

DISSERTATION

PATHOGENESIS OF EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY GOPINATH S. PALANISAMY ENTITLED, PATHOGENESIS OF EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS, BE ACCEPTED AS FULFILLING, IN PART, REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PATHOGENESIS OF EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS

Tuberculosis is an important infectious disease of humans that can be modeled in a number of small laboratory animal species. In humans and guinea pigs, infection with the causative agent *Mycobacterium tuberculosis*, incites a chronic inflammatory response in the lung (pulmonary) and other (extra-pulmonary) tissues and organs of the body. Granulomatous inflammation can become organized into a distinct inflammatory mass referred to as a granuloma. Granuloma formation is thought to represent a favorable host response that functions to contain the infection, thus preventing spread within or between susceptible hosts. If infected cells within the granuloma die, intracellular bacteria are released and become entrapped in an extra-cellular microenvironment where they persist for long periods of time protected from drug therapy and the host immune response. The mechanism responsible for granuloma cell death (necrosis) is unknown but is important to understand as it represents a unique microenvironment for drug-tolerant bacilli to persist. One potential mediator of granuloma necrosis is the generation of cell and tissue damaging oxygen free radicals, also known as reactive oxygen species (ROS), a hypothesis tested in these studies. We used the guinea pig model of human tuberculosis to test what influence bacterial strain had on the development of pulmonary and extra-

pulmonary granuloma necrosis. Our studies showed that the virulence of clinical isolates of *M. tuberculosis* was reflected in more severe and widely disseminated disease in experimentally infected guinea pigs and was a better predictor of virulence than the bacterial burden determined by culture. These data provide supporting evidence that the extent of lesion necrosis correlated with the severity of disease and is an important determinant in the clinical outcome of tuberculosis. We concluded that both host and pathogen factors contribute to the pathogenesis of lesion necrosis during *M. tuberculosis* infection.

To determine the host factors that contribute to the pathogenesis of lesion necrosis, we focused on the role ROS generation has in the pathogenesis of lesion necrosis in experimental tuberculosis and explored whether this adverse response could be controlled therapeutically or through vaccination of guinea pigs with *M. bovis* BCG prior to virulent challenge. We found that depletion of host antioxidant defenses was a major determinant in the imbalance between the generation of ROS and host antioxidant capacity in this tuberculosis model. Moreover, we attributed the decreased expression of key antioxidant proteins to a defect in the function of a critical antioxidant transcription factor, nuclear factor-erythroid 2-related factor 2 (Nrf2). We were able to partially restore Nrf2-mediated antioxidant defenses therapeutically in *M. tuberculosis* infected guinea pigs with the antioxidant drug N-acetylcysteine. We also established that low density lipoproteins were among the host macromolecules that are oxidized during the chronic inflammatory response typical of tuberculosis. Oxidized low density lipoproteins (OxLDL), known to be rich in cholesterol, accumulated in macrophages during infection and elevation of OxLDL levels was accompanied by increased expression of the OxLDL

scavenger receptors CD36 and LOX-1. The significance of these data are that through the use of the guinea pig tuberculosis model, we have uncovered a previously unrecognized mechanism by which the host and pathogen interact to create a unique microenvironment that allows difficult to treat *M. tuberculosis* to persist. The characterization of these host-pathogen interactions may lead to the development of novel adjunct therapies aimed at preventing the adverse effect of *M. tuberculosis* infection in humans.

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DEDICATION

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Chapter One

“In the future, the battle against this plague of mankind (tuberculosis) will not just be considered with an uncertain something but with a tangible parasite, about whose characteristics a great deal is known and can be explored”

Robert Koch, 1882

General Hypothesis

My general hypothesis in the study of experimental tuberculosis in guinea pigs based on the literature discussed here is as follows:

In the guinea pig model of tuberculosis, lesion necrosis is a correlate of virulence and is mediated by free radicals. Deficient antioxidant defense and oxidation of host lipoprotein during oxidative stress conditions contribute to disease pathogenesis.

Specific aims

Aim 1: Demonstration of the extent of lesion pathology and necrosis as a measure of mycobacterial virulence and comparison of virulence of various clinical strains of *Mycobacterium tuberculosis* in the guinea pig model.

Aim 2: Demonstration of oxidative stress and defect of an antioxidant transcription factor Nrf2-mediated defense mechanism in the guinea pig model of tuberculosis. Investigate the therapeutic reversal of the Nrf2 defect, to assess the role of oxidative stress in the development of lesion necrosis.

