not show germ-line receptor rearrangement and their effector functions are governed by the combinations of activating and inhibitory receptors. The functions of NK cells allow the recognition of altered self at a cellular, rather than the molecular level of the T cell receptor and immunoglobin. Direct cytotoxicity via perforin or the secretion of Th1 cytokines is mediated by the activated NK cells. NK cells may directly lyse the pathogens or can lyse infected monocytes (76).

During early infection, NK cells are capable of activating phagocytic cells. A likely mechanism of NK cytotoxicity is apoptosis. NK cells produce IFN-γ and can lyse mycobacterium pulsed target cells (77).

In a recent study, the role of NK cells in early innate resistance to infection with *M. tuberculosis* was evaluated in mice, after an aerosol infection. A steady increase in NK, but not NK T cells, subsets were observed within the lung over the first three weeks of infection. NK cells increased the expression of cell surface activation markers, were IFN-γ positive and labeled positive for intracellular perforin after *in vitro* stimulation. Despite this, the depletion of NK cells using lytic antibodies had no influence on the pulmonary bacterial load. This suggests that in response to an infection with *M. tuberculosis*, lung NK cells can become activated, but are not an essential protective mechanism (78).

Acquired Immunity Against *Mycobacterium tuberculosis* Infection

A classic example where cell mediated immunity is the protective response to a pathogen is *Mycobacterium tuberculosis*. This is due to the fact that the bacteria live within cells, mainly macrophages; thus T cell effector mechanisms, rather than antibody, are required to control or eliminate the bacteria.

Research has been initially focused on the $CD4^+$ T cell response to *M. tuberculosis*, but the interest in the role(s) the $CD8^+$ T cells play in the immune response to the bacteria has been increasing.

The most common outcome of infection with *Mycobacterium tuberculosis* is MHC-II presentation of mycobacterial antigens to $CD4^+$ T cells since vacuoles within macrophages are the primary site of *M. tuberculosis* within the cell. The adaptive acquirement of a tuberculosis-specific, Th1-type cellular immune response is required for growth inhibition of the bacteria. A central role for the interaction between Th1 $CD4^+$ lymphocytes and macrophages is supported by ample experimental evidence. In this model, *M. tuberculosis*-derived antigens are processed and presented via macrophage-associated MHC-II. Antigen recognition by $CD4^+$ T cells then leads to the release of pro-inflammatory cytokines, that in turn limit *M. tuberculosis* growth intracellularly.

Several studies have demonstrated that $CD4^+$ T cells have an important role in the protective response against *M. tuberculosis*. The requirement of $CD4^+$ T cells to control infection in the murine model has been shown by studies of antibody depletion of $CD4^+$ T cells (79), adoptive transfer (53,80,81) or through the use of gene disrupted mice (31,82). $CD4^+$ T cells are

approximately two times more abundant than $CD8^+$ T cells are at sites of *M. tuberculosis* infection in both mice and humans (83,84). The increased susceptibility of both acute and reactivated tuberculosis, in humans, through the loss of $CD4^+$ T cells has been demonstrated with co-infection of HIV. It has been shown that IFN-γ is produced in infected subjects in response to a wide variety of mycobacterial antigens, from studies of $CD4^+$ T cells. The primary effector function of $CD4^+$ T cells is believed to be the production of IFN-γ and TNF-α, which is sufficient to activate macrophages which in turn can control or eliminate intracellular organisms (85).

Along with IFN-γ, IL-17 producing $CD4^+$ T cells are increased in population due to tuberculosis infected DCs and are also induced *in vivo* during infection. Following aerosol infection IL-17-producing-antigen specific cells are induced in mice. The presence of IL-23 is required by these cells and most of the IL-17 response is in the lung (86). There is a small divergence in the early inflammatory response, in the murine model, when IL-17 is absent and subsequently in the majority of the IL-17 response in this model (86). It is not known whether these cells are protective or damaging. However, it has been shown that IL-17 producing T cell population is limited by IFN-γ, and that an important factor in limiting mycobacteria-associated immune-mediated pathology may be this counter-regulation pathway (87).

$CD4^+$ T cells have another role in activating or maturing APCs. Enhanced antigen presentation and co-stimulatory activity results from the interaction of CD40L on CD4 T cells with CD40 on macrophages or DCs (88). This interaction may be important in *M. tuberculosis* infection. Optimal priming of T cells and control of tuberculosis infection by aerosol requires CD40, but not CD40L, in the murine model (89). The importance that $CD4^+$ T cells have on

priming and the maintenance of $CD8^+$ T cell effector and memory functions has been demonstrated indirectly in mycobacterial infections (90,91).

Antigens presented by MHC-II molecules on APCs, such as DCs and macrophages are the target of $CD4^+$ T cells recognition. A vacuole, part of the endocytic system, is the location of MHC-II molecules that are loaded with antigenic peptides. In contrast, $CD8^+$ T cells recognize MHC-I molecules. MHC-I molecules are loaded with antigens transported from the cytoplasm into the endoplasmic reticulum. Initially it seemed highly likely that MHC-I molecules would have a hard time recognizing antigens for $CD8^+$ T cells since tuberculosis mainly lives within a vacuole, rather than the cytoplasm of a cell.

$CD8^+$ T cells, along with $CD4^+$ T cells, have been shown to be required for the control of tuberculosis infection, from early studies using antibody-mediated T cell subset depletion (79). Early studies also suggested that adoptive transfer of purified immune $CD8^+$ T cells reduce the numbers of *M. tuberculosis* bacteria in the spleens of infected mice, even though these cells have a lower efficiency compared to $CD4^+$ T cells (81). Further data implicates the role of $CD8^+$ T cells in the control of tuberculosis infection which is supported by the development of gene-disrupted mice (92).

Mice genetically deficient in β_2-microglobulin (β_2m), which lack functional MHC Class I molecules and consequently $CD8^+$ T cells, were unable to control infection, particularly in the lung, and died early due to tuberculosis (93). It was initially assumed that susceptibility of $\beta_2m^{-/-}$ mice was due to a drastically reduced total number of peripheral $CD8^+$ T cells, but what is now known is that the defect in $\beta_2m^{-/-}$ mice compromises other molecules.

The contributions of classical or nonclassical MHC Class I–dependent $CD8^+$ T cell populations in protection against tuberculosis, was confirmed when a series of gene-disrupted mouse strains were compared for susceptibility to intravenous *M. tuberculosis* infection, as measured by survival time and bacterial loads. Among the strains tested, the most susceptible mice were the $\beta_2m^{-/-}$, followed by $TAP1^{-/-}$ (transporter associated with antigen processing), $CD8\alpha^{-/-}$, $perforin^{-/-}$, and $CD1d^{-/-}$ mice (94). The conclusion was that classically restricted (TAP1-dependent) $CD8^+$ T cells contribute to *in vivo* protection against *M. tuberculosis*; however, the role of $CD8^+$ T cells in protective immunity was not limited to perforin-dependent cytotoxicity (94). The β_2m-/- mice developed granulomas that were initially devoid of lymphocytes. As infection progressed, lymphocytes did accumulate, but they failed to infiltrate the macrophage-dominated lesions, implicating a novel and undefined β_2m-dependent mechanism influencing early lymphocyte accumulation (95).

This confusion, in regards to MHC-I/$CD8^+$ T cells role in tuberculosis control, has been clarified by recent studies. In one report, mice specifically deficient in classical MHC-Ia molecules were more susceptible to *M. tuberculosis* infection than were wild-type mice, although not as susceptible as ß2$m^{-/-}$, providing strong evidence for the role of MHC-I classically restricted $CD8^+$ T cells in resistance to *M. tuberculosis* (96). More recently it was demonstrated that apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1. In this latter report, investigators showed how the mycobacteria induced apoptosis in macrophages, causing the release of apoptotic vesicles that carry mycobacterial antigens to uninfected antigen-presenting cells, facilitating the presentation through MHC-I. Furthermore, they showed that

inhibition of apoptosis reduced transfer of antigens to bystander cells and activation of $CD8^+$ T cells (51).

More evidence for the role of $CD8^+$ T cells is supported in studies comparing wild-type and gene-knockout mice deficient in both $CD4^+$ and $CD8^+$ T cells, MHC I or MHC II. When these mice were infected via aerosol and monitored for survival and ability to control infection, the results showed that in contrast to $CD4^+$ T cells, $CD8^+$ T cells were dispensable and not essential for the control of infection (97). Mice devoid of $CD4^+$ T cells died earlier from tuberculosis than did $CD8^+$ T cell–deficient mice, but the absence of both $CD4^+$ and $CD8^+$ T cells resulted in even greater susceptibility. The fact that mice devoid of $CD8^+$ T cells still succumb to *M. tuberculosis* infection, despite the development of fully functional $CD4^+$ T cell responses, argues that $CD8^+$ T cells may play an important role in controlling chronic infection. Furthermore, depletion of $CD8^+$ T cells resulted in reactivation of latent tuberculosis in a murine model, suggesting that this T cell subset may also be essential for controlling latent tuberculosis (98). These results are corroborated by experiments comparing the course of the infection in control and CD8 knockuot (CD8-KO) mice (92). Researchers showed that the role of $CD8^+$ T cells was not during the early stage of the infection, when the bacterial load is initially contained, but during the chronic phase of the disease. The gradual loss of resistance in CD8-KO mice was associated with an increased lymphocytic influx of $CD4^+$ T cells that despite the capacity to produce IFN-γ, were not sufficient to control the bacterial load.

$CD8^+$ T cells can function as a source of type-1 cytokines such as IFN-γ and TNF-α or they may exert their protective effect by killing infected macrophages within the tissues. Macrophage activation requires IFN-γ and TNF-α and both $CD4^+$ and $CD8^+$ T cells have the

ability to produce these cytokines at the infection site (85). $CD8^+$ T cells can kill infected macrophages through a perforin-mediated mechanism. Infected macrophages are killed by perforin, which is a protein found in the granules of $CD8^+$ T cells that creates a hole in the infected macrophage membrane, allowing the entry of toxic proteins,that include granzymes or granulysin. Apoptosis of macrophages can also be induced by ligation of Fas - ligand on activated $CD8^+$ T cells, to the Fas molecule on infected macrophages.

There are differences between memory, effector, and naive T cell subsets. The following are some of the ways in which memory $CD4^+$ and $CD8^+$ T cells differ from naive and effector T cells: memory cells express distinctive activation markers and intracellular proteins that distinguish them from naive and effector T cells; memory cells have lower activation thresholds and effector functions different from naive T cells (99); memory cells express chemokine and adhesion receptors that allow them to hone to infected tissues and organs throughout the body (100).

Memory starts when an antigen enters the body and is carried in afferent lymphs to draining lymph nodes or is taken up by peripheral DCs which are then stimulated to migrate to the lymph nodes. Once in the lymph nodes antigen is processed into short peptides and presented by DCs on MHC-II and MHC-I molecules to initiate the response of naive $CD4^+$ and $CD8^+$ T cells (59). The presence of co-stimulatory molecules on the surface of DCs is required for the initiation of an effective immune response. These molecules include CD40, CD80, and CD86, bound to their T-cell counter-receptors, along with CD154, CD28 and CD152 generate an effective immune response. Stimulatory cytokines and chemokines are produced by DCs and other cells of the innate immune response. PAMPS determine the type of signals produced by

innate immune cells, PAMPS stimulate these cells of the innate immune system by conserved pattern-recognition receptors. The fate of the newly generated T cells are determined by these signals (101).

An important determinant of memory duration appears to be the extent of the initial T cell response (102), while at the same time memory is also somewhat determined by the number and affinity of the naive precursors (103). Clonal expansion is initiated by the delivery of a TCR signal that has the appropriate co-stimuli, which has different affects on $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells stimulated *in vitro* for 24 hours and then transferred into antigen-free hosts undergo at least seven divisions and acquire effector and memory functions (104). $CD8^+$ proliferation and differentiation is influenced by co-stimuli and cytokines (105). Continuous antigen exposure is required for the proliferation and differentiation of $CD4^+$ cells *in vitro* and *in vivo* (106). Even with constant antigen exposure, the frequency of responding $CD4^+$ T cells are lower, and the size of individual $CD4^+$ clones smaller when compared to those of $CD8^+$ cells (37).

The generation of T cell memory seems to begin when clonal contraction occurs when studied in viral infection models. The peak response to antigen is reached within 1-2 weeks, where a phase of contraction follows, where most of the short-lived antigen-specific effector T cells die due to apoptosis. After the loss of these short-lived effector T cells, a population of longer-lived memory cells remains (107). $CD4^+$ cells have slower kinetics and do not expand as much as $CD8^+$ cells do. $CD4^+$ cells have a pattern of contraction that is biphasic where $CD8^+$ cells show massive and rapid expansion and collapse, which is followed by life-long preservation of a stable memory population, at least in experimental animal models (37).

Expression of the anti-apoptotic protein B cell leukemia/lymphoma 2 (BCL2) is required for memory T cell survival (108,109). Early in the encounter of T cells with antigen-presenting cells, death receptors and proapoptotic proteins such as CD95 and BCL2-like protein 11 (BIM), that are influenced by chemokines and cytokines, are rapidly upregulated, leading to T-cell death.

$CD8^+$ T cells require the help of $CD4^+$ T cells along with signals from the innate immune system. $CD8^+$ effectors can be produced in a primary response in the absence of $CD4^+$ T cells, though memory $CD8^+$ cells generated in the absence of $CD4^+$ T cells make poor responses when re-stimulated and die by activation-induced cell death, mediate via the TNF-related apoptosis-inducing ligand (TRAIL).

Based on their homing characteristics and their effector functions, two types of memory T cells have been described within the $CD4^+$ and $CD8^+$ T cell populations. T effector-memory (T_{EM}) and T central-memory (T_{CM}) cells differ both functionally and in their migratory properties and can be distinguished based on their CD62L and CCR7 expression in humans (49). T_{CM} cells express CCR7 and CD62L, while T_{EM} cells do not express CCR7 or CD62L. In both humans and mice, $CD4^+$ T_{CM} and $CD8^+$ T_{CM} cells mainly reside within lymphoid organs (110,111). While $CD4^+$ T_{EM} cells reside in peripheral tissue to provide protection against re-infection, and rapidly produce IFN-γ or IL-4 once TCR stimulation occurs. $CD8^+$ T_{EM} cells are also located within the peripheral tissue and exhibit immediate cytokine secretion and perforin killing activity (49,110,111). Immediate protection against reinfection/reactivation is provided by T_{EM} cells, while T_{CM} cells reside within the lymphoid tissue where they can rapidly expand and differentiate to re-supply the effector T cells at peripheral sites.

Studies using transgenic and knockout mice along with blocking antibodies have shown the importance of IL-7 and IL-15 for the survival of memory cells. IL-15 is essential for memory $CD8^+$ T cell survival, and although there is no absolute need for IL-7, it can compensate for the absence of IL-15 (112). In mouse experiments where the host is antigen free or lacks MHC-I molecules and $CD8^+$ memory cells are transferred, the number of $CD8^+$ memory cells slowly decline over time. These results shown that CD8 memory maintenance does not require the presence of antigen. In contrast, CD4 memory cell survival requires both TCR signals and IL-7 (113).

Chapter 2 - Evaluation of clinical isolates of *Mycobacterium tuberculosis* in the murine model

Introduction

Globally the tuberculosis epidemic continues unabated, affecting over nine million people a year, with more than half a million of these cases being resistant to multiple drugs. Multiple drug resistant tuberculosis (MDR-TB) is becoming a growing problem to the world's population. Despite this growing problem, very little research is being focused on MDR-TB. One basic question not yet addressed is how drug resistance affects virulence levels. Drug resistance in *M. tuberculosis* strains is believed to be a result of a gene point mutation or a gene deletion. It is thought by many that these mutations and/or deletions result in reduced virulence level for MDR strains. This hypothesis originates from the classical studies of Mitchison, who observed that a significant number of isoniazid-resistant *M. tuberculosis* strains isolated from patients tended to be of low virulence in the guinea pig model of infection (114).

An early study conducted in the murine model by Ordway and colleagues showed that multiple strains that were either resistant to one drug or multiple drugs had high bacterial lung burdens in the first 20 days of infection (115). At this point it cannot definitely be said if drug resistance is connected with either lower or higher virulence levels.

Multiple drug resistance strains of tuberculosis are not the only cause of alarm for the continuing epidemic. Many strains that are associated with outbreaks around the world are being classified as either high or low transmission strains. High transmission strains are thought to be associated with increased rates of infections and higher virulence, the latter driving the former.

Low transmission strains are the opposite; while they have been known to cause disease the numbers of cases where these strains have been identified are fewer.

The study of clinically relevant strains is important, since researchers need to know how these isolates behave in vivo; moreover, it is these strains that newly developed drugs and vaccines have to combat. Instead, however, virtually all experiments being conducted to test new vaccines and new chemotherapy regimens for *M. tuberculosis* utilize laboratory strains, such as H37Rv or Erdman. It is becoming increasingly evident that laboratory strains are somewhat less virulent, do not cause as severe pathology, and have different immune responses in comparison to clinical isolates of *M. tuberculosis*.