Aim 3: Demonstration of the accumulation of oxidized host lipoprotein systemically and locally within granulomas via receptor-mediated uptake pathway in macrophages and evaluation of the functional significance of the accumulation of oxidized lipoprotein in macrophages.

Review of Literature

Tuberculosis – A brief historic view

Tuberculosis is one of the oldest recorded diseases that afflicted ancient civilizations from prehistoric times (Bedeir 2003). A paleomorphologic study hypothesized the presence of a neural form of tuberculosis named “meningitis tuberculosa” nearly 500,000 years ago, based on the gross pathological changes present in a *Homo erectus* skull recovered from Turkey (Kappelman et al 2008). A more convincing evidence for the prehistoric presence of tuberculosis comes from a paleomicrobiological study that demonstrated *Mycobacterium tuberculosis* (*M. tuberculosis*) DNA in fossils recovered from a cave in Wyoming of a Bison which was estimated to be from Pleistocene epoch roughly 17,000 years ago (Donoghue et al 2004). The ravage this disease had on Egyptian civilization has been confirmed by Zeihl-Neelsen staining and polymerase chain reaction (PCR) besides the paleopathological evidence in samples collected from the mummies that were from around 1000 BC (Nerlich et al 1997; Zimmerman 1979). History has conferred numerous names to tuberculosis. *Phthisis* (to waste), *scrofula*, *The King’s Evil* (when English and French kings were thought to have powers to heal TB with their touch), *lupus vulgaris* (skin form), *Pott’s disease* (spinal tuberculosis), *White Plague* and *Consumption* are some intriguing names (Harms 1997). Interestingly, Hippocrates considered tuberculosis to be inherited and non-contagious (Chadwick & Mann 1978).

The term “tubercle” was coined by a Dutch anatomist, Sylvius, who noticed them consistently in lungs and other areas of affected patients. In 1702, a few pathological aspects of miliary tuberculosis were described by Manget (Harms 1997). Rene Laennec

(1781-1826), an eminent French physician, in an unpublished manuscript, described a detailed pathological review of pulmonary and extra-pulmonary tuberculosis (Keers 1981). Gaspard Bayle (1774-1816), a colleague of Laennec reported six types of pulmonary tuberculosis after studying 900 post-mortem specimens including: tubercular, glandular, ulcerous, calculous, cancerous and melanosis phthisis (Bayle 1810). Pioneering studies of experimental tuberculosis were performed on guinea pigs by the physicians John Burton Sanderson and Wilson Fox who noticed similarities between human and guinea pig disease (Fox 1868; Sanderson 1867). In describing normal anatomy of the lymphatic system in a two volume description of his work, Edmund Klein included a thorough microscopic description of experimental tuberculosis in the guinea pig (Klein 1874; 1875).

A series of ground-breaking studies by two German physicians followed. In the late 19th century, Robert Koch discovered a staining technique to detect *M. tuberculosis* in sputum smears. He realized that the presence of the organism did not necessarily mean the causal association, so he proposed a set of criteria in order to objectify the causal relationship which became “Koch’s postulates” (Koch 1882). For most of his studies on tuberculosis, Koch used guinea pigs extensively. Paul Ehrlich incorporated some modifications to Koch’s method of staining tubercle bacillus and the technique was further modified by Franz Ziehl and Friedrich Neelsen. Today known as “Ziehl-Neelsen method”, this technique still remains in use and is an indispensable diagnostic tool for tuberculosis.

The Bacilli

Mycobacteria are rod-shaped bacteria and measure approximately $0.2\text{-}0.6\mu \times 1.0\text{-}10.0\mu$. They are aerobic, non-motile and non-spore forming organisms. They are catalase positive and form filaments in culture, which are rapidly fragmented (Prescott et al 1999). The genus *Mycobacterium* (Family - Mycobacteriaceae; Order - Actinomycetales and Suborder - Corynebacterineae) includes 70 different species that exhibit a wide array of lifestyles. The large majority of these are saprophytic inhabitants of soil and water. However, a few species in this genus are intracellular pathogens of higher vertebrates that are responsible for easily the most devastating disease ever to affect mankind. The tuberculosis complex is comprised of *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*. *M. tuberculosis* causes disease in humans whereas *M. bovis* primarily causes disease in animals although cross infections can occur (Espinal & Raviglione 2003). Tuberculosis caused by *M. bovis* through milk contamination has almost been completely eliminated due to efficient pasteurization methods that are in practice.

Global Epidemiology of Tuberculosis

Tuberculosis has probably killed around 100 million people over the past century (Iseman 2000). It is one of the leading causes of death from an infectious disease (Frieden et al 2003). Prevalence of the infection with *M. tuberculosis* varies according to demographic factors such as age, sex and country as well as socio-economic factors such as crowding, poverty, confinement, etc.

Tuberculosis was declared by the WHO a decade ago as a global health emergency due to rising mortality rates (Espinal & Raviglione 2003). According to the

World Health Organization (WHO), one-third of the world population, or 1.7 billion people are latently infected with *M. tuberculosis*. Worldwide an estimated 9.27 million new cases of tuberculosis were present in 2007, which was an increase from 8.8 million in 2005. Most cases are present in Asia (55%) and Africa (31%). As of 2007, Sub-Saharan Africa has the highest incidence rate (363 per 100,000 population), but the most populous countries of Asia have the largest numbers of cases; India, China, Indonesia, Bangladesh, and Pakistan together account for more than half of the global burden (WHO 2009). Among the 15 countries with the highest estimated tuberculosis incidence rates, 13 are in Africa, a phenomenon linked to high rates of HIV coinfection.

In the United States, tuberculosis cases steadily declined at a rate of about 5.8% per year from the mid 1950s to 1985 (Reider 1989). However, the trend reversed in 1985 and reached an annual increase rate of 20% in 1992 (Cantwell et al 1994). More than 90% of the increased cases were concentrated in five states: California, Florida, New Jersey, New York, and Texas. Racial, ethnic minorities and foreign born individuals accounted for the majority of the new cases, coinciding with immigration from high-prevalence countries and emergence of drug-resistance. In 2000, an Institute of Medicine report declared the primary reason for this increase was the failure of the public health infrastructure and tuberculosis control programs in the United States (Geiter 2004). But, tuberculosis is on the decline again in the United States with 15 consecutive years of decreasing numbers of cases, with only about 13,000 cases and a case rate of 4 per 100,000 in 2007 (WHO 2009).

In the developed nations, tuberculosis mortality has decreased to very minimal levels. Conversely, in resource-limited countries, despite of the availability of effective

treatment options, the mortality rate is still high. Recent estimates suggest that mortality from tuberculosis to be around 1.3 million deaths (20 per 100,000 people) in 2007 among HIV-negative cases and about 0.5 million among HIV-positive cases (WHO 2009).

Influence of HIV/AIDS on Tuberculosis Epidemiology

The ability of HIV to undermine the immune system is one of the primary risk factors for progression of latent *M. tuberculosis* infection to the clinical disease (Reider 1989). The impact of HIV on the tuberculosis pandemic is remarkable (Espinal & Raviglione 2003). Out of an estimated 2 million human fatalities due to HIV, 23% were co-infected with *M. tuberculosis*. *M. tuberculosis* infection progresses faster to full-blown tuberculosis in HIV co-infected individuals than people who are infected with *M. tuberculosis* alone (Girardi et al 2000; Sonnenberg et al 2001). The estimated numbers of HIV-positive tuberculosis cases and deaths in 2007 is twice as much compared to the previous years, although aggressive data collection could explain this increase to an extent (WHO 2009). HIV-positive individuals are approximately 20 times more likely to develop tuberculosis in countries that have an HIV epidemic (WHO 2009).

Multidrug-resistant and extensively drug-resistant tuberculosis

Mycobacteria have acquired drug-resistance to various antibiotics since the introduction of antibiotic drug therapy in 1950s and the drug-resistance poses a serious threat to global tuberculosis control. Multidrug-resistance (MDR) is defined as resistance to at least two of the first line drugs, usually isoniazid and rifampin. Extensive drug-resistance (XDR) is defined as additional resistance to any fluoroquinolone and at least

one of the three injectable second line drugs: capreomycin, kanamycin and amikacin. Drug resistance can arise due to the lack of patient compliance, monotherapy and poor-quality of the drugs prescribed. Besides HIV/AIDS, tuberculosis control has been made more challenging by MDR-TB (Espinal & Raviglione 2003). Global notification data from 2007 provides a number of roughly 30,000 MDR-TB cases mostly in European countries and South Africa. This number is considered to represent only 8.5% of estimated smear positive cases of MDR-TB (WHO 2009). According to a recent study on XDR-TB, only one among the 53 persons lived past 16 days of positive sputum testing in a rural hospital in South Africa (Gandhi et al 2006). In 2008, 55 countries have reported at least one case of XDR-TB (WHO 2009).