In this study the growth of two sets of isogenic pairs of *M. tuberculosis* was investigated in a mouse infection model, in order to determine if acquired drug resistance increased or decreased the virulence of the drug resistant strain. Virulence was defined as being the severity of the tuberculosis disease. In further studies the virulence and pathogenicity, with pathogenicity being defined as changes that lead to the severity of disease (TB), of two strains was examined with the strains being selected for apparent high versus low transmission patterns, recently seen in an outbreak of tuberculosis within the Chinese community in San Francisco, CA, USA. All the isolates found in this outbreak were typed as W – Beijing, and as noted above very little is known about the family of W-Beijing strains of tuberculosis, except that these strains mostly seem to have significant levels of virulence, usually at higher levels than typically seen in other strains. It has also been observed that these strains can spread quickly, causing large community-wide TB outbreaks. There is also a concern that W-Beijing strains are being positively selected by the current vaccine, BCG, rendering the vaccine ineffective against these

strains, and thus causing large community outbreaks of these strains (116). In areas of the world, such as China, where BCG is given to a large portion of the population, those that come to the clinic and are diagnosed with tuberculosis are found to be infected with W – Beijing strains. Whereas areas in the world that have just recently begun to do widespread BCG vaccinations, such as Viet Nam, patients coming into the clinic and being diagnosed with tuberculosis are infected with a variety of tuberculosis strains. Many W-Beijing strains are also found to be multiple drug resistant (MDR), or even extensively drug resistant (XDR).

Materials and Methods:

Animals: Specific pathogen free female 6-8 week old C57BL/6 mice were purchased from Charles River Laboratories, Wilmington, MA. They were kept under barrier conditions in Animal Bio-Safety Level III (ABSL-III) laboratory and were provided with sterile bedding and given sterile chow and water ad libitum. Specific pathogen free status was verified by testing sentinel mice within the colony. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University.

Experimental Infections: *M. tuberculosis* strains CSU 9, CSU 21, CSU 23, CSU 27(University of New Mexico, Albuquerque, NM) and Midori 3507 and 4334 (University of Southern California, San Francisco, CA) were grown in 7H9 media containing OADC and Tween-80 to mid-log phase and then frozen in aliquots at - 80°C until needed. For low dose aerosol infections, bacterial stocks were diluted in 5 ml of sterile distilled water to 2 x 10^6 CFU/ml and placed in a nebulizer attached to an airborne infection apparatus (Glass-Col, Terre

Haute, IN). Mice were exposed to an aerosol infection in which approximately 100 bacteria were deposited in the lungs of each animal. Bacterial counts in the lung and spleen were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar (Becton, Dickinson, and Company, Sparks, MD) and counting CFU after three weeks of incubation at 37°C.

Preparation of Cells: Mice were euthanized by CO_2 asphyxiation and cervical dislocation, and the thoracic cavity was opened. The lungs were cleared of blood by perfusion through the pulmonary artery with 10 ml of ice-cold phosphate buffered saline (PBS) (Mediatech Inc, Manassas VA) containing 50 U/ml of heparin (Sigma, St. Louis, MO). Lungs were aseptically removed and placed in medium. The dissected lung tissue was incubated with incomplete DMEM containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 µg/ml;Sigma-Aldrich) for 30 minutes at 37°C. The digested lungs were further disrupted by gently pushing the tissue through a cell strain (BD Biosciences, Sparks, MD). Red Blood Cells were lysed with Gey's Solution, washed, and resuspended in complete DMEM. Total cell numbers were determined by flow cytometry using Invitrogen CountBright absolute liquid counting beads, as described by the manufacturer (Invitrogen, Eugene, OR).

Flow Cytometry for surface markers and intracellular cytokines: For flow cytometry analysis, single cell suspensions of lungs from each mouse were resuspended in 1x PBS (Mediatech Inc, Manassas, VA) containing 0.1% sodium azide. Cells were incubated in the dark for 30 minutes at either 4°C or 37°C with predetermined optimal titrations of specific antibodies. Cell surface expression was analyzed for CD4, CD44, CD62L, CD8, CD25, and Foxp3, and the cytokines analyzed were IL - 17 and IFN - γ. All antibodies and reagents were purchased from

BD Pharmingen (BD Pharmingen, San Jose, CA), eBioscience (eBioscience, San Diego, CA), or Biolegend (Biolegend, San Diego, CA). All samples were analyzed on a Becton Dickinson LSR II instrument, and data were analyzed using FACSDiva v.6.1.1 software. Cells were gated on lymphocytes based on characteristic forward and side scatter profiles. Individual cell populations were identified according to the presence of specific fluorescence-labeled antibodies. All the analysis was performed with an acquisition of a minimum of 100,000 events.

Determination of bacterial CFU in lung and spleen: at 7, 30, 60, and 90 days post infection, five mice from each group, were euthanized by CO_2 inhalation and cervical dislocation. Lungs and spleen were aseptically removed. Spleens were removed, cut in half, with one half being used for CFU enumeration and the other being placed in 10% formalin (Fisher Scientific, Fair Lawn, NJ) for histology. The upper right lung lobe was removed and placed in 10% neutral buffered formalin for histology and the lower right lobe was for CFU determination. Serial dilutions of organ homogenates were plated on nutrient 7H11 agar with counting of CFU being completed after three weeks of incubation at 37°C.

Histology: At each time point post infection, approximately half of the spleens and the upper right lung lobe were preserved in 10% formalin and subsequently embedded in paraffin, sectioned, and stained with hematoxylin and eosin staining. All pictures of the lungs were taken with an Olympus BX41 microscope at 2x magnification.

Statistics: A student's t test was performed in GraphPad Prism 4 to determine any statistically significant differences.

Section 1: Studies examining two isogenic pairs of M. tuberculosis in the mouse, to determine if acquired drug resistance increased or decreased the virulence of the drug resistant strain

Results

In these studies the isogenic pair CSU 21 and CSU 27 was evaluated. CSU 21 is susceptible to the front line drugs, isoniazid (INH), rifampin (RIF), ethambutol (EMB), pyrazinamide (PZA), and streptomycin (STM). CSU 27 the other strain in this isogenic pair, but resistant to isoniazid. The second pair studied was CSU 23 and CSU 9. CSU 23 is drug susceptible to the front line drugs, while CSU 9 is resistant to rifampin. The hypothesis to be tested was whether these mono - resistant changes influence the subsequent virulence of each strain.

Organ Bacterial Burdens of lungs and spleens for isogenic pairs

CSU strains 21 and CSU 27 had a lung bacterial burden of approximately 2 logs at day 7. At the rest of the time points there was a significant difference in the bacterial burdens. CSU 27 continued to grow in the lungs, with an organ burden of approximately 5 logs at day 90. In contrast, CSU 21 had a lung burden of approximately 2 logs at day 30 also, but then grew poorly, increasing to only 3 logs at day 60 with the levels staying stable through day 90, as indicated in Figure 1A.

The spleen bacterial burden for CSU 27 has the largest rate of growth within the first 30 days, resulting in approximately 4.5 logs. The burden then decreased from day 30 to day 60, and then increased again at day 90 to approximately 4.5 logs again. The growth pattern for CSU 21 was more stable, with a gradual increase in the organ bacterial burden until reaching approximately 3 logs at day 90 (Figure 1B).

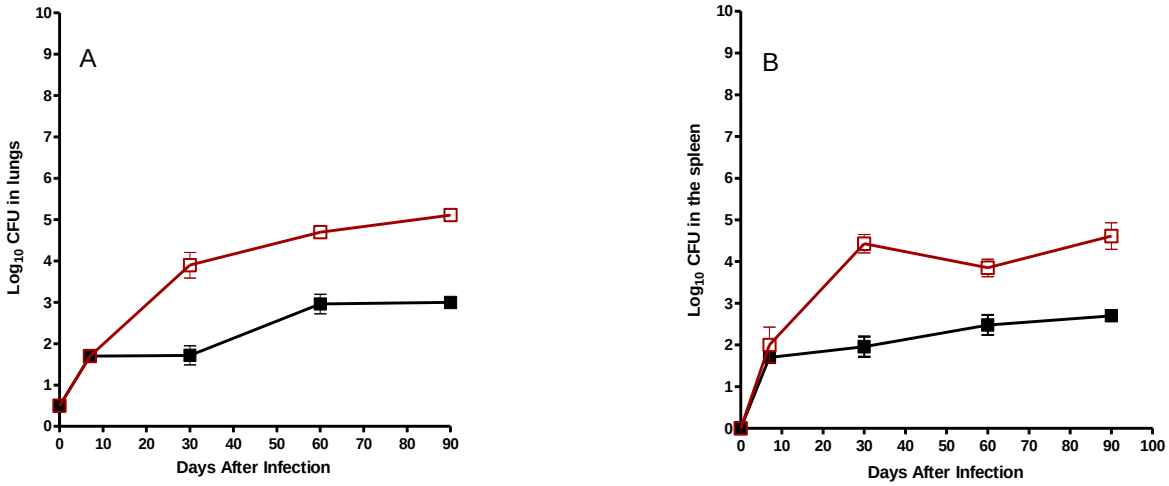

Fig. 1 bacterial counts in lung (A) and spleen (B) of mice infected with CSU 21 (■) and CSU 27 (□) at days 7, 30, 60, and 90. The results are expressed as average (n =5) bacterial loads in each group expressed as the $\log_{10}$ number of CFU (± SEM).

Of the four different strains in the two isogenic pairs studied, CSU 23 had the highest bacterial burden in the lungs. At day 7 there was a burden of a little more than 2 logs, then gradually increased to a burden of approximately 6 logs at day 90. The bacterial burden in mice infected with CSU 9 increased to approximately 4 logs at day 30, decreased at day 60, then increased again at day 90 to approximately 5 logs (Figure 2A).

In the spleens for CSU 23 and CSU 9 the bacterial burdens were very similar to one another throughout the entire experiment. Both of the strains started at roughly 2 logs at day 7 and then increased. CSU 9 obtained approximately 4 logs at day 30 before being contained, which is evident by the stable growth. At day 30 CSU 23 had a lower burden, of nearly 3 logs, and continued to grow to roughly 5 logs before being contained as depicted in Figure 2B.

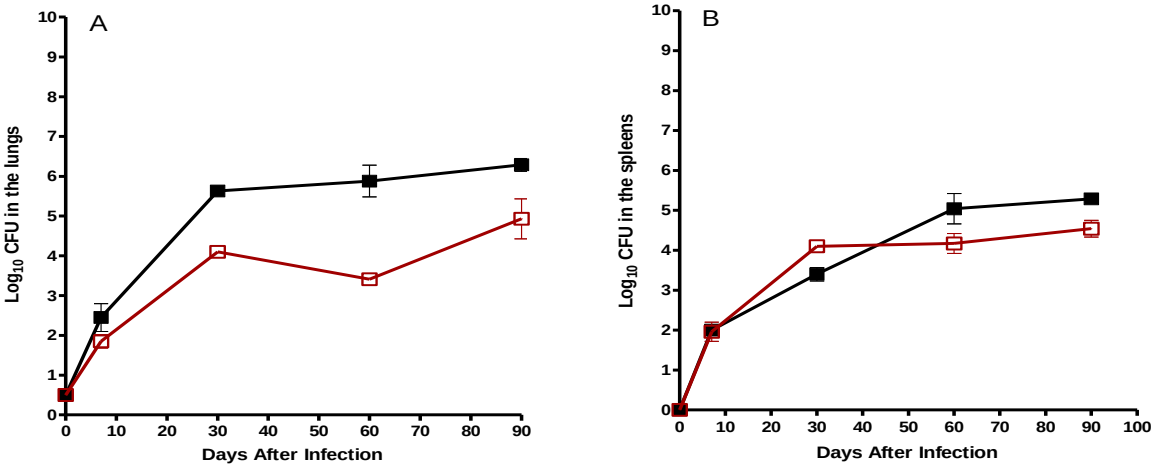

Fig. 2 bacterial counts in lung (A) and spleen (B) of mice infected with CSU 9 (□) and CSU 23 (■) at days 7, 30, 60, and 90. The results are expressed as average (n =5) bacterial loads in each group expressed as the $\log_{10}$ number of CFU (± SEM).

Flow cytometry analysis of the lungs for the isogenic pairs

Levels of $CD4^+CD44^+CD62L^{lo}$, CD4 effector T cells in the infected mice

$CD4^+CD44^+CD62L^{lo}$ cells were gated as indicated in Figure 3, below.

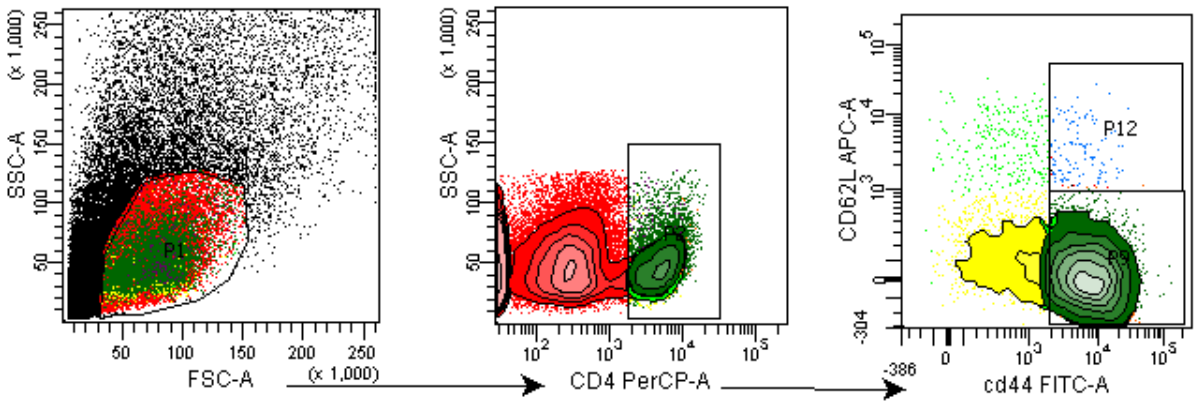

Fig. 3 gating strategies for $CD4^+CD44^+CD62L^{lo}$ and $CD4^+CD44^+CD62L^{hi}$ cellular populations.

CSU 21 and CSU 27 had similar levels of $CD4^+CD44^+CD62L^{lo}$ (CD4 effector T cells) at day 7, 30, and 60, which can be seen in Figure 4. From day 60 to day 90 this cell population increased from both groups, but the increase was a lot more dramatic for CSU 21, with it reaching a level of 3×10^6 cells, compared to a level of 1.8×10^6 cells for CSU 27.

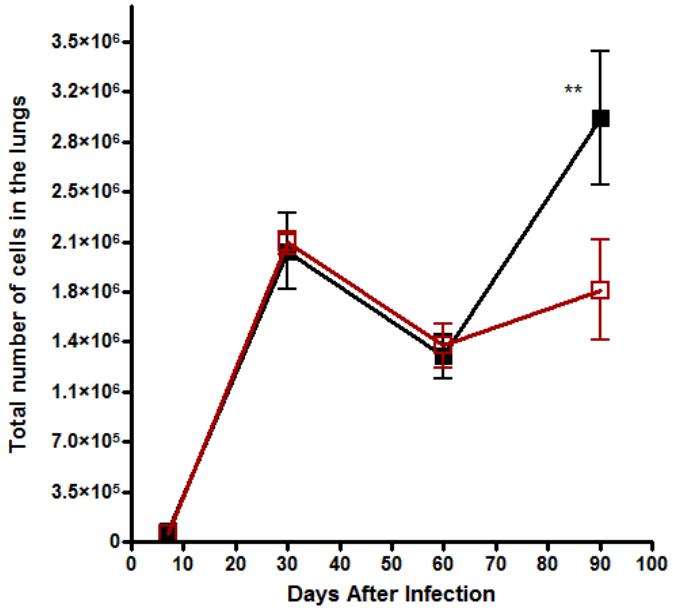

Fig. 4 changes in $CD4^{+}CD44^{+}CD62L^{lo}$ in lungs over the course of the study for CSU 21 (■) and CSU 27(□). ** P<0.05

The pattern that was observed for the effector subset, $CD4^{+}CD44^{+}CD62L^{lo}$, of CD4 T cells of CSU 21 and CSU 27 was not evident for both CSU 23 and CSU 9. CSU 23 had a similar pattern of increasing from day 7 to day 30, decreasing to day 60, and then increasing once again to day 90. The cellular levels at day 30 and 90 were very similar, being approximately 2.8×10^6 at day 30 and 2.5×10^6 at day 90. CSU 9 did not follow this cellular influx pattern. The cell influx for this strain into the lungs had a gradual increase that reached approximately 1.8×10^6 cells at day 90 (Figure 5).

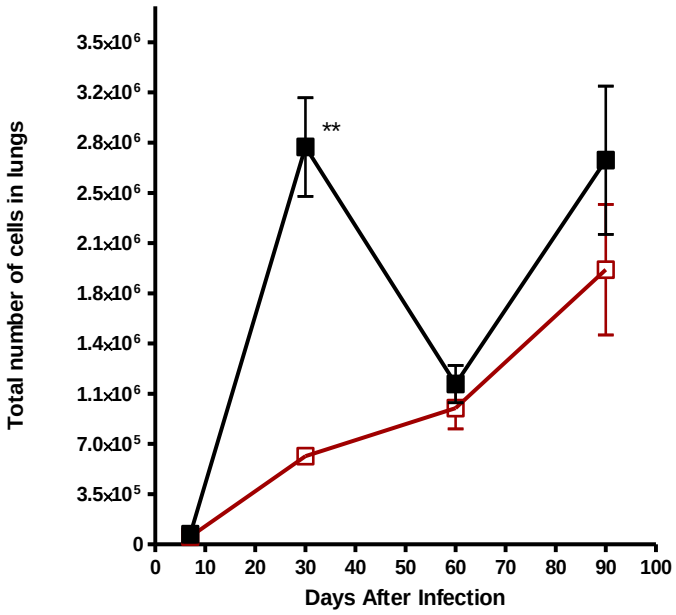

Fig. 5 changes in $CD4^+CD44^+CD62L^{lo}$ in lungs over the course of they study for CSU 9 (☐) and CSU 23 (■). ** P<0.05

Levels of $CD4^+CD44^+CD62L^{hi}$, CD4 memory T cells in the infected mice

$CD4^+CD44^+CD62L^{hi}$, CD4 memory T cells were gated as shown in Figure 3.