Diagnosis of Tuberculosis

Of all the people exposed to *M. tuberculosis*, only around 5% develop active tuberculosis disease and nearly 95% of individuals are asymptomatic and become asymptomatic or latent carriers. Latent tuberculosis infection (LTBI) itself progresses to active disease in approximately 5% to 10% of infected persons. The rate of progression is determined by the immune status of individuals affected. It is estimated that 2 billion people are living with LTBI worldwide (Dye et al 1999). LTBI is consequently a major barrier to the control and elimination of tuberculosis.

Tests for Latent Tuberculosis Infection

Tuberculin Skin Test (TST)

Robert Koch first described tuberculin, a broth culture filtrate of tubercle bacilli in 1891 (Edwards & Edwards 1960). Koch's original intent to use it as a curative was unsuccessful, but the characteristic local reaction he observed at the site of injection in tuberculosis patients led to its usage as a diagnostic tool. A standardized version of tuberculin, the purified protein derivative (PPD), made by Seibert and Glenn in 1939 still remains a global standard for PPD (ATS 2000a).

TST has been used nearly for a century and is the only universally accepted test for the diagnosis of LTBI. Infection with *M. tuberculosis* results in a cell-mediated immune response giving rise to sensitized *M. tuberculosis* antigen-specific- CD4⁺ and CD8⁺ T lymphocytes (Orme & Cooper 1999). Stimulation by *M. tuberculosis* antigens causes these T cells to release interferon-gamma (IFN- γ). TST elicits such a response in previously sensitized individuals. In such individuals, an intra-dermal injection of PPD evokes a delayed-type hypersensitivity (DTH) response mediated by sensitized T cells resulting in cutaneous induration. The limitation of TST comes from the fact that PPD is a culture supernatant cocktail of nearly 200 antigens, many of which are shared by other mycobacteria including many non-tuberculous mycobacteria (NTM) and *M. bovis* (Harboe 1981; Lalvani et al 2001). Hence, the DTH response to PPD may signify infection with *M. tuberculosis*, infection with NTM (Judson & Feldman 1974) or vaccination with BCG (Snider 1985). This cross-reactivity limits the specificity of the TST in many populations (Fine et al 1999a). Anergy caused by an immuno-compromised state (HIV infection or drug-induced) may lead to false-negative results (ATS 2000b). False-negative results also may occur in individuals with active disease (Huebner et al

1993; Poulsen 1950). Despite such limitations, due to the lack of viable alternatives TST remains in widespread use to screen LTBI (Graham et al 1992).

New Diagnostic Tests for LTBI

The limitations of TST have led to concerted efforts to develop novel diagnostics with more specificity. IFN γ release assays (IGRAs) address the TST's limitations on specificity. QuantiFERON-TB Gold (QFN-Gold) tests (Cellestis Limited, St. Kilda, Australia) and the T SPOT-TB test (Oxford Immunotec, Oxford, UK) are two examples of IGRAs. The basis of these tests is the detection of either the release of IFN- γ on stimulation of sensitized T cells by *M. tuberculosis* antigens *in vitro* (QuantiFERON) or detection of the T cells themselves (T SPOT-TB) (Brodie & Schluger 2005). Similarly a whole-blood assay detects IFN- γ in response to PPD in bovine tuberculosis cases (Wood et al 1990). This method was adapted to a sandwich immunoassay and was shown to be both sensitive and specific in field comparisons with the intra-dermal tuberculin test (Rothel et al 1990; Wood et al 1992). This sandwich whole blood IGRA has been approved for use in Australia for the diagnosis of bovine tuberculosis (Streeton et al 1998). This test also has been shown to be effective in the diagnosis of human tuberculosis (Streeton 1995). QFN-Gold uses the *M. tuberculosis* specific RD1 antigens (RD1 is a 9.5 kb genomic DNA segment of *M. tuberculosis/M. bovis*, which is deleted in all strains of *M. bovis* BCG vaccines) ESAT-6 and CFP-10 in an ELISA format and the T SPOT-TB test uses the same antigens in an ELISPOT assay that identifies antigen-specific IFN- γ -secreting CD4⁺ T cells (Brodie & Schluger 2005). The IGRAs, in general,

have more sensitivity and specificity than TST and are not affected by the BCG vaccination status of the individuals.