In CSU 21 and CSU 27 there was a slight influx into the lungs of $CD4^+CD44^+CD62L^{hi}$ cells reaching a level of roughly 1 x 10^5 cells at day 30 from the onset of infection. This particular cell population decreased a little to day 60 then had a large increased in the cells coming into the lungs. As with the CD4 effector numbers for CSU 21, there was a large increase of the cellular subset for CSU 21 compared to CSU 27 at day 90 (Figure 6).

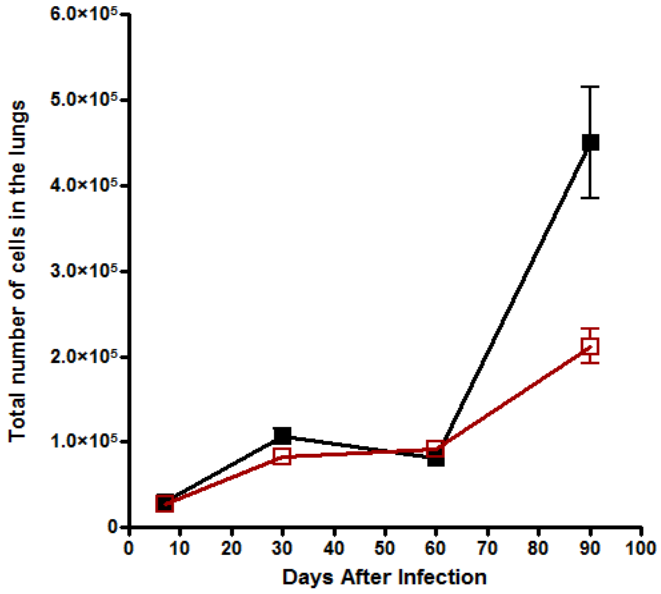

Fig. 6 changes in $CD4^{+}CD44^{+}CD62L^{hi}$ in lungs over the course of the study for CSU 21 (■) and CSU 27 (□).

In the CD4 T cell memory cell population, CSU 23 and CSU 9 induced similar levels to one another, also in line with CSU 21 and CSU 27. There was a slight influx of this subset into the lungs at day 30 that decreased, then increased once again. The levels for $CD4^{+}CD44^{+}CD62L^{hi}$ cells for the two strains were close to one another as see in Figure 7.

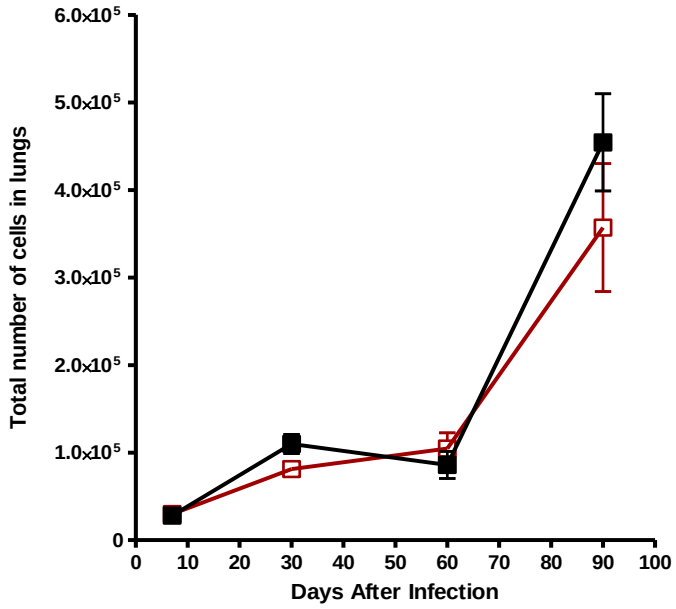

Fig. 7 changes in $CD4^{+}CD44^{+}CD62L^{hi}$ in lungs over the course of they study for CSU 9 (□) and CSU 23 (■).

Levels of $CD8^{+}CD44^{+}CD62L^{lo}$, CD8 effector T cells in the infected mice

To show the appropriate population(s) of CD8 effector T cells – $CD8^{+}CD44^{+}CD62L^{lo}$ the gates in Figure 8 were used.

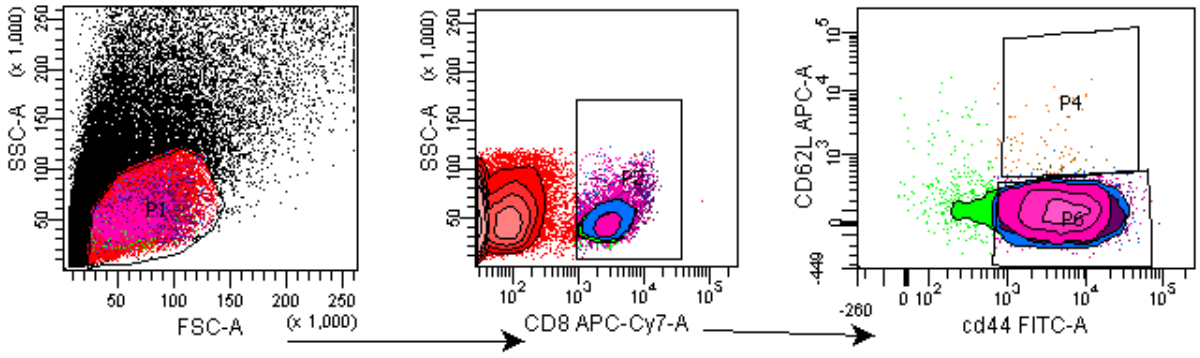

Fig. 8 gating strategies for $CD8^{+}CD44^{+}CD62L^{lo}$ and $CD8^{+}CD44^{+}CD62L^{hi}$ cellular populations

The cellular subset of $CD8^{+}CD44^{+}CD62L^{lo}$ T cells reached a level of nearly 1 x 10^{6} cells in the lungs at day 30 for CSU 27, then declined to a level of 7.5 x 10^{5} cells at day 90. In CSU 21 the day 30 level of this cellular subset was about 7.5 x 10^{5} cells, held steady until day 60, where it then increased to about 1.3 x 10^{6} cells at day 90 (Figure 9).

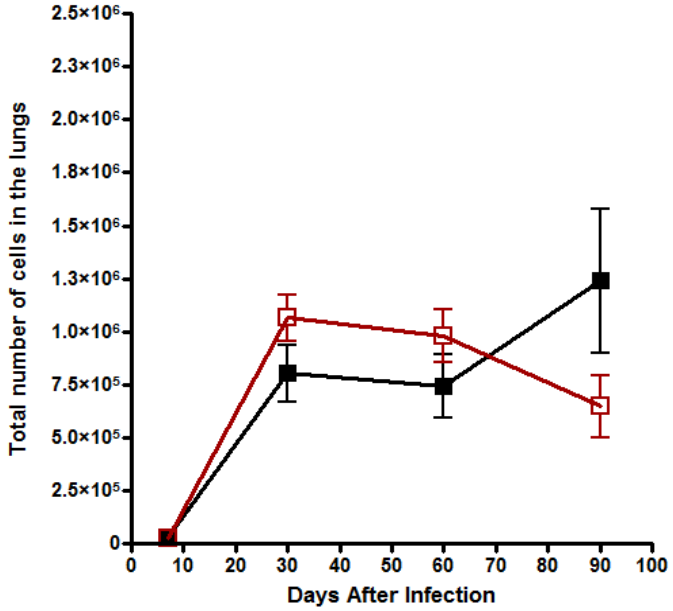

Fig. 9 changes in $CD8^{+}CD44^{+}CD62L^{lo}$ in lungs over the course of the study for CSU 21 (■) and CSU 27 (□).

CSU 23 had a large influx of CD8 effector cells into the lungs at day 30, reaching a level of approximately 1.3 x 10^{6} cells, then declined to a level of 6.0 x 10^{5} cells at day 60, where it then held steady until day 90. The cellular influx for $CD8^{+}CD44^{+}CD62L^{lo}$ cells with strain CSU 9 was similar to the influx pattern seen with $CD4^{+}CD44^{+}CD62L^{lo}$ with this strain. As seen in Figure 10 the cellular levels in the lungs gradually increased to roughly 6 x 10^{5} cells at day 90.

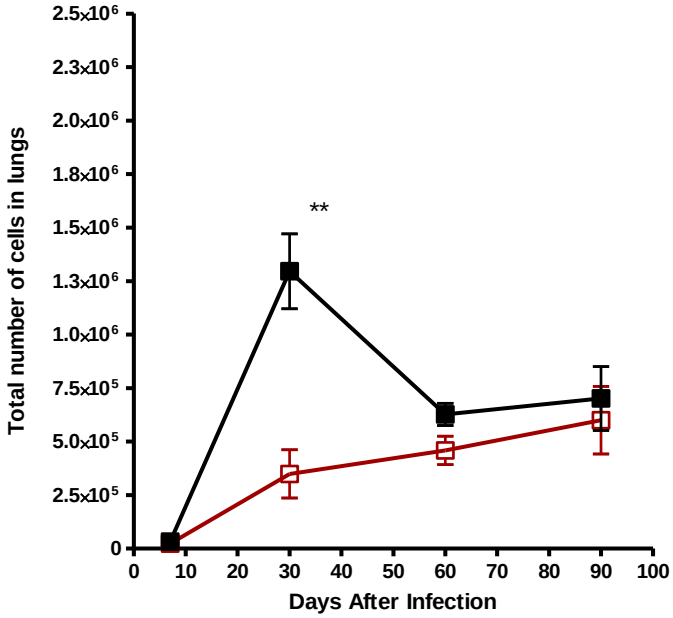

Fig. 10 changes in $CD8^{+}CD44^{+}CD62L^{lo}$ in lungs over the course of they study for CSU 9 (□) and CSU 23 (■). ** P<0.05

Levels of $CD8^{+}CD44^{+}CD62L^{hi}$, CD8 memory T cells in the infected mice

$CD8^{+}CD44^{+}CD62L^{hi}$, $CD8^{+}$ T memory cells were gated in forward and side scatter schemes to show the populations as seen in Figure 8.

The pattern for CD8 T memory cells ($CD8^{+}CD44^{+}CD62L^{hi}$) for cellular influx into the lungs for CSU strains 21 and 27 was the opposite as seen in the influx patterns/levels for CD4 T memory cells. Where the CD4 memory cells for these strains had low cellular levels until day 60 and then increased, as indicated in Figure 10 to the levels for CD8+ T memory cells for CSU 21 and CSU 27 were high in the beginning and then either held constant for CSU 21 or decreased in CSU 27 (Figure 11).

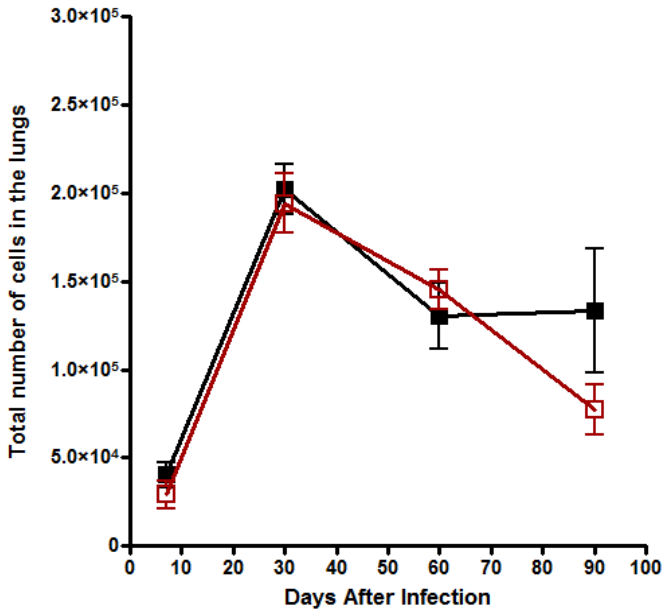

Fig. 11 changes in $CD8^{+}CD44^{+}CD62L^{hi}$ in lungs over the course of the study for CSU 21 (■) and CSU 27 (□). .

CSU 23 had a similar cell influx pattern as CSU 21 and CSU 27, a large peak at day 30, decrease until day 60 and constant cellular levels until day 90. CSU 9 did not follow the pattern of the other three strains. This strain had a steadily increasing population level until day 60 where it reached a level of approximately 1.5×10^5 cells then had a slight decline until day 90 (Figure 12).

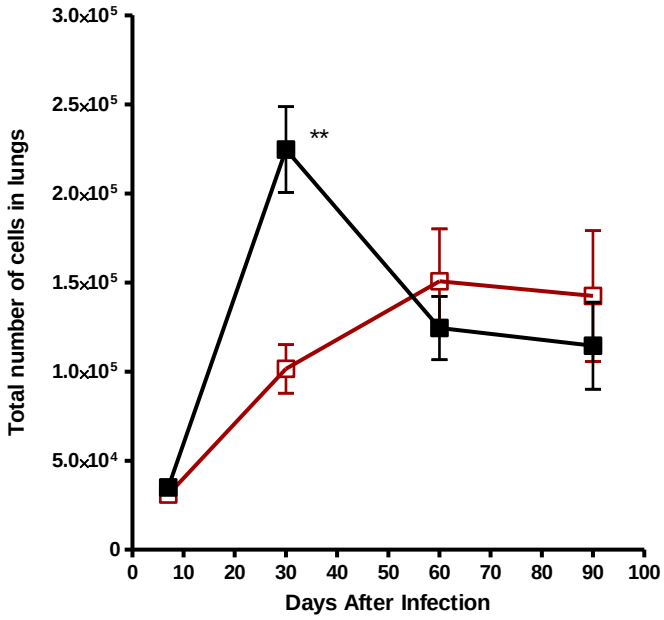

Fig. 12 changes in $CD8^{+}CD44^{+}CD62L^{hi}$ in lungs over the course of they study for CSU 9 (□) and CSU 23 (■). ** P<0.05

Levels of $CD4^{+}IFN\text{-}\gamma^{+}$ cells in the infected mice

The appropriate lymphocyte population (s) to show $CD4^{+}IFN\text{-}\gamma^{+}$ cells were gated as shown in Figure 13.

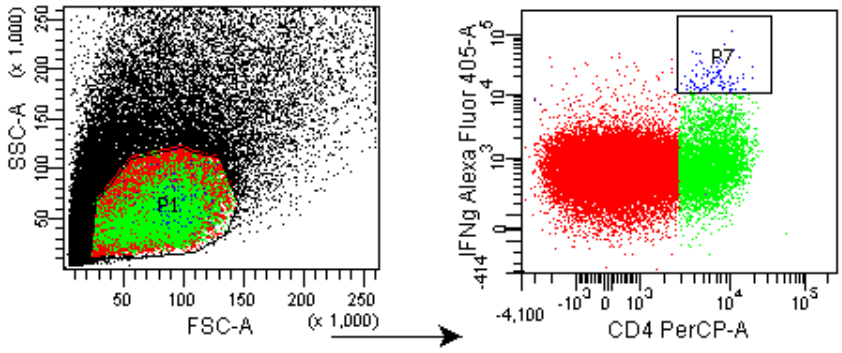

Fig. 13 gating strategies for $CD^{+}IFN\text{-}\gamma^{+}$ cellular population

$CD4^+IFN\text{-}\gamma^+$ levels for CSU 21 and CSU 27 peaked at day 30 at approximately 7.5 x 10^5 cells and then dropped to a level of roughly 5 x10^5 cells at day 60. There was a slight increase for CSU 21 at day 90, while CSU 27 declined to close to 2.5 x 10^5 cells at day 90 (Figure 14).

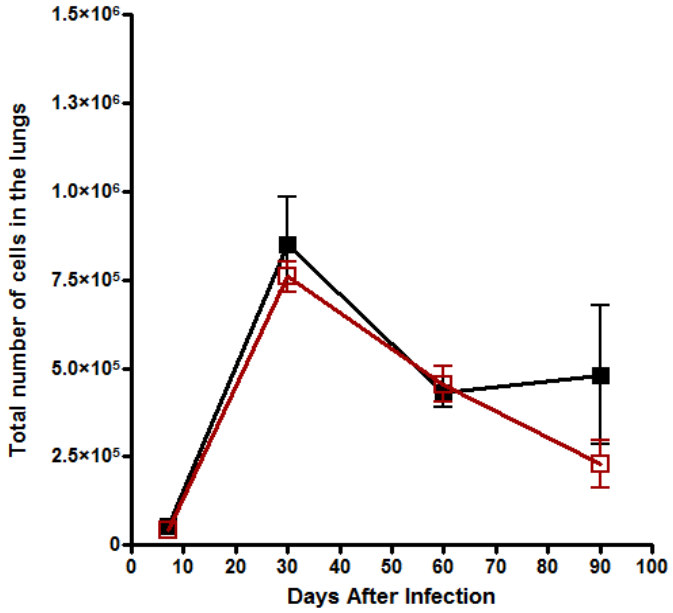

Fig. 14 changes in $CD4^+IFN\gamma^+$ in lungs over the course of the study for CSU 21 (■) and CSU 27 (□).

CSU 23 had a rapid influx of $CD4^+IFN\text{-}\gamma^+$ cells at day 30 with a level of approximately 1 x 10^6 that then steadily declined to roughly 4 x 10^5 cells at day 90. CSU 9 had a constant influx of $CD4^+IFN\text{-}\gamma^+$ cells into the lungs throughout the experiment, reaching a level of approximately 5 x 10^5 cells at day 90 (Figure 15).

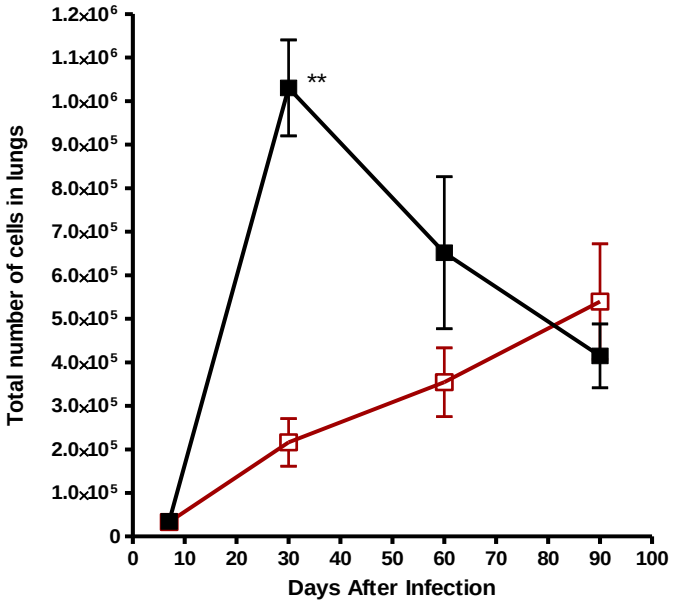

Fig. 15 changes in $CD4^{+}IFN\text{-}\gamma^{+}$ in lungs over the course of the study for CSU 9 (□) and CSU 23 (■). * P<0.05

Levels of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells in the infected mice

T regulatory cells, $CD4^{+}CD25^{+}Foxp3^{+}$, were gated as indicated in Figure 16.

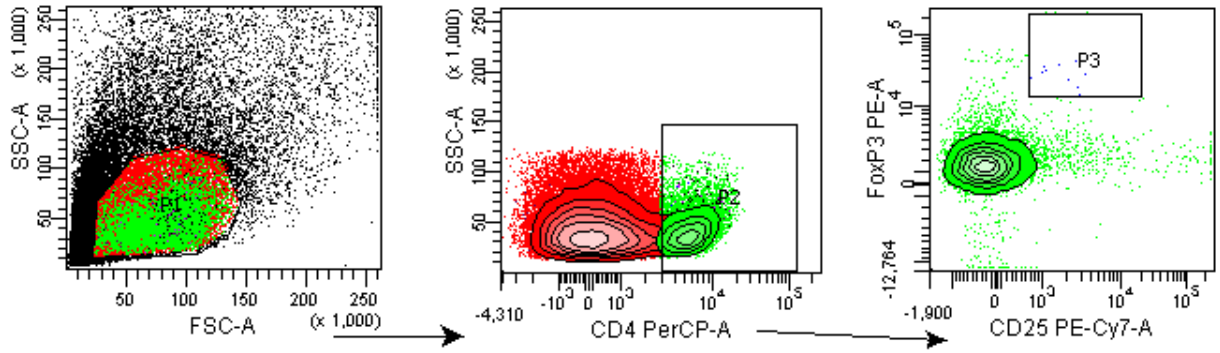

Fig. 16 gating strategies of $CD4^{+}CD25^{+}Foxp3^{+}$ cellular population

The levels for $CD4^+CD25^+Foxp3^+$ regulatory T cells did not start to increase for CSU 21 and CSU 27 until day 60, with levels being near 5×10^4. Strain CSU 21 increased to a level of approximately 6×10^5 at day 90, while CSU 27 had very low levels of Treg cells in comparison, as indicated in Figure 17.

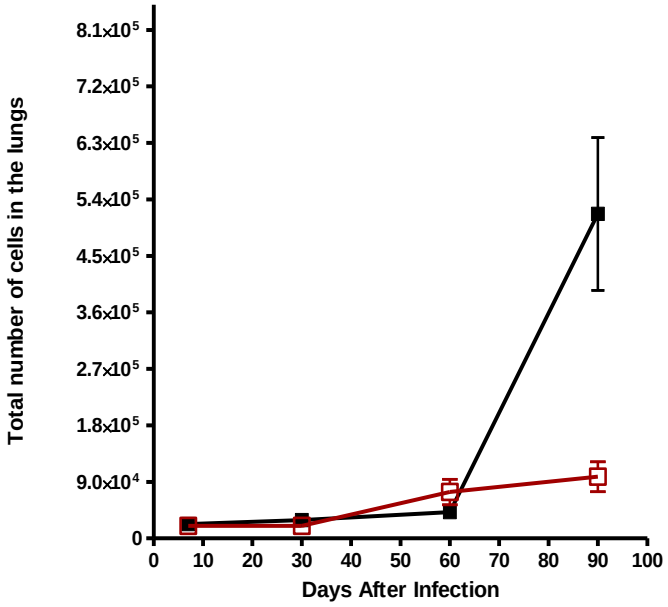

Fig. 17 changes in $CD4^+CD25^+Foxp3^+$ in lungs over the course of the study for CSU 21 (■) and CSU 27 (□).

In CSU 23 and CSU 9, a difference in cellular influx numbers for Tregs was evident at day 30, but was not really obvious until day 60 as evident in Figure 18. At day 60 CSU 23 had a Treg level of approximatly 2.3×10^5 cells in the lungs, whereas the Treg cell level for Strain CSU 9 was roughly 7.5×10^4 at the same time point. Both strains continued to have a cellular influx of T regulatory cells into the lungs, though CSU 23 had a lot higher levels.

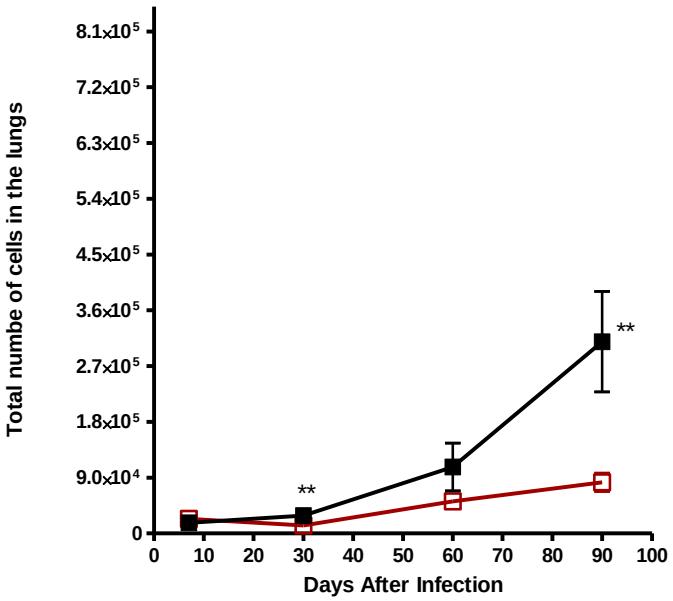

Fig. 18 changes in $CD4^{+}CD25^{+}Foxp3^{+}$ in lungs over the course of the study for CSU 9 (□) and CSU 23 (■). ** P<0.05

Levels of $CD4^{+}IL\ 17^{+}$, T helper 17 (Th17) cells in the infected mice

TH17 cells, $CD4^{+}IL17^{+}$ cells, were properly gated as observed in Figure 19.

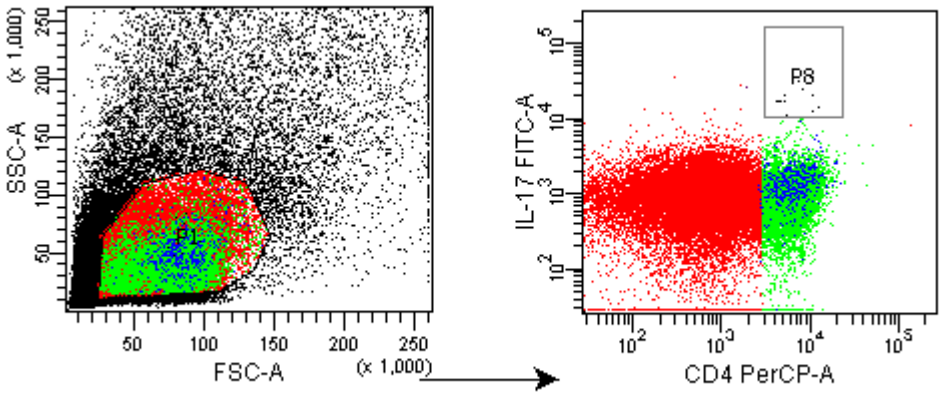

Fig. 19 gating strategies of $CD4^{+}IL\ 17^{+}$ cellular population

The cellular influx pattern(s) for Th17 ($CD4^{+}IL17^{+}$) cells for CSU 21 and 27 were similar to that seen for the Treg cell subset for these two strains. CSU 21 had a level of roughly 2.5 $x10^{5}$ cells at day 30, decreased at day 60, and then dramatically increased at day 90 with a cellular influx level of approximately of 2.8 x 10^{6} cells at day 90. The TH17 cells levels for CSU 27 were similar from day 7 to day 30, where they then started to increase to a level of approximately 7.5 $x10^{5}$ cells at day 90 (Figure 20).

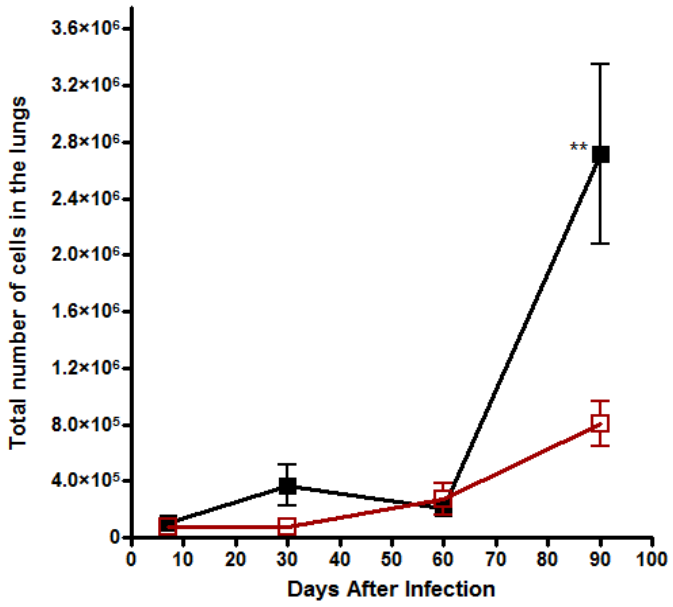

Fig. 20 changes in $CD4^{+}IL\text{-}17^{+}$ in lungs over the course of the study for CSU 21 (■) and CSU 27 (□). **P<0.05

The TH17 levels for both CSU strains 23 and CSU 9 were constant from day 7 to day 30 where an increase in the cellular influx of TH17 cells occurred for both strain. The rate of increase for CSU 23 was progressive for the whole experiment, resulting in a level of approximately 9 x 10^5 cells at day 90. Levels at day 90 for CSU 9 were close to 5 x 10^5, significantly lower than the level for CSU 23 (Figure 21).

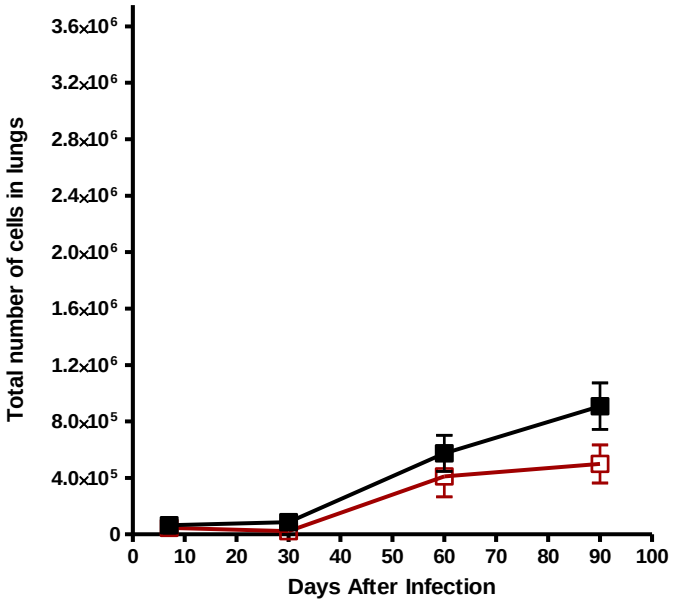

Fig. 21 changes in $CD4^+IL\text{-}17^+$ in lungs over the course of the study for CSU 9 (□) and CSU 23 (■).

Histopathological analysis of isogenic pairs

In both CSU 21 and CSU 27 there was no significant pathology present at day 7. There was evidence of infiltration of histiocytic cells at day 30 (Figure 22F). The histolytic cells are primarily foamy macrophages. For CSU 21 these cells have started to coalesce together. At day 30 there is evidence of lymphocytes infiltration, lymphocytes continue to increase at day 60 (Fig. 22C and G). The lymphocytes also start to form foci at day 60. There is no appreciable change between days 60 and 90 for either strain.

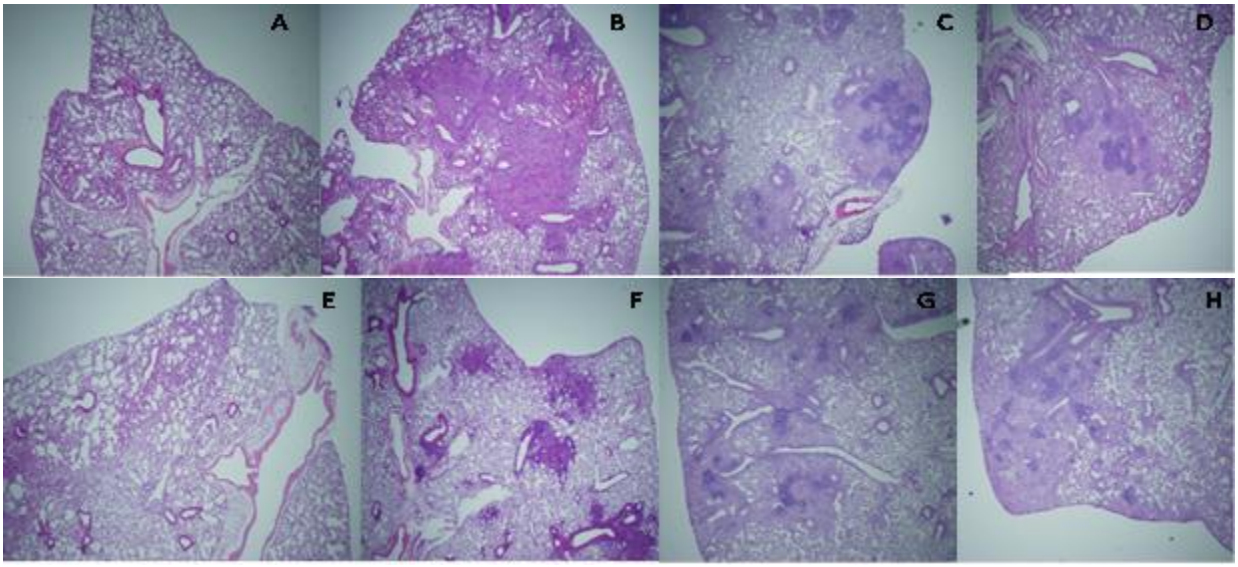

Fig. 22 Increase lung pathology of mice infected with CSU #21 (A-D) and CSU #27 (E-H). A,E d7, B,F d30 C,G, d60 D,H d90. Total original magnification is 2x.

Isolates CSU 9 and CSU 23 follow a similar pattern to what was seen in CSU 21 and CSU 27. At day 7 there is no evidence of any significant pathological change. There is a distinct change in pathology between days 7 and 30 for both of the strains. Histiocytic cells, such as foamy macrophages, start to infiltrate the lung tissue pictured. While the majority of cells present at day 30 are foamy macrophages, there is evidence of some lymphocytes also. The

number of lymphocytes present in the lung tissue increased from day 30 to day 60. At day 60 the lymphocytes start to aggregate together. In CSU 9 and CSU 23 it was noticed that the number of lymphocytes present in the tissue decreased from day 60 to day 90, otherwise there was no noticeable change between these time points as indicated in Figure 23.

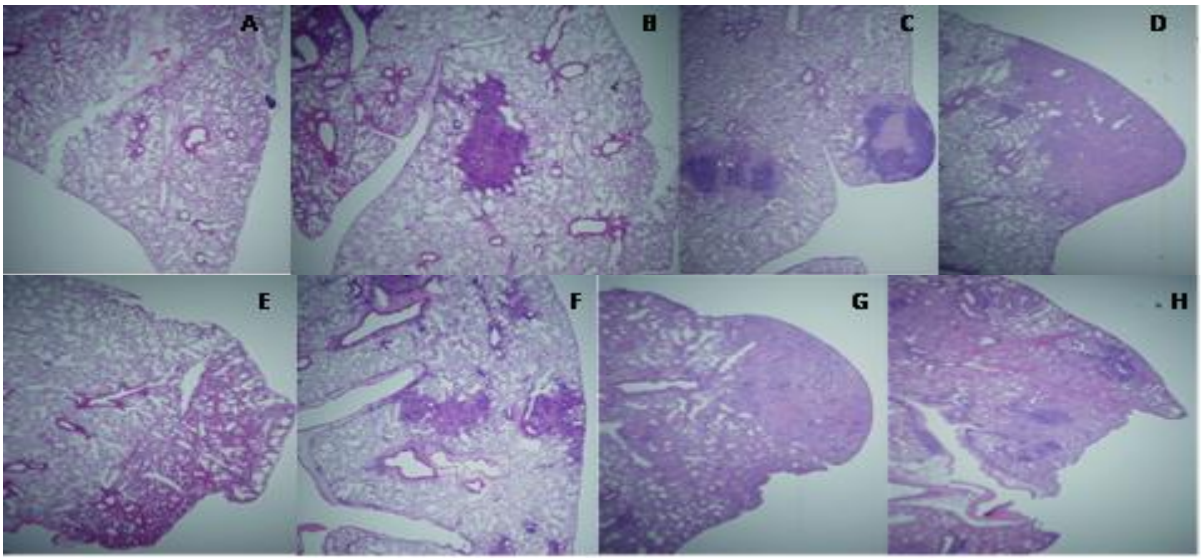

Fig. 23 Increase lung pathology of mice infected with CSU #9 (A-D) and CSU #23 (E-H). A,E d7, B,F d30 C,G, d60 D,H d90. Total original magnification is 2x.