Tests for Active Tuberculosis Disease

Sputum-based Diagnosis

Sputum smear based diagnosis is currently the most widely used diagnostic test for active tuberculosis. The technique used to obtain the respiratory sample strongly influences the ability to detect pulmonary tuberculosis. The sensitivity of expectorated sputum ranges from 34% to 80% with cavitory disease having the highest sensitivity (ATS 2000a; Cohen et al 1998). In patients unable to expectorate or who had smear-negative sputum samples, sputum induction has been used successfully for obtaining a suitable sample for culture even in resource-poor settings (Li et al 1999; Parry et al 1995). Light microscopy is commonly used for smear evaluation. Fluorescent microscopy provides an alternate method for the evaluation with a nearly 10% increase in sensitivity. Fluorescence microscopy using light-emitting diode (LED) as a light source is a cost-effective means of adapting this technique in economically challenged nations (Van Deun et al 2008).

Cultures

As cultures require less than a hundred organisms to detect *M. tuberculosis*, the sensitivity and specificity of culture method is very high, ranging from 80% to 93% and 98% respectively (ATS 2000a; Dalovisio et al 1996). Moreover, cultures are useful for speciation and drug-susceptibility testing (ATS 2000a). Broth media (e.g., BACTEC 460TB and BACTEC MB9000, the Mycobacterial Growth Indicator Tube) combined

with DNA probes can provide results in less than 2 weeks as opposed to 4-6 weeks for methods based on the standard solid media (Sharp et al 1997).

Nucleic acid amplification assays (NAA)

NAA assays amplify *M. tuberculosis*-specific nucleic acid sequences using a nucleic acid probe. NAA assays enable direct detection of the organisms in clinical specimens. NAA assays offer quick, sensitive, and specific detection of *M. tuberculosis* (ATS 2000a; Brodie & Schluger 2005). FDA has approved two NAA assays for commercial use: the AMPLICOR *M. tuberculosis* (Roche Diagnostic Systems, Inc., Branchburg, NJ), and the Amplified *M. tuberculosis* Direct (MTD) Test (Gen-Probe, Inc., San Diego, CA). The AMPLICOR assay uses DNA polymerase chain reaction (PCR) to amplify nucleic acid targets. The COBAS AMPLICOR is an automated version of the AMPLICOR *M. tuberculosis*. The MTD test uses a transcription-mediated amplification technique to detect *M. tuberculosis* specific rRNA (Brodie & Schluger 2005). The sensitivity and specificity of the NAA assays currently in commercial use is at least 80% and 95% respectively in most studies, and these assays can detect as few as 10 bacilli in a sample (ATS 2000a).

Diagnosis of Drug Resistance

Traditional methods of drug-susceptibility testing rely on cultures of *M. tuberculosis* inoculated with antibiotics and can take several weeks. Novel methods can detect drug-resistance rapidly. One approach takes advantage of genotypic abnormalities by identifying mutations primarily in the region of the *M. tuberculosis* rpoB gene

associated with most rifampin-resistant strains of *M. tuberculosis*. Combining the assays that spot genetic mutations with PCR allows rapid detection of the drug-resistant mutations from various specimens (Hirano et al 1999; Torres et al 2000). Thin layer agar (TLA) method relies on microscopic examination of growth on a solid media with or without the presence of anti-tuberculosis drugs to evaluate drug-resistance (Robledo et al 2008). Microscopic observation of drug susceptibility (MODS) can detect isoniazid and rifampicin resistance in as few as 9 days and involves observation of liquid culture in 24-well plates using an inverted microscope (Ejigu et al 2008).

Treatment of Tuberculosis

Until 1928, when Alexander Flemming launched the antibiotic era, the treatment for tuberculosis relied on supportive care and moving the patient to a dry and clean air environment such as mountains.

First-line Drugs

Isoniazid is used for the treatment of both latent and active tuberculosis and works by inhibiting the synthesis of mycolic acid which is a crucial component of mycobacterial cell wall. Its side effects, such as hepatitis, can be alleviated by the use of vitamin B₆ (Snider 1980).

Rifampin is a potent agent against actively dividing intracellular and extracellular organisms and has activity against semi-dormant bacilli. It works primarily by inhibiting bacterial DNA-dependent RNA polymerase and thus blocking RNA transcription (Blumberg et al 2003). Rifabutin is a rifamycin with properties similar to those of

rifampin. It is more active than rifampin against mycobacteria *in vitro*; however it has low bioavailability.