Section 2: Studies examining the virulence of high and low transmission strains

Organ Bacterial Burdens of lungs and spleens for the W – Beijing strains 4334 (high transmission) and 3507 (low transmission)

In a further set of experiment the virulence of two W – Beijing strains was compared; low transmission strain 3507 and 4334 high transmission.

The two W – Beijing strains, had similar growth patterns to each other as evident in Figure 24A. At day 7, 3507 was about a log higher than 4334 in the lungs, but they both peaked at day 30 with approximately 7.5-8 logs in the lungs. From this point the burdens decreased until becoming stable.

In the spleens the two W – Beijing strains were nearly identical in the organ bacterial burdens as depicted in Figure 24B. Both of the strains had a burden of approximately 2 logs at day 7 and ended with a burden of roughly 6 logs in this organ at day 90.

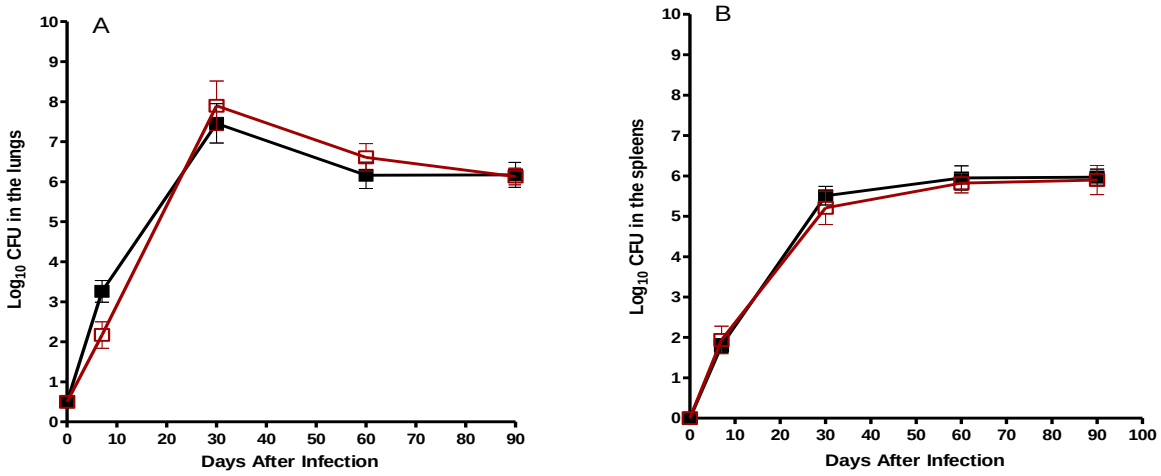

Fig. 24 bacterial counts in lung (A) and spleen (B) of mice infected with 3507 (■) and 4334 (□) at days 7, 30, 60, and 90. The results are expressed as average (n =5) bacterial loads in each group expressed as the $\log_{10}$ number of CFU (± SEM).

Flow cytometry analysis of the lungs for the W – Beijing strains 4334 (high transmission) and 3507 (low transmission)

Levels of $CD4^+CD44^+CD62L^{lo}$, CD4 effector T cells in the infected mice

At day 30 3507 had higher levels of CD4 T effector ($CD4^+CD44^+CD62L^{lo}$) cells in the lungs than 4334 did, approximately 4.0 x 10^6 and 2.0 x 10^6 respectively. The number of cells for this cellular subset decreased to approximately 2.5 x 10^6 cells at day 60 and then increased to roughly 3.5 x 10^6 at day 90 for 3507. After the large influx of this subset for 4334, there was a gradual increase of CD4 effector T cells, until day 90 with a level close to 2.5 x 10^6 cells (Figure 25).

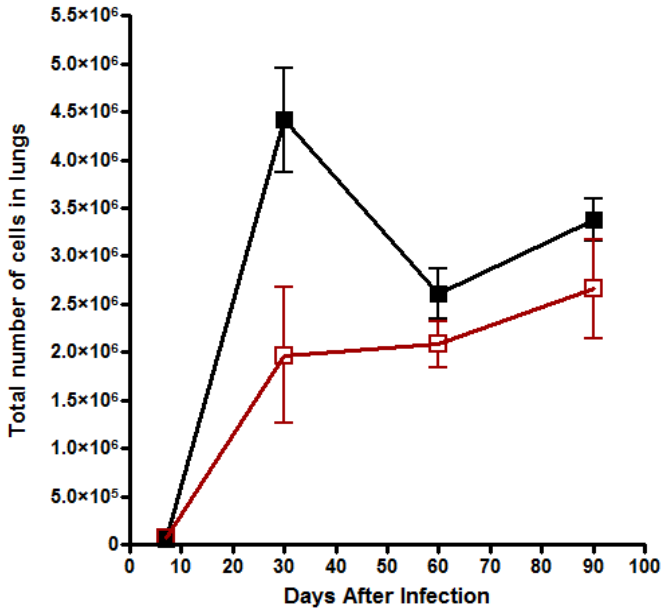

Fig. 25 changes in $CD4^+CD44^+CD62L^{lo}$ in lungs over the course of the study for 3507 (■) and 4334 (□). .

Levels of $CD4^{+}CD44^{+}CD62L^{hi}$, CD4 memory T cells in the infected mice

CD4 memory T cells levels for 3507 and 4334 reached levels ranging from 1 x 10^5 and 1.5 x 10^5 at day 30, where 4334 stayed the same and 3507 decreased to roughly 1 x 10^5 cells in the lungs at day 60. Both strains increased dramatically at day 90, with 3507 reaching levels of around 4.5 x 10^5 and 4334 with levels of approximately 3 x 10^5 at day 90 (Figure 26).

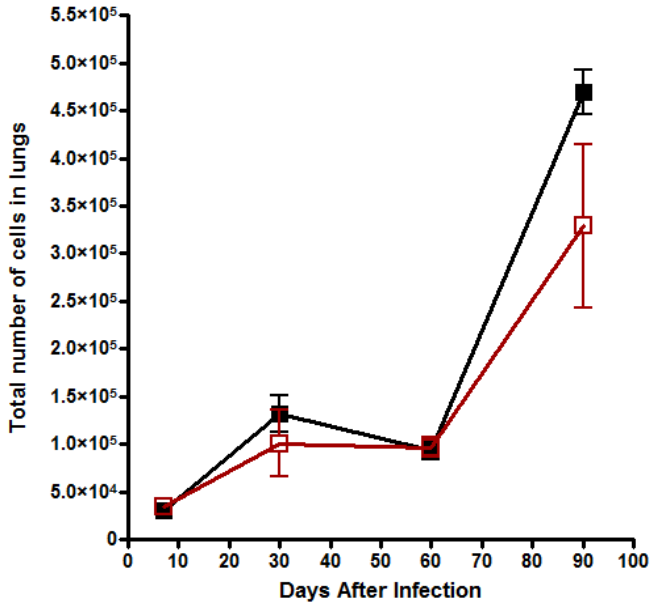

Fig. 26 changes in $CD4^{+}CD44^{+}CD62L^{hi}$ in lungs over the course of they study for 3507 (■) and 4334 (□).

Levels of CD8 $CD8^{+}CD44^{+}CD62L^{lo}$, CD8 effector T cells in the infected mice

$CD8^{+}CD44^{+}CD62L^{lo}$ cell levels for both W – Beijing strains, 3507 and 4334, increased to levels of roughly 1.8 x 10^6 and 1.5 x 10^6 respectively at day 60. 3507 then declined to approximately 1.6 x 10^6 at 90, with 4334 reaching a level of CD8 effector T cells of roughly 1.1 x 10^6 in the lungs at day 90 (Figure 27).

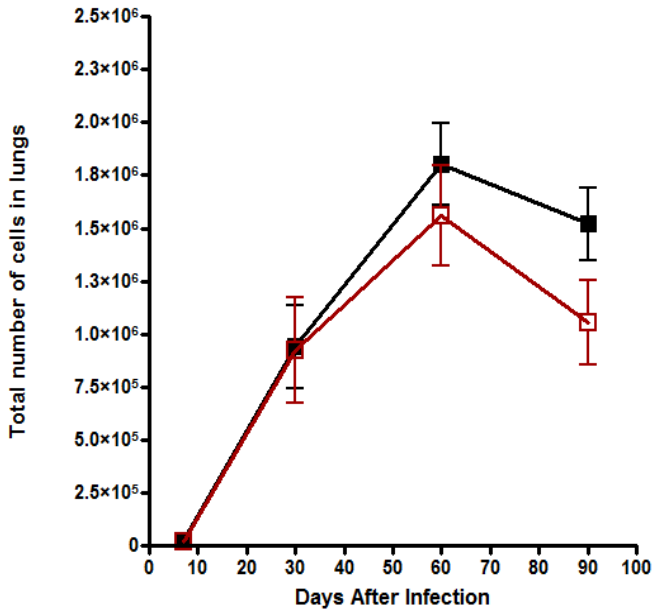

Fig. 27 changes in $CD8^+CD44^+CD62L^{lo}$ in lungs over the course of the study for 3507 (■) and 4334 (□).

Levels of $CD8^+CD44^+CD62L^{hi}$, CD8 memory T cells in the infected mice

Total numbers of $CD8^+CD44^+CD62L^{hi}$ T cells for 4334 reached its peak at day 30, with nearly 1 x 10^5 cells and then held constant for the remainder of the experiment. 3507 had the same levels of cells as 4334 for both day 7 (a little less than 5 x 10^4 cells) and day 30 (approximately 1 x 10^5) and then CD8 memory T cell levels for this strain continued to increase until day 90, reaching a level close to 2 x 10^5 cells (Figure 28).

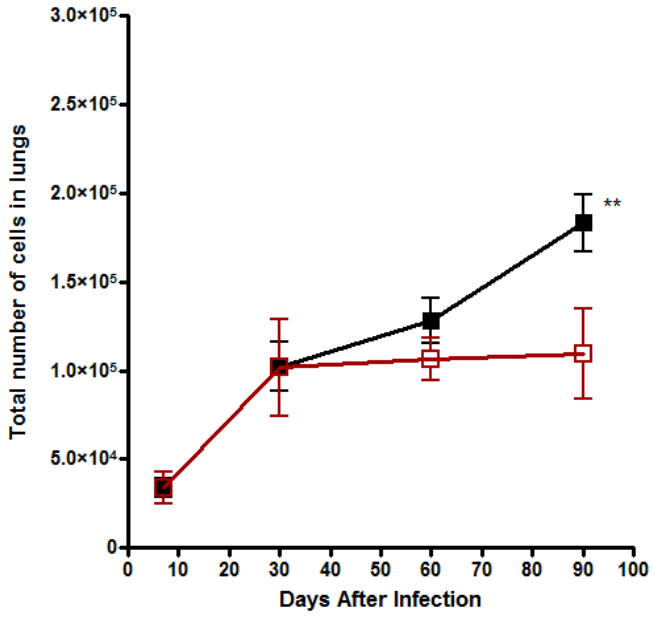

Fig. 28 changes in $CD8^+CD44^+CD62L^{hi}$ in lungs over the course of the study for 3507 (■) and 4334 (□). ** P<0.05

Levels of $CD4^+IFN\text{-}\gamma^+$ cells in the infected mice

Levels of IFN-γ producing $CD4^+$ T cells reached a peak of approximately 9 x 10^5 cells for strain 3507 at day 30, and then had a sharp decline in cell numbers, until reaching an approximate level of 4 x 10^5 cells at day 90. As indicated in Figure 29, the levels of $CD4^+IFN\text{-}\gamma^+$ cells increased to roughly 5 x 10^5 cells for 4334 at day 30, where they then stayed constant until dropping to a level near 4 x 10^5 at day 90.

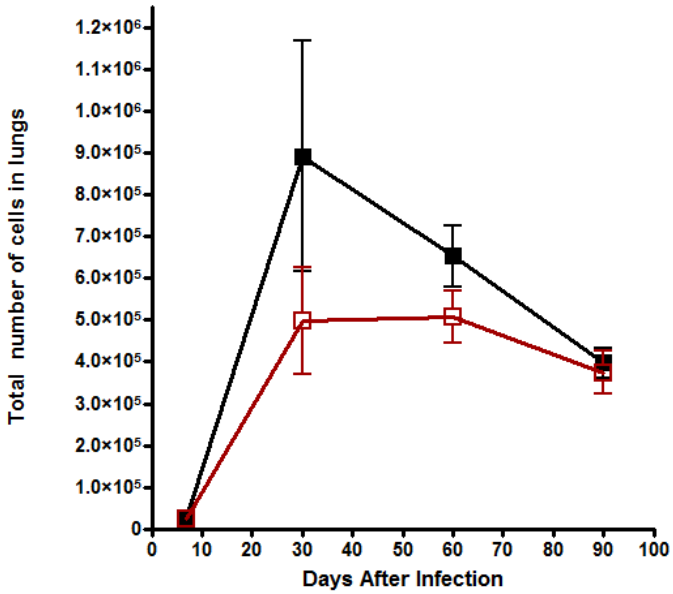

Fig. 29 changes in $CD4^{+}IFN\text{-}\gamma^{+}$ in lungs over the course of the study for 3507 (■) and 4334 (□).

Levels of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells in the infected mice

3507 had higher levels of T regulatory cells earlier in the infection than 4334 did. At day 30, 3507 had an approximate level of 1 x 10^5 Treg cells, with this level increasing to approximately 1.3 x 10^5 at day 60, then dropping back to roughly 1 x 10^5 cells at day 90. Whereas it took longer for Treg cells to be induced for 4334, the resulting levels of day 90 were higher, with a level of approximately 2 x 10^5 cells (Figure 30).

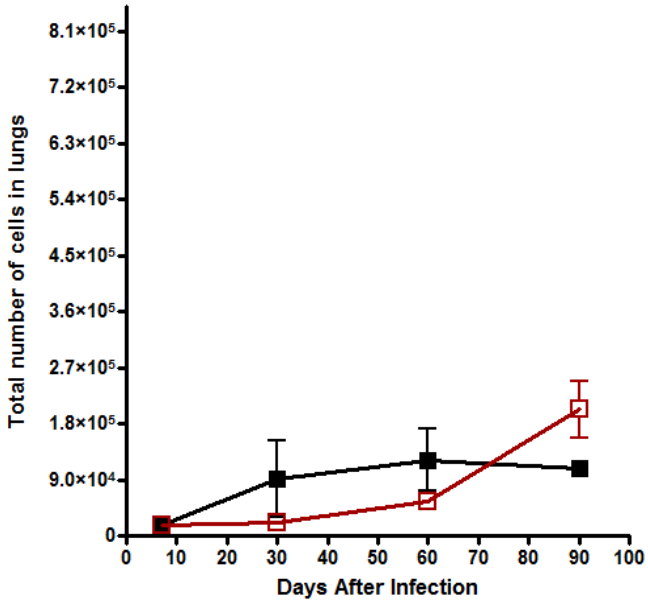

Fig. 30 changes in $CD4^+CD25^+Foxp3^+$ in lungs over the course of they study for 3507 (■) and 4334 (□).

Levels of $CD4^+IL17^+$, T helper 17 (TH17) cells in the infected mice

Strains 3507 and 4334 had similar levels of $CD4^+IL17^+$ cells at days 7, 30, and 60. At day 60 the two strains continued to increase in the total cells in the lungs, but the rates of influx were different. 4334 had a final cell total of approximately of 2.2 x 10^6 at day 90, whereas 3507 had roughly 1.5 x10^6 cells in the lungs at this time (Figure 31).

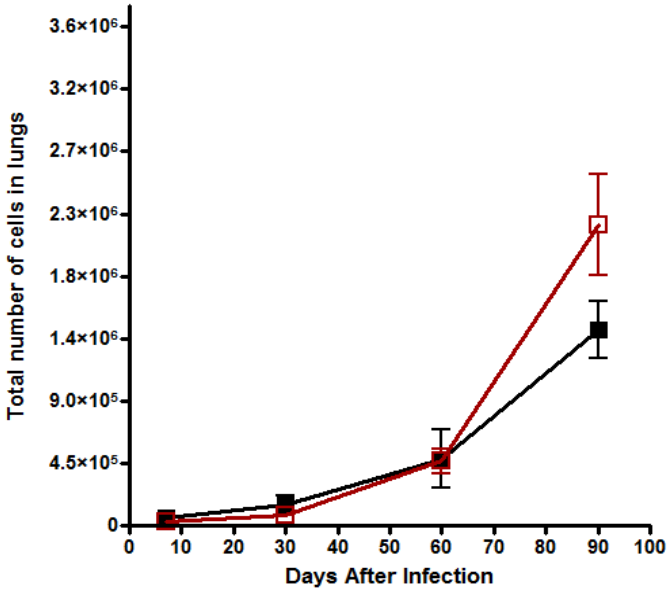

Fig. 31 changes in $CD4^{+}IL\text{-}17^{+}$ in lungs over the course of the study for 3507 (■) and 4334 (□). .

Histopathological analysis of W – Beijing strains 4334 (high transmission) and 3507 (low transmission)

3507 and 4334 had no significant pathology at day 7. There was an increase of histiocytic cells into the lungs at day 30. Of the tissue sections pictured in Figure 32 the infiltration of these histiocytic cells take up a good portion of the tissue. In 3507 (Fig. 32B) there is evidence of lymphocytes starting to aggregate together compared to 4334 (Fig. 32F). Another distinct change in pathology occurs between day 30 and day 60 for 3507 and 4334, with more lymphocytes present. There is a stalling of any pathological change between day 60 and day 90.

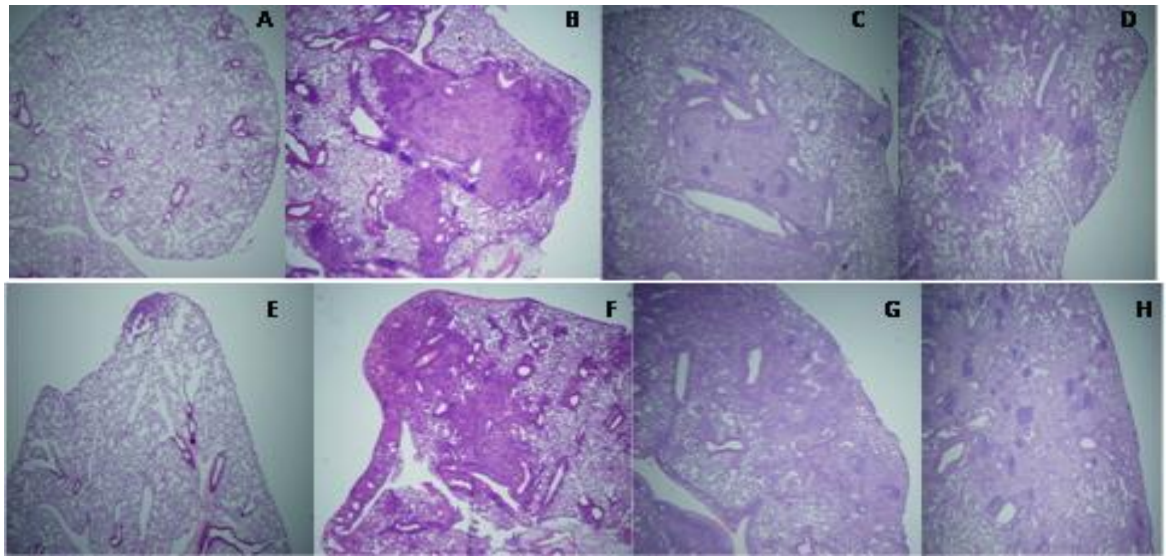

Fig. 32 Increase lung pathology of mice infected with 3507 (A-D) and 4334 (E-H). A,E d7, B,F d30 C,G, d60 D,H d90. Total original magnification is 2x.

Discussion

The purpose of this study was to begin to answer some fundamental questions about the impact of drug resistance on the virulence for *M. tuberculosis*. An early hypothesis, based on the classical studies of Mitchison who studied the effects of INH resistant clinical isolates in the guinea pig model of tuberculosis, was that resistant tuberculosis strains had lower virulence levels (114). A murine study conducted by Ordway et al however found no clear relationship between multi-drug resistant bacteria and virulence levels (117), with most strains showing considerable virulence.

The drug resistant strains that were studied here were highly defined isogenic pairs, where one strain had drug resistance while the other isogenic strain in the pair was drug susceptible. In the first study in which CSU 27 was used, which is resistant to INH, and CSU 9 which is resistant to RIF. The results of this study are similar to those of Ordway with drug resistant strains exhibiting a range of virulence. CSU 27, which is drug resistant, had a higher organ burden in both the lungs and spleens studied than its isogenic pair at days 30, 60, and 90. In contrast, in the other iosgenic pair studied, a lower virulence level for the drug resistant strain was observed. CSU 9 had easily a log difference in the organ burden for the lungs as days 30, 60, and 90. The organ burden for the spleens between CSU 9 and CSU 23 were much closer at all time points.

There is a scarcity of data pertaining to the growth kinetics of multiple drug resistant bacteria. For the murine model, there are only a couple of other laboratories, besides Ordway, that have published their results. Dave and colleagues compared the growth patterns of multi drug resistance against susceptible isolates of *M. tuberculosis* in mouse lungs and had similar

findings to Ordway. This group found no significant relationship between drug resistance and survival time/bacterial burden (118). Timm and colleagues reported studies in cultured mouse macrophages and their results support previous findings about challenging the view that MDR bacteria are less fit than drug susceptible bacteria (119). Future studies regarding the growth kinetics of multiple drug resistant bacteria in vivo are essential. Such studies are important to understanding the biological differences that are significant for pathogenesis, alternative methods of treatment, new vaccine and chemotherapy agent development and for the better understanding of the host-parasite relationships.

As an indication of protective immunity to each infection, it was found that the levels of $CD4^{+}IFN\gamma^{+}$ cells in mice infected with each of the strains was different. In the isogenic pair consisting of CSU 21 and CSU 27 the cell totals were approximately the same at all the time points, except between day 60 and 90 where a decrease in numbers in the lungs of mice infected with the drug-resistant strain CSU-27 was observed, along with a slight increase in mice infected with strain CSU 21. In the other isogenic pair studied there were clear differences in the numbers of IFN –γ producing $CD4^{+}$ T cells. Mice infected with CSU 23 had a spike in numbers at day 30 which then decreased, whereas the numbers of $CD4^{+}$ $IFN\gamma^{+}$ cells in mice infected with CSU 9 showed gradual increase throughout the study.

For approximately the first 30 days of infection, CD4 effector T cells were highly expressed, then the levels diminished. When the levels of this cell population increased again at day 60, expression of the memory phenotype for CD4 cells was also at high levels. Before day 60, memory CD4 cells were present, just not at the same levels seen with the CD4 effector T cells. CSU 9 had a different expression pattern for $CD4^{+}CD44^{+}CD62L^{hi}$ cells than the other

strains, with a progressive increase in cell numbers in the lung compared to large spikes in cell numbers. There were not as many CD8 effector T cells in mice infected with any of the strains, compared to CD4 effector T cells. CSU 9 had a similar cellular influx pattern for CD8 effector T cells as it did for CD4 effector T cells. The number of CD8 memory cells for strains CSU 21, CSU 27, and CSU 23 peaked earlier than CD4 memory T cells did, though once the CD8 memory T cells peaked there was no further influx of these cells into the lungs. The CD8 memory cells for CSU 9, like both subsets of CD4 and CD8 effector T cells, progressively increased in the lungs over time.

In the analysis of Treg and TH17 cells it was observed that the drug susceptible bacteria within each isogenic pair had a higher number of total cells, at each time point, compared to the drug resistant bacteria. The inflammatory Th17 cells are believed to be one of the first subsets of cells present at the site of infection. The inflammation that is caused by these cells is important in recruitment of other cells, such as antigen presenting cells that interact with incoming CD4 or CD8 effector T cells. As the immune response continues to expand, memory cells of either the CD4 or CD8 variety start to be detected. Memory cells are faster acting than effector cells since they have already seen a particular antigen and do not have to go through all the stages of development. When memory cells are present, inflammation is still high, so it is now thought that regulatory T cells are induced to help suppress the level of this inflammation. There is also increasing evidence that these cellular subsets are suppressive against one another. Currently, it is hypothesized that Treg cells most likely suppress Th17 cells through the production of the immunosuppressive cytokine IL-10.

Drug resistant strains are not the only cause for concern in regards to the tuberculosis epidemic. The spreading of W – Beijing strains, in general, are also causing increasing concern. Very little is known about the W – Beijing family of strains. Early reports by Manca et al suggest that the higher virulence of these strains was due to their inability to induce TH1 immunity and produce IFN-γ. They found that at several later time points in an infection with HN878 (a W – Beijing strain) T cell proliferation and IFN-γ production in response to mycobacterial antigens was significantly lower in spleen cells than mice infected with NHN5 (a non W – Beijing strain). They hypothesized that the increased mortality of mice infected with HN878 appeared to be associated with a decreased ability of their spleen and draining lymph node cells to proliferate and produce IFN-γ in response to M. tuberculosis antigen. Through cytokine tests they also found a diminished IL-12 mediated Th-1 type response (120). It has since been reported by Ordway that W – Beijing strains actually do produce IFN-γ and are potent inducers of TH1 immunity (121).

In the study reported here, we were given the opportunity to study two strains from a recent outbreak in San Francisco, CA, USA that differed in their ability to be transmitted. Strain 4334 is considered to be a high transmission strain, while 3507 is thought to be a low transmission strain. It is a reasonable assumption that transmission rates, especially of W – Beijing strains, may well be related to virulence levels, with higher transmission strains being of higher virulence

This hypothesis was not however supported by the results of this study. When the bacteria burdens for both the lungs and spleens were analyzed, no statistically significant difference was observed between the two strains at any of the time points observed. The total

number of inflammatory TH17 cells seen in mice infected with strains 3507 and 4334 was only different at day 90. Strain 4334, the high transmission strain, had higher levels of TH17 cells, but this was not a significant difference, with the P value being greater than 0.05. The low transmission strain, 3507, had higher numbers of Treg cells up to day 60, after which the numbers of these cells declined, compared to an increase in these cells in mice infected with the highly virulent 4334 strain.

These results also support the previous findings of Ordway in regards to the ability of W – Beijing strains to induce the production by host T cells to produce IFN γ (121). After the initial increase in IFN – γ producing cells in the lungs, those in 3507 infected mice decreased to a level of 4×10^5 cells at day 90, whereas those in 4334 infected mice remained constant between days 30 and 60, and then decreased to the same level of cells as seen in mice infected with 3507.

CD4 and CD8 effector T cell populations for mice infected with either 3507 or 4334 were decreasing at the time that T regulatory cells were increasing in the lungs of the infected mice. The populations of CD4 and CD8 effector cells, though, did not follow the same influx patterns. In the CD4 effector T cell population for mice infected with 3507 and 4334 there was a spike at day 30 and then a slight increase in total cell numbers found in the lungs. For the CD8 effector T cell population the spike occurred later in the infection and did not increase again. The memory cell population of CD4 and CD8 T cells was also different to one another. For the CD4 memory T cell population there was a spike at day 30 with an approximate level of 1.5×10^5 cells, a decrease in numbers, which was followed by a dramatic increase in total cell numbers between days 60 and 90. The memory population for CD8 T cells spiked earlier in the infection for both 3507 and 4334, and either continued to increase for 3507 or remained constant for 4334. The

memory and effector populations for CD4 cells followed a similar influx pattern of spiking at day 30, decreasing, and then increasing again, though the second increase for the memory cells was a lot higher than the effector cell population.

W – Beijing strains are worrisome because they are the cause of many community wide outbreaks throughout the world and they can spread rapidly. Of all the six isolates evaluated, the W – Beijing strains, 3507 and 4334, had some of the most pathological changes in the lung tissues. The worsened severity of the pathology noted in these two strains may be related to the number of T regulatory cells infiltrating the lungs. It has been recently shown by Ordway and colleagues that the emergence of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells can evade the protective immunity of BCG vaccination, in a model studying two well defined W – Beijing strains, an observation that has serious implications for new vaccine development (115). Given the possibility that widespread BCG vaccination programs may have eradicated strains of lower fitness, but failed to get rid of W – Beijing strains (high fitness) thus selected for the W – Beijing strains, it is currently unclear if any BCG-based vaccination will be effective in slowing the spread of W – Beijing based infections.

Chapter 3 – Evaluation of isogenic pairs of M. tuberculosis in the guinea pig model, in order to determine in acquired drug resistance increased or decreased virulence of the drug resistant strain

Introduction

Globally the tuberculosis epidemic continues unabated, affecting over nine million people a year, with more than half a million of these cases being resistant to multiple drugs. In comparison, the case rates in the United States continue to decline. In 2009, there were 11,545 reported cases of tuberculosis, which was a decline to 3.8 cases per 100,000 of the population, which is the lowest rate of decline since 1953, when reporting of the disease was initiated (25). The Center for Disease Control and Prevention and the National Institutes of Health in the United States had a 2010 national goal of TB elimination (defined as <0.1 case per 100,000 population), however this goal was not met. The unmet goal could be due to the fact that foreign borne people and racial/ethnic minorities are disproportionately affected by the tuberculosis disease. In 2009, 59% of the total United States TB cases occurred in foreign-born persons. Foreign-born Hispanics and Asians together represented 80% of reported TB cases in foreign-born persons, and accounted for 48% of the national total (1).

This population group is cause for concern because these people come to the United States already infected, and it is now being discovered that a portion of this population is infected with W-Beijing strains of tuberculosis. As discussed above, very little is known about the family of W-Beijing strains of tuberculosis, except that these strains have varying levels of virulence, usually at higher levels than typically seen with other strains. It has also been observed

that these strains can spread quickly, causing large community-wide TB outbreaks. There is concern that W-Beijing strains are being selected by the widespread use of the current vaccine, bacilli Calmette-Guérin (BCG), rendering the vaccine ineffective against these strains, and thus causing large community outbreaks of these strains (116). As noted above, many W-Beijing strains are also found to be multiple drug resistant (MDR), or even extremely drug resistant (XDR).

As discussed above multiple in vivo studies in the murine model of tuberculosis have since been conducted that show a variety of results in regards to drug resistance and virulence levels. The overall conclusion of the murine studies is that drug resistance does not always lend itself to lower virulence levels of the strains in question. Even less data exists in regards to the guinea pig model of infection. The guinea pig model of tuberculosis infection differs from the mouse model in that infected guinea pigs generate necrosis of the lungs, similar to humans, whereas mice do not produce necrotic lesions of the lungs. Palanisamy and colleagues (122) asked the same question in regards to drug resistance and virulence levels using the guinea pig model. This group's results were similar to the classical studies performed by Mitchison and opposed the finding of the murine model, in finding that MDR-TB strains produce less severe disease *in vivo* (122).

The study of clinically relevant strains in vivo is important, so that researchers know how these isolates behave. The majority of studies regarding vaccine and chemotherapy regimens for *M. tuberculosis* development utilize laboratory strains. Laboratory strains, such as H37Rv and Erdman, are somewhat less virulent, do not have as severe pathology, and have different immune responses in comparison to clinical isolates of *M. tuberculosis*. For this reason, our laboratory

strongly feels that clinical isolates should be used in development studies, since these are the actual strains people are infected with and the effect of the new vaccine or chemotherapy agent/regimen against clinical isolates needs to be known.

In this study the responses of three sets of isogenic pairs of *Mycobacterium tuberculosis,* that were collected from patients in Spain and Costa Rica, were compared using a guinea pig model. I was interested to see how the organ bacterial burden and histopathology of each strain behaved in the guinea pig model, and if there were any similarities between the drug susceptible and drug resistant strains. In two of the pairs studied, one strain was resistant to at least one drug while the other strain in the pair was susceptible to all the front line drugs. In the pair from Spain one of the strains (CSU 12) was susceptible to the front line drugs – isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin, while the other strain (CSU 10) was resistant to all the drugs listed above except for pyrazinamide. One of the isogenic pairs from Costa Rica had similar drug susceptibility/resistant properties as the pair from Spain, being that one of the strains in the pair was susceptible to all the drugs (CSU 26), while the other strain (CSU 22) had resistance to at least one of the drugs, and in this case it was resistant to rifampin. The remaining pair studied, which came from Costa Rica, each of the strains tested had drug resistance and susceptibility. In this pair one of the strains was resistant to only one drug (CSU 7, rifampin) while the remaining strain in the pair (CSU 24) had resistance to multiple drugs – isoniazid, rifampin, and streptomycin (Table 1).

As shown below, the results of this study are in line of the findings with both Mitchison and Palanisamy, in regards to the drug resistant bacteria being less virulent in vivo. In the three pairs tested, two of the drug resistant strains were less virulent than the drug susceptible strain in

the pair. It was also observed that the W – Beijing strains had a variety of virulence levels at both time points tested.

Table 1 isogenic strain characteristics. Isoniazid (H), Rifampin (R), Streptomycin (S), Ethambutol (E), Pyrazinamide (Z). 0: Susceptible 1: Resistant

			Susceptibility				
Strain	**Collection Location**	**Lineage**	**H**	**R**	**S**	**E**	**Z**
CSU 12	Spain	Beijing	0	0	0	0	0
CSU 10			1	1	1	1	0
CSU 26	Costa Rica	Beijing	0	0	0	0	0
CSU 22			0	1	0	0	0
CSU 7	Costa Rica	LAM9	0	1	0	0	0
CSU 24			1	1	1	0	0

Materials and Methods:

Animals: Female out bred Harley guinea pigs (approximately 500 g in weight) were purchased from Charles River Laboratories (North Wilmington, MA) and were held under barrier conditions in an Animal Bio-Safety Level III (ABSL-III) laboratory. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University.

Experimental Infections: Isolates for this study were received from Dr. M. Burgos (University of New Mexico, Albuquerque, NM) and were associated with outbreaks/human infections in Spain and Costa Rica. These strains are three pairs of isogenic pair strains – CSU 12 and CSU 10, CSU 7 and CSU 24, and CSU 22 and CSU 26. Each strain has both drug susceptibility and drug resistance to at least one of the front line drugs. All of these strains were grown in 7H9 media containing OADC and Tween – 80 to mid – log phase and then frozen in aliquots at -80°C until needed. For low dose aerosol infections, bacterial stocks were diluted in 15 ml of sterile distilled saline to 1 x 10^5 CFU/ml and placed in a nebulizer attached to a Madison Chamber aerosol generation device (Madison Exposure Chambers, LLC, Bayfield, WI). Guinea pigs were exposed to an aerosol infection in which approximately 20 bacilli of each strain tested were deposited in the lungs of each animal.

Bacterial counts in the lung, medistainal lymph node, and spleen were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar (Becton, Dickinson, and Company, Sparks, MD) and counting CFU after three weeks of incubation at 37°C. Histopathology was also used to determine location and type of lesions in the lungs.