Pyrazinamide is most active in acid environments, especially within macrophages (Steele & Des Prez 1988). Pyrazinoic acid, the active form of pyrazinamide, has been shown to inhibit mycobacterial fatty acid synthetase I which is critical for fatty acid synthesis (Zimhony et al 2000).

Ethambutol is active against both intracellular and extracellular organisms. It disrupts arabinogalactan (a cell wall component) synthesis by inhibiting the enzyme arabinosyl transferase. Disruption of the arabinogalactan synthesis leads to increased permeability of the cell wall. This drug is given as part of the initial regimen in cases in which isoniazid resistance is suspected. The main side effect of ethambutol is optic neuritis (Varughese et al 1986).

Second-line Drugs

Cycloserine inhibits alanine racemase, the enzyme involved in the synthesis of UDP-N-acetylmuramyl-pentapeptide, a precursor of bacterial cell wall peptidoglycan (Neuhaus & Lynch 1964). Its side-effects include dose-related neurologic or psychiatric disturbances (Hershfield 1999).

Streptomycin functions by impeding bacterial protein synthesis. Ototoxicity and nephrotoxicity are linked with administration of this drug, especially in elderly patients (Blumberg et al 2003). Amikacin, kanamycin, and capreomycin are three aminoglycosides that are second-line agents used in treatment of patients who have drug-

resistant tuberculosis (Finegold 1959). Side effects include ototoxicity and nephrotoxicity.

An isonicotinic acid derivative, Ethionamide, interferes with bacterial peptide synthesis. Ethionamide often causes gastrointestinal side effects, such as abdominal pain, nausea, vomiting and anorexia (Hershfield 1999).

The fluoroquinolones are less effective than the first-line agents in treating tuberculosis and are mainly used in the treatment of drug-resistant disease (Kennedy et al 1993). They do not have many serious adverse reactions. Four fluoroquinolones are used in the treatment of tuberculosis: ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin and sparfloxacin.

Drugs in development

Ciofazimine, linezolid, thioacetazone, imipenem-cilastin, diarylquinolone (TMC-207 aka R207910), nitroimidazopyrans (PA-824 and OPC-67683) and diamine (SQ-109) are some of the novel drugs that are in various stages of pre-clinical or clinical trials (Lenaerts et al 2008; Ralph et al 2009).

Combating Tuberculosis - The DOTS strategy

The DOTS strategy (Directly Observed Therapy Short-course) as advocated by the WHO and the Stop-TB Partnership are a package of programs and policies designed to lessen the burden of tuberculosis through passive case-finding and treatment of the cases. DOTS has five major components: political commitment, good quality diagnosis, good quality drugs, short-course chemotherapy under direct observation and systematic

monitoring and accountability. DOTS hopes to achieve a cure-rate of over 90%.

According to several authors, without active case-finding and programs to prevent the disease it will be difficult to significantly impact tuberculosis. Despite the demonstration in all countries that DOTS has more than tripled the treatment success rate from 23% to 77%, the expansion of this strategy has been slow (Dye 2003).

Tuberculosis Vaccines

Bacillus Calmette Guerin (BCG)

Leon Charles Albert Calmette and Camille Guerin, the French microbiologists, isolated an attenuated strain of *M. bovis* after more than a decade of effort. This attenuated strain of tubercle bacillus was unable to produce tuberculosis in any laboratory animal (Calmette 1923). This strain, later named BCG, was initially given as an oral vaccine but adopted for intra-dermal usage in 1950.

The BCG vaccine has been used for nearly a century as the only vaccine to control tuberculosis. BCG vaccination is recommended by WHO for all infants under 1 year of age and annually 100 million newborn children are given this vaccine and globally nearly 3 billion individuals are already BCG vaccinated (Fine et al 1999b). The shortcoming is that the protective efficacy of BCG varies greatly, from 0 to 80% (Colditz et al 1994a; Fine et al 1999b). Even though the overall efficacy is low, the BCG vaccine protects against childhood tuberculosis. The immunity conferred by BCG wanes with age, resulting in insufficient protection against adult pulmonary tuberculosis (Fine et al 1999b).