Determination of bacterial CFU in lung, lymph node, and spleen: at 30 and 60 days post infection, five guinea pigs from each group, were euthanized by a sedation mixture containing 30 mg/ml of ketamine and 20 mg/ml of xylazine followed by an intracardial stick of 500 µl of pentobarbital sodium. The right cranial lung lobe, the mediastinal lymph node, and the spleen were aseptically removed. Spleens and the medistainal lymph node were removed, cut in half, with one half being used for CFU enumeration and the other being fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 10x phosphate buffered saline(PBS) (Gibco, Carlsbad, CA) for histology. Bacterial loads of organs tested were determined by plating serial dilutions of organ homogenate on nutrient rich 7H11 agar (Becton, Dickinson, and Company, Sparks, MD) plates and counting the number of CFU after three weeks of incubation at 37°C. The bacterial load for each organ was calculated and converted to logarithmic units.

Histology: The left caudal lung lobe, a portion of the mediastinal lymph node, and a portion of the spleen were collected at days 30 and 60 after infection and fixed in 4% paraformaldehyde in 10x phosphate buffered saline (PBS). The tissues were embedded in paraffin wax and 4µm thick sections were cut and stained with hematoxylin and eosin. All pictures were taken with an Olympus BX41 microscope at 2x magnification.

Statistics: A student's t test was performed in GraphPad Prism 4 to determine any statistically significant differences at specific time points.

Results

Comparison of bacterial burdens in the lungs, lymph nodes, and spleens

At day 30, in the lungs of guinea pigs infected with CSU 12 and CSU 10 there was a difference in bacterial burdens of 0.39 logs. The lung burden of both groups decreased to day 60. The bacterial burdens for the lymph nodes of guinea pigs infected with CSU 12 and CSU 10 followed a similar pattern to the lungs. The lymph nodes started with a burden of 6.67 logs for CSU 12 and 6.02 for CSU 10 at day 30, but then dropped to 5.14 logs and 4.23 logs for CSU 12 and CSU 10, respectively, at day 60 (Table 2). The biggest difference in bacterial burdens for these particular strains was with the spleens. At day 30 after infection, there was a difference of 1.32 logs between CSU 12 and CSU 10. The burden for the two groups declined between days 30 and 60, with the difference between the groups almost doubling to 2.59 logs (Figure 1).

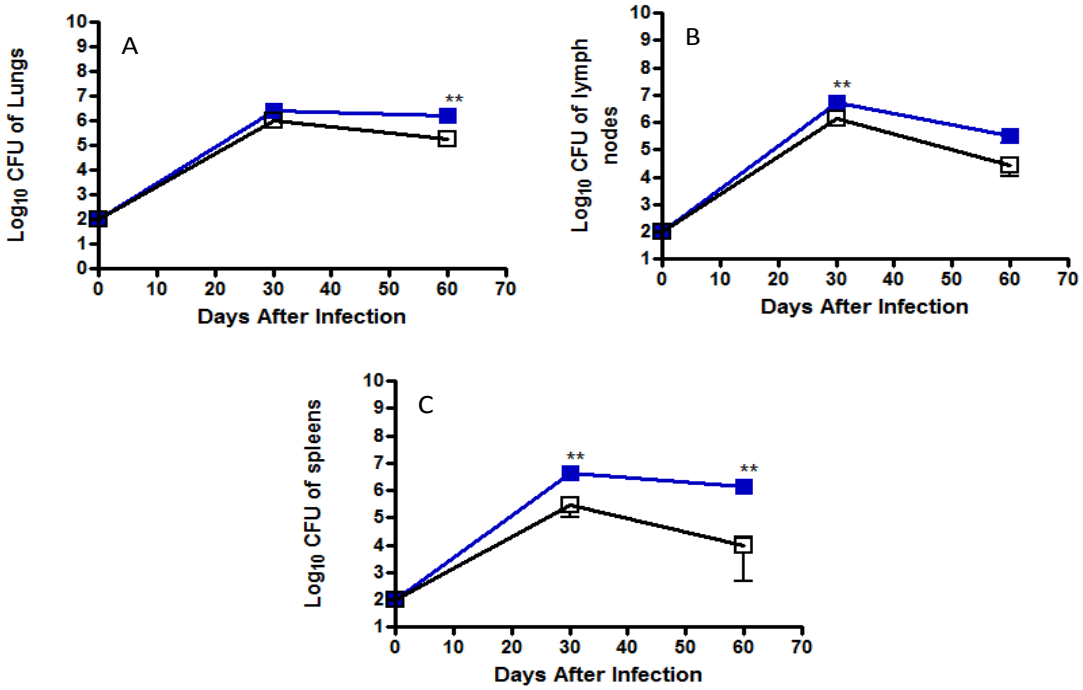

Fig. 1 bacterial counts in right cranial lung lobe (A), medistainal lymph nodes (B), and spleen (C) of guinea pigs infected with CSU 12 (■) and CSU 10 (□) at days 30 and 60. The results are expressed as mean bacterial loads in each group expressed as the $\log_{10}$ number of CFU (± SEM). ** $P<0.05$

The growth pattern observed in the lungs, lymph nodes, and spleens of strains CSU 12 and CSU 10 was only observed in the lymph nodes of strains CSU 7 and CSU 24. In the lungs and spleens of strain CSU 7 the burden of the organs increased from day 30 to day 60, while strain CSU 24 decreased. The spleens, similar to that seen in strains CSU 12 and CSU 10, had the biggest difference at both time points. At day 60 there was a difference of 2.70 logs between strains 7 and 24 in the spleens. (Figure 2)

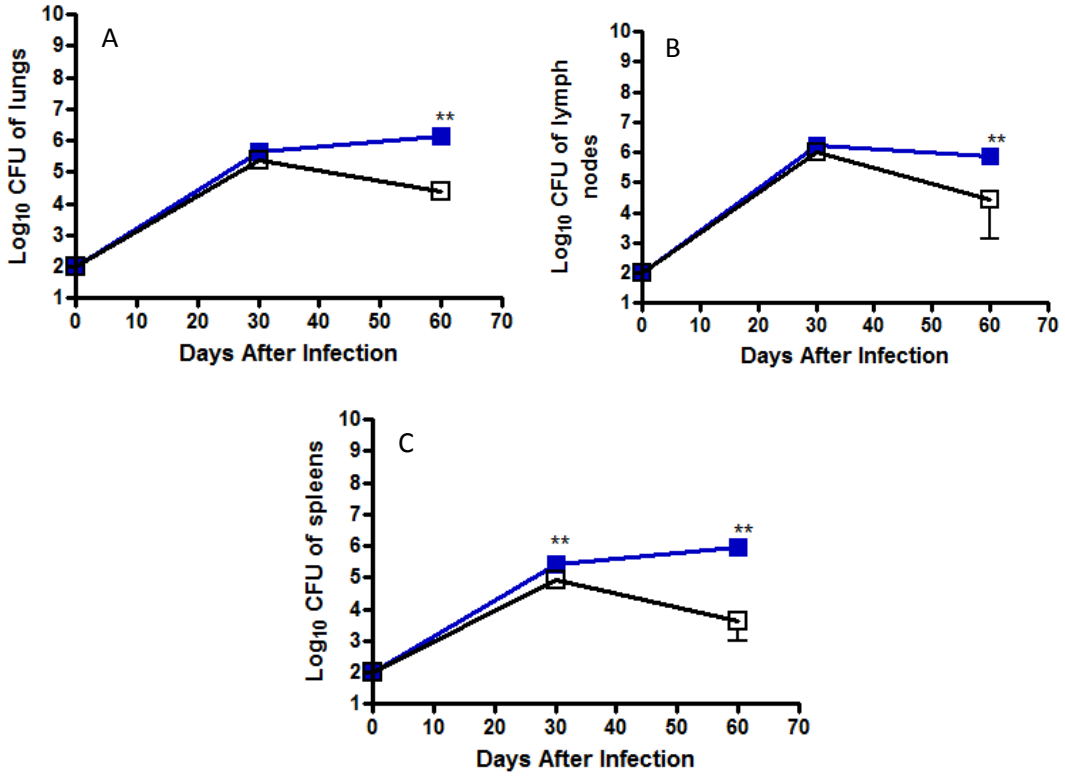

Fig. 2 bacterial counts in right cranial lung lobe (A), medistainal lymph nodes (B), and spleen (C) of guinea pigs infected with CSU 7 (■) and CSU 24 (□) at days 30 and 60. The results are expressed as mean bacterial loads in each group expressed as the $\log_{10}$ number of CFU (± SEM). ** $P<0.05$

The differences in the growth curves of strains CSU 26 and CSU 22 were not as dramatic as strains CSU 12, CSU 10, CSU 7, and CSU 24. The biggest difference, as with the other strains tested, was in the spleen at day 60, with a difference of 1.07 logs. When the four other strains were compared, the two strains of each isogenic pair against one another; it was observed that one of the strains clearly had a bigger bacterial burden in each of the organs than the other strain in the pair at both time points. This was not always the case between strains CSU 26 and CSU 22, as indicated in Figure 3. In the lungs at day 30 strain CSU 22 had a higher burden than

strain CSU 26, but then at day 60 the two strains had a bacterial burden within 0.05 logs of each other. This pattern was not seen in the spleens and lymph nodes though. CSU strain 26 clearly outgrew CSU 22 in the spleen and lymph nodes.

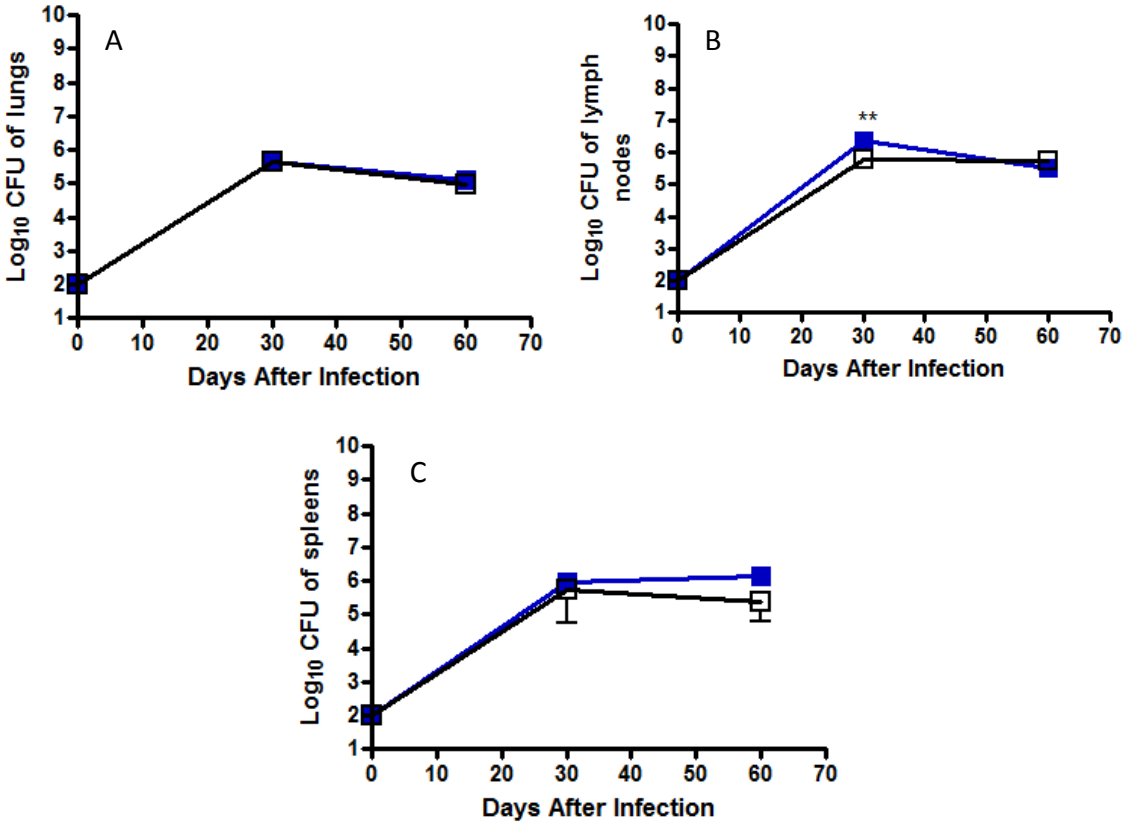

Fig. 3 bacterial counts in right cranial lung lobe (A), medistainal lymph nodes (B), and spleen (C) of guinea pigs infected with CSU 26 (■) and CSU 22 (□) at days 30 and 60. The results are expressed as mean bacterial loads in each group expressed as the $\log_{10}$ number of CFU (± SEM). ** $P<0.05$

Table 2. Mean bacterial counts in right cranial lung lobe, mediastinal lymph node (half), and spleen (half) of guinea pigs. * n/N, number of guinea pigs that yielded viable CFU data (n) over the total number of guinea pigs in that group (N)

			Log_{10} CFU ± SEM			
Group	**Lung Lobe**	**n/N**	**MLN**	**n/N**	**Spleen**	**n/N**
day 30 CSU 1	6.23 ± 0.20	5/5	6.67 ± 0.09	5/5	6.48 ± 0.18	5/5
day 30 CSU 1	5.84 ± 0.20	5/5	6.02 ± 0.17	5/5	5.16 ± 0.25	5/5
day 30 CSU 7	5.59 ± 0.11	5/5	6.21 ± 0.05	5/5	5.38 ± 0.12	5/5
day 30 CSU 2	5.32 ± 0.11	5/5	5.89 ± 0.15	5/5	4.82 ± 0.18	5/5
day 30 CSU 2	5.26 ± 0.42	5/5	6.33 ± 0.07	5/5	5.78 ± 0.23	5/5
day 30 CSU 2	5.54 ± 0.18	5/5	5.75 ± 0.11	5/5	4.98 ± 0.38	5/5
day 60 CSU 1	6.14 ± 0.15	4/5	5.14 ± 0.43	4/5	6.00 ± 0.23	4/5
day 60 CSU 1	5.27 ± 0.03	5/5	4.23 ± 0.33	4/5	3.41 ± 0.68	3/5
day 60 CSU 7	6.09 ± 0.11	5/5	5.82 ± 0.09	5/5	5.91 ± 0.10	5/5
day 60 CSU 2	4.26 ± 0.23	4/5	4.40 ± 0.69	3/5	3.21 ± 0.49	1/3
day 60 CSU 2	4.97 ± 0.16	5/5	6.15 ± 0.14	5/5	6.15 ± 0.14	5/5
day 60 CSU 2	4.92 ± 0.08	5/5	5.60 ± 0.19	4/5	5.08 ± 0.35	5/5

Histological Analysis of the Lungs

At day 30 CSU 10 had very few lesions present. The lesions that were evident were primary lesions, which were small and had a centralized region of necrosis. In CSU strain 12 there was evidence of both primary and secondary lesions in the lungs sampled. Most of the secondary lesions present were small in size. At day 60 there were some secondary lesions in CSU 10, but most of the lesions seen were primary lesions. Almost all of the primary lesions observed had evidence of calcification in the central region of necrosis. A couple of the animals had primary and secondary lesions that had coalesced. In the lungs of CSU 12 both primary and secondary lesions of varying sizes were present. Many of the lesions – primary: primary, secondary : secondary, primary : secondary had fused with one another (Figure 4).

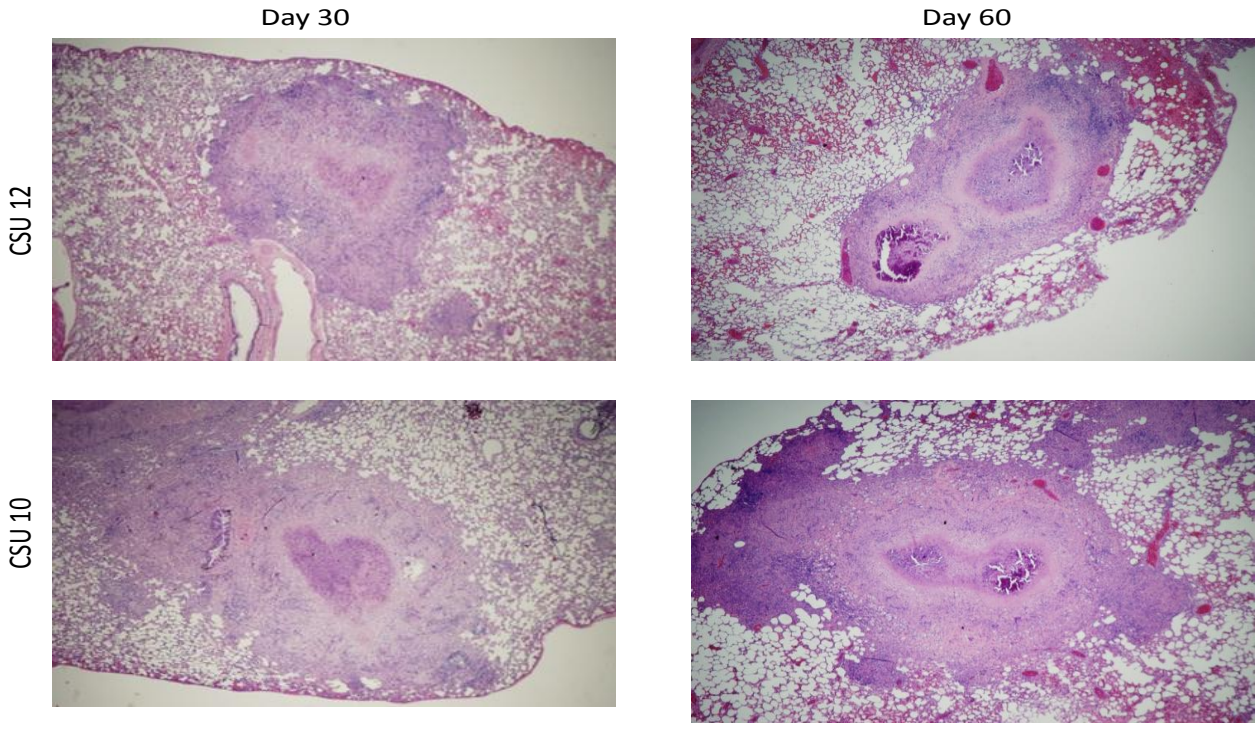

Fig. 4 Progression of lung pathology of guinea pigs infected with CSU 12 and 10. Total original magnification was 2x.