Variability in BCG vaccine efficacy

Continuous attenuation of BCG preparations over time, environmental factors, sub-optimal delivery of vaccine, genetic and nutritional differences between populations have been thought to contribute to the variability in BCG vaccine's protective efficacy (Fine 1995). The observed performance deficiencies between BCG from earliest recorded trials and the later trials support the attenuation theory of BCG (Colditz et al 1995; Colditz et al 1994b). Recombinant BCG vaccines that supplement the lost genes back to the genome (Horwitz et al 2000) and genetically modified attenuated strains of *M. tuberculosis* (Lewis et al 2003; Sampson et al 2004) were designed to address such attenuation deficiencies, however the new vaccines based on mycobacterial vectors will likely have flaws that are inherent to BCG, if other theories on the failure of BCG are true (Doherty 2005; Orme 2005c).

Sensitization by environmental mycobacteria and latent infection prior to BCG vaccination are widely thought to be the reasons for the low protective efficacy of BCG vaccination in adults in the tropical regions of the world. The immune responses from these individuals would likely either mask the responses to BCG vaccination or inhibit *M. bovis* BCG replication before it can induce immunity (Fine 1989; Orme & Collins 1984b; Palmer & Long 1966; Rook et al 1981). This concept has been demonstrated in the mice model in which BCG vaccination of environmental mycobacteria-sensitized mice was unable to confer protection against *M. tuberculosis* infection (Brandt et al 2002). Moreover, calves that had high interferon-gamma (IFN- γ) response to environmental *M. avium* strain-antigens prior to BCG vaccination were less protected when challenged with virulent *M. bovis* (Brandt et al 2002; Buddle et al 2002).

The lack of efficient local induction of immunity within the lungs by immunization through the intradermal route has been considered to be another plausible explanation for the failure of BCG vaccine. Several new vaccine strategies are focused on optimizing vaccine efficacy by mucosal administration and new adjuvant/delivery systems (Aldwell et al 2003; Doherty et al 2002; Lyadova et al 2001; Orme 2005c). The shift in immune response towards a Th2 type has also been shown to significantly reduce the protective efficacy of BCG (Elias et al 2005; Malhotra et al 1999).

New Tuberculosis Vaccines

An NIH funded program in Colorado State University (CSU) performed preclinical testing of over 130 vaccine candidates (Orme 2005a). The candidates include live attenuated *M. tuberculosis* vaccines, recombinant BCG and vaccinia-vectored vaccines, DNA vaccines, subunit vaccines and fusion proteins, killed BCG and *M. bovis* combined with novel adjuvants and delivery systems.

Live Vaccines

Live *M. vaccae* and *M. microti* were variable in their protective efficacy (de Bruyn & Garner 2003). *M. tuberculosis* with defects in mycobacterial lipids (*M. tuberculosis* drrC) has been shown to be more protective than BCG when administered in mice (Pinto et al 2004). Several *M. tuberculosis* mutants including the ones that lack genes for mycolic acid synthesis have been tested at CSU with encouraging results. Currently several double knockout strains of *M. tuberculosis*, for example of the genes

involved in the pantothenate pathway and the RD1 region, are being evaluated (Orme 2005a).

Recombinant BCG

Introducing additional copies of existing genes or reintroducing the lost genes to improve BCG efficacy has garnered widespread interest. One recombinant BCG (rBCG30) that over-expresses immunodominant antigen Ag85B, has shown good protection in animal models (Horwitz & Harth 2003). A phase I clinical trial with rBCG30 was started in 2004 (Haile & Kallenius 2005). In one of these clinical studies, rBCG30 has been shown to induce a more significant *M. tuberculosis*-specific immunity in human volunteers compared to BCG (Hoft et al 2008). BCG complemented with ESAT-6, which is missing in BCG, showed better protection in mice (Pym et al 2003). However the complementation of RD1 genes ESAT-6 and CFP-10 to BCG did not improve the efficacy of BCG significantly (Orme 2006). Additional candidates shown to be effective in animal models are rBCGs secreting either the listerolysin molecule (Orme 2005a) or cytokines such as interleukin 2, interferon gamma, or granulocyte-macrophage colony-stimulating factor (Moreira et al 2000; Murray et al 1996).