CSU strain 7 had evidence of both primary and secondary lesions at day 30. The primary lesions were of varying size, while the secondary lesions were usually small in size. It was observed that the smaller lesions were located peripherally, whereas the larger ones were more centrally located in the lungs. Only primary lesions were observed at day 30 in CSU 24 with all the lesions being roughly the same size. The lesions in CSU 24 had differing levels of necrosis present. At day 60 for CSU 7 the lesion size for both primary and secondary lesions had increased. High levels of calcification were present for the primary lesions. There really was not much difference between day 30 and day 60 for CSU 24. All the primary lesions were small, with varying levels of necrosis. There was no evidence of calcification for any lesions (Figure 5).

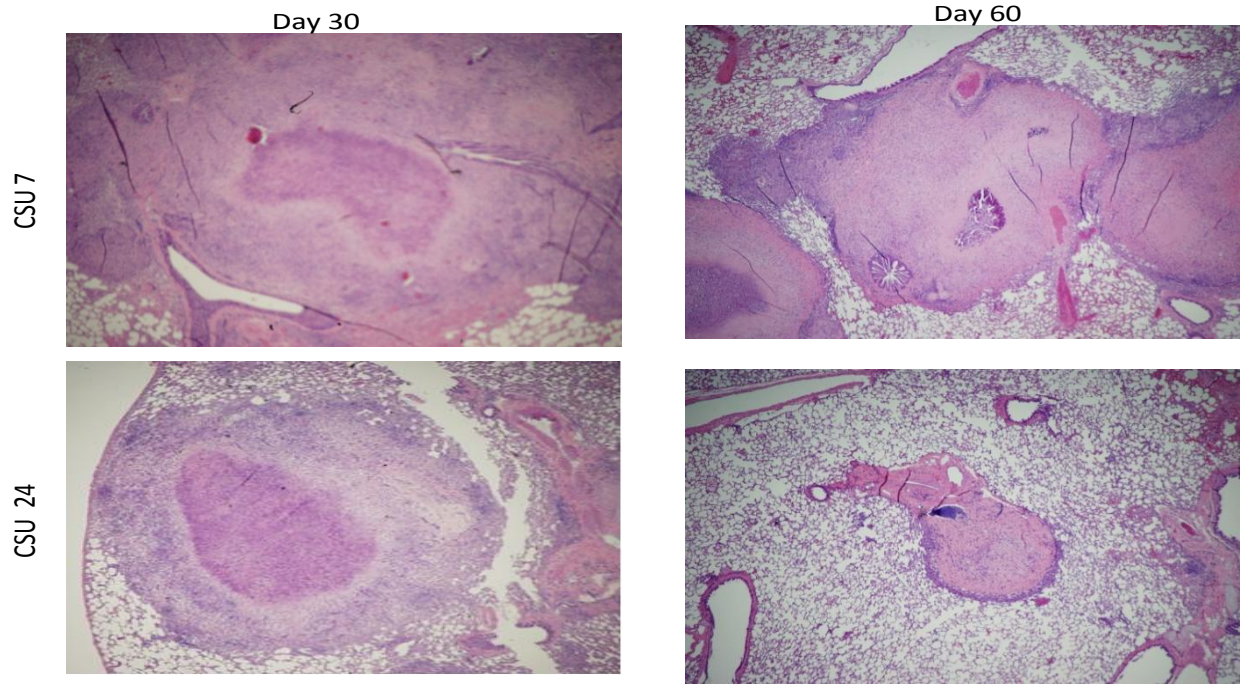

Fig. 5 Progression of lung pathology of guinea pigs infected with CSU 7 and 24. Total original magnification was 2x.

At day 30 CSU 22 had evidence of both primary and secondary lesions that were similar in size and number. CSU 26 at this time point only had evidence of primary lesion, though the size and number of the primary lesions varied, within an animal and from animal to animal. There was a range of small lesions that had no necrosis to mid – sized lesions with necrosis present. At day 60 CSU 22 had a mix of primary and secondary lesions that did not increase much in size from day 30. Many of the primary lesions had calcification present. There were no secondary lesions observed at day 60 for CSU 26, only primary lesions. The primary lesions were relatively small in size, with some of the lesions having calcification present in the centralized region of necrosis (Figure 6).

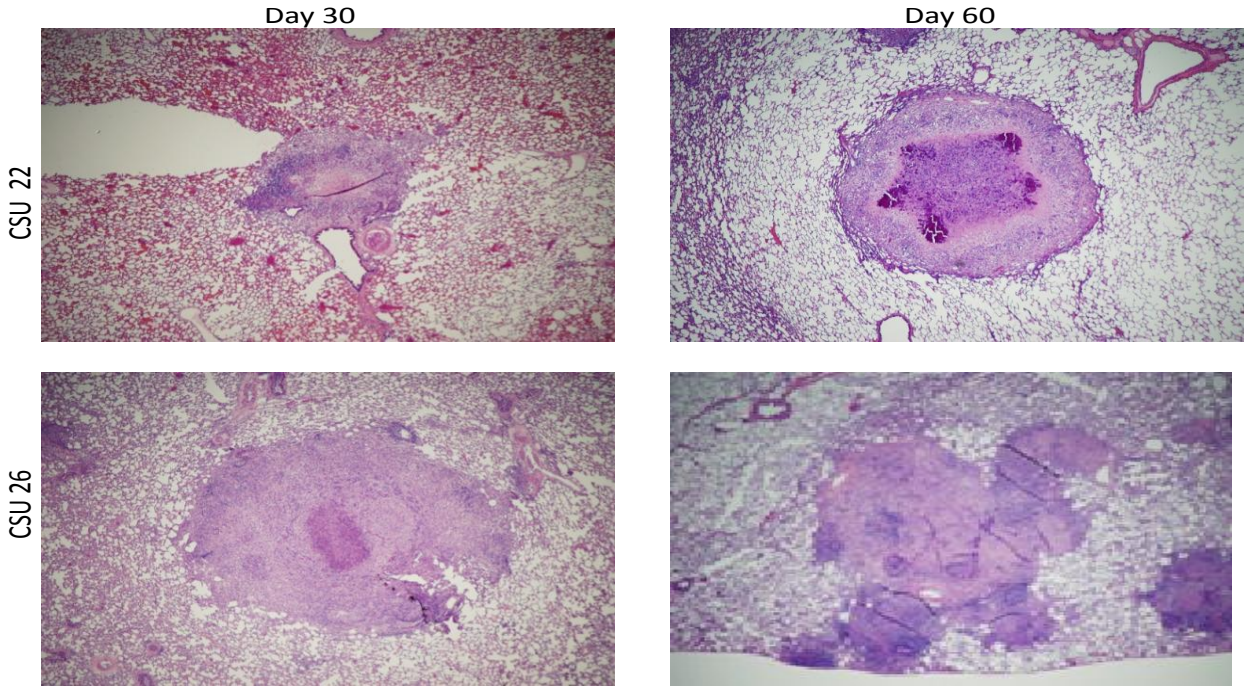

Fig. 6 Progression of lung pathology of guinea pigs infected with CSU 26 and 22. Total original magnification was 2x.

CSU 7 had the worst lesion progression, at both day 30 and day 60, for all the strains tested. At the two time points there were both primary and secondary lesions present. The lesions observed at these particular time points were the largest of all the strains. At day 30 the primary lesions had the largest amount of necrosis, while at day 60 this group had the largest amount of lesions with high levels of calcification present.

Discussion

The results of this study show that in the guinea pig model the drug resistant bacteria are less virulent. In the three pairs tested, two of the drug resistant strains (CSU 22 and CSU 24) were less virulent than the drug susceptible strain in the pair. It was also observed that the W – Beijing strains had a variety of virulence levels at both time points tested

Of the strains studied, two of the three isogenic pairs were of the W – Beijing family. The third pair of isogenic strains studied was of the LAM9 lineage. The two W – Beijing pairs studied were from different areas of the world. CSU 10 and 12 were collected from patients in Spain while CSU 22 and 26 were from Costa Rica. The LAM9 strains CSU 7 and 24 were also collected from patients in Costa Rica.

Prior to being tested in the guinea pig model of infection, six of the clinical isolates were tested for drug susceptibility/resistance to/against isoniazid, rifampin, streptomycin, ethambutol, and pyrazinamide. Each of the pairs studied came from one patient and had varying levels of drug susceptibility/resistance. Overall, it was observed that one of the strains had a higher level of resistance, meaning that it was resistant to more drugs, than the other strain in the pair. The

W – Beijing pair collected from Spain, CSU 10 and CSU 12, had the highest level of drug resistance. CSU 12 was susceptible to all the drugs tested, while CSU 10 was resistant to four of the five drugs tested (susceptible to pyrazinamide). The LAM9 pair collected from Costa Rica had the next highest level of drug resistance. CSU 7 was susceptible to all the drugs tested except for rifampin, which it was resistant to. CSU 24 was resistant to isoniazid, rifampin, and streptomycin while being susceptible to ethambutol and pyrazinamide. CSU 7 and CSU 24 was the only pair tested where each of the strains tested was resistant to at least one drug. The other W – Beijing pair tested only had one strain being susceptible to one drug- rifampin for strain CSU 22. The comparison of the drug susceptibility/resistance profiles of the evaluated strains show that W – Beijing are multi-drug resistant, but also that they have varying levels of drug resistance and are not the only lineage capable of being drug resistant.

Of the limited data that exists for the W – Beijing family of tuberculosis strains, it is known that these strains have a varying level of virulence. This study supports these findings. When the bacteria burdens for day 30 were evaluated it was observed that CSU 12 had the highest burdens in the observed organs, right cranial lung lobe, mediastinal lymph node, and spleen. In regards to the lung data at day 30, the next highest burden was in a W – Beijing strain, the pair to CSU 12 (CSU 10), but then the data became mixed. It was observed that one of the non W – Beijing strains had the third highest burden followed by a W – Beijing strain. This shows that the two strains in the pairs do not act identical to one another. The results for the lymph node and spleen at day 30 were similar to the lungs, with just more mixed results of how all the strains act, as can be seen in table 2. Varying levels of virulence for the W – Beijing and non W – Beijing strains were also observed at day 60 in the organs examined.

Classical studies conducted by Mitchison (114) showed that isoniazid resistant isolates had a lower virulence level when tested *in vivo*. More recently Palanisamy et al (122) did a similar study and also found drug resistant strains had a lower virulence level in the guinea pig. The isolates tested by Palanisamy were resistant to isoniazid along with several other drugs and were all clinical isolates from around the world (122). In the pairs containing CSU 12 and CSU 10 and CSU 7 and CSU 24 it was observed that the more drug resistant strain, CSU 10 and CSU 24 respectively, had a lower bacterial burden in all three of the organs evaluated at days 30 and 60. In the isogenic pair containing CSU 22 and CSU 26, with CSU 22 being the resistant strain, the results were more mixed. CSU 22 was not always the strain with the lower burden in the lung, lymph node, and/or spleen at either day 30 or 60, which can be seen in Table 2 or Figure 3. The results of CSU 22 and CSU 26 are curious, is the pattern of CSU 22 not always being less virulent due to the fact that it is only resistant to one drug, so overall this pair has a higher drug susceptibility profile? Having said that however, the results of this study support the findings of both Mitchison and Palanisamy.

In general there is very little data that exist in regards to the relationship of drug resistance and virulence levels *in vivo*, though when one is looking for data regarding the guinea pig model, the data becomes even scarcer. The studies that exists that looked into the relationship of drug resistance and virulence levels of tuberculosis strains tested in the guinea pig generally seem to agree that drug resistant strains produce less severe disease. When the murine model data evaluating this relationship is analyzed it is noted that there is no difference. Investigators utilizing the murine model find there is no discernable relationship between drug resistant profiles and levels of virulence. However, this may reflect the lack of lung necrosis in this species.

Chapter 4

Concluding Remarks

While the incidence of tuberculosis may be declining in certain regions of the world, there are other regions where the number of yearly reported cases is increasing alarmingly. One of the main reasons for the increased incidence rates in these regions, such as Sub-Saharan Africa, is the spread of the W – Beijing family of *M. tuberculosis*. Limited data exists for these strains, since the W – Beijing family is one of the newer identified lineages of tuberculosis. What we do know is that almost all reported cases of W – Beijing tuberculosis infections, in both humans and experimental animals, suggests higher virulence than non W – Beijing strains. This family is the cause of many community wide outbreaks, not only in the Asian and African continents, but also worldwide. A majority of the W – Beijing cases reported in others areas of the world except for Asia are actually caused by a person that was initially infected on the Asian continent and then traveled, causing the spread of the disease and that particular strain. Many of W – Beijing strains are MDR, and the drugs used to treat MDR-TB are very expensive and are toxic to the patient; so many patients do not complete a full treatment course.

Currently there is an efficacy rate for the BCG vaccination between 0-80 percent, depending on where one lives in the world. One reason for this is the effect environmental mycobacteria have on vaccination. An emerging concern with the BCG vaccination and the W – Beijing strains is that people now think that these strains are potentially being selected by BCG vaccination; moreover, studies at Colorado State University (CSU) now indicate that BCG has no sustained protective effect against W – Beijing strains tested in the mouse model. Murine data from CSU has shown that there is no BCG protection after 60 days when infected with

W – Beijing tuberculosis. In histologic examination of the lungs of vaccinated animals, reduced lung consolidation and smaller and more organized granulomas in the vaccinated mice after 30 days was shown. Effector T cell responses were increased in the vaccinated mice infected with HN878 but these diminished in numbers after day 30 of the infections while there was an increase in $CD4^+$ $Foxp3^+$ T cells in the lungs, draining lymph nodes and the spleen. This data raises the question about the soundness of doing clinical trials of new vaccines for tuberuculosis, many of which are recombinants of BCG, in areas that have a high prevalence of W – Beijing tuberculosis infections.

The guinea pig data obtained here from the W – Beijing strains along with the other lineage examined supports the notion that there is a variety of virulence levels for the individual strains. Overall it was observed that at least one of the W – Beijing strains had a higher virulence level than any of the other strains being evaluated. It was also observed that each strain in the isogenic pair acted independently of each other. When the W – Beijing strains in the murine model were observed, it was hard to tell if the higher transmission strain caused a more severe disease in the animal than the low transmission strain tested. When the W – Beijing strains in the murine model were compared to the non W – Beijing strains it was noted that the W – Beijing strains were not any more virulent than the other lineages evaluated. The two W – Beijing strains evaluated had a bacterial burden around 6 logs at day 90, where three of the non W – Beijing strains had bacterial burdens at day 90 being between five and six logs.

In the guinea pig model used W – Beijing isolates were used that had drug resistance to at least one of the first line drugs. The strain evaluated in the guinea pig that had the most drug resistance was a W – Beijing strain. There was not any exact data on the drug resistant profiles

for the W – Beijing strains tested in the murine model, it was only known that they were drug sensitive.

A long standing question in regards to drug resistant strains of bacteria is does the isolate lose any virulence when becoming resistant to a drug? Classical studies conducted by Mitchison in the guinea pig concluded that drug resistant strains in general were less virulent. A more recent study conducted in the guinea pig, using different isolates that had different drug resistant profiles, conducted by Palanisamy (122) support the findings of Mitchison (114), in that less severe pathology was seen in animals infected with drug resistant strains. Studies questioning the relationship between virulence levels of bacteria and drug resistant profiles have been tested in other animal models, including the murine model. These studies have also shown that many of the clinical isolates were very virulent, especially when compared to the laboratory strains of H37Rv and Erdman. Ordway (117) tested a variety of drug resistant strains in the mouse and found no truly discernable relationship between drug resistance and virulence. In some of the strains tested there were high bacterial burdens within the first 20 days of infection, but then in other drug resistant strains the burdens were low. Other laboratories have had similar findings as Ordway when utilizing the murine model and questioning this particular relationship.

The results that were obtained with the murine data support all the previous studies reported. The strains in which the drug resistance was known had a variety of virulence levels. In one of the isogenic pairs test in the murine model the drug resistant strain had a log less in the lungs at days 30, 60, and 90. In the other isogenic pair the drug resistant strain had a higher bacterial burden then the drug susceptible strain.

The data obtained from the guinea pig model of infection supports the classical studies of Mitchison and the more recent studies conducted by Palanisamy. Out of the three isogenic pairs evaluated, it was observed that two of the pairs the drug resistant strains were less virulent than the drug susceptible strains. The two drug resistant strains, CSU 10 and CSU 24 consistently had lower burdens throughout the entire study. It is interesting to note the differences observed between the guinea pig and murine models.

The knowledge obtained from evaluating clinical isolates *in vivo* is vital. All vaccines and chemotherapy development must initially be tested in animal models. Most current animal model studies for the development of vaccines and chemotherapy utilize laboratory strains. This practice of using laboratory strains in the development of treatment and/or prevention methods needs to be questioned. Laboratory strains do not behave in the same fashion as clinical isolates do; there are differences both in pathology and the subsequent immune response. Any new vaccine or chemotherapy needs to be tested utilizing clinical isolates in animal models because the clinical isolates are what infect humans, not the laboratory strains. If there are going to be any complications with the new vaccine or chemotherapy agent with the clinical isolate(s), such as the fact they may not induce a particular cellular subset, it is better to learn this while conducting *in vivo* experiments in relevant animal models before moving to extremely expensive clinical trials in humans.

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