Vaccinia-vectored Vaccines

Recombinant adenoviral or vaccinia virus live vectors (which can trigger Th1 dominated immune response) modified to carry immunodominant genes from *M. tuberculosis* are some of the vaccine candidates which have shown good protection in animal models (McShane et al 2001; Mollenkopf et al 2001). The recombinant,

replication-deficient, vaccinia virus expressing Ag85A (MVA85A) has been tested successfully in preclinical trials and was the first prophylactic vaccine candidate to enter a phase I clinical trial (McShane et al 2004). The results show increased levels of antigen-specific, IFN- γ -secreting T cells in the naïve healthy volunteers and in groups who had been BCG vaccinated earlier (Haile & Kallenius 2005). Side effects have been relatively minimal and this vaccine is currently under multiple phase II clinical trials in South Africa (Sander et al 2009). Aeras-402 Ad35, a replication deficient recombinant adenovirus 35 expressing Ag85a, Ag85B and TB10.4 is under initial phase I clinical testing in United States and South Africa (Radosevic et al 2007).

DNA Vaccines

DNA vaccines, containing plasmids coding for mycobacterial antigens, such as members of the mycolyl-transferase family (Ag85 complex) and heat shock proteins 60, 65, 70 (Hsp60, 65, 70) (Ferraz et al 2004; Lowrie & Silva 2000) have been shown to be protective in laboratory animal models (Huygen 2003). These vaccines have been tested as post-exposure vaccine candidates with mixed results (Orme 2006). DNA vaccines not only generate specific Th1 T cell responses, but also CD8⁺ T cell-mediated cytotoxicity, which is important in the protection against *M. tuberculosis*. However, the DNA vaccines, so far, have not been highly immunogenic in humans. Co-immunization with cytokines or formulation of DNA in cationic lipids or other types of adjuvants are being studied to increase vaccine efficacy (D'Souza et al 2002; Zaks et al 2006).

Subunit Vaccines

Secreted proteins of *M. tuberculosis* when used with strong adjuvants provide potential vaccine candidates (Hubbard et al 1992; Pal & Horwitz 1992). ESAT-6, Ag85B, hsp60, and a hybrid protein that is a fusion of ESAT-6 and Ag85B are some of the most prominent subunit vaccine candidates. Another vaccine candidate, consisting of a 72 kDa polyprotein or a fusion protein based upon the Mtb32 and Mtb39 antigens of *M. tuberculosis* (Mtb72F), has shown protective capacity as an adjunct to the BCG vaccine in mouse, guinea pig and non-human primate models (Brandt et al 2004; Reed et al 2009). This vaccine also entered clinical trials in 2004. The initial phase I clinical trial showed Mtb72f to be well tolerated and highly immunogenic in humans (Von Eschen et al 2009).

Killed Vaccines

Killed BCG has been shown to give only slight or no protection, in line with the consensus that bacteria has to replicate in the host to be protective (Weiss 1959). However, recently a heat-killed BCG vaccine used in combination with the Eurocine™ L3 adjuvant has been shown to improve the efficacy of BCG when given as a nasal prime boost vaccine (Haile et al 2005). Similarly formalin killed *M. bovis* has been shown to improve the survival of *M. tuberculosis* infected guinea pigs (Chambers et al 2004).

Immunopathology of Tuberculosis

Mycobacterium tuberculosis, the causative agent of tuberculosis is uniquely adapted for surviving and persisting in host phagocytic cells. Its ability to survive

phagocytosis is critical to establish the infection. Upon primary infection, *M. tuberculosis* enters and survives in alveolar macrophages. Later, the bacilli disseminate from the lung via lymphatic or blood circulation to establish systemic infection or reinfection of the lungs. Using a rabbit model, Arthur Dannenberg and Max Lurie had elegantly described pathogenesis of tuberculosis in four stages (Dannenberg 1994b). These stages are described below

Stage 1: Transmission and Infection

When a potential host inhales the infectious aerosol droplet generated by coughing, the bacilli travel through airway and are deposited in terminal alveoli where they are engulfed by resident alveolar macrophages. If the macrophages are capable enough, as is the case with 95% of individuals in a population, they engulf and destroy tubercle bacilli through innate immune responses. The progression of the mycobacterial infection depends on the mycobacterial virulence and the immune status of the host. In humans, the outcome varies from relatively mild and transient symptoms to a widely disseminated disease. If the bacillus survives the initial clash with macrophages, it initiates the primary infection.

Stage 2: Proliferation and Dissemination

The surviving bacilli multiply inside the alveolar macrophages and this multiplication eventually destroys the cell. During this stage, infected macrophages secrete various cytokines and chemokines attracting other immune cells including additional alveolar macrophages, dendritic cells (DCs), peripheral blood monocytes

(PBMCs), lymphocytes and neutrophils. When dying macrophages release the bacilli, the newly recruited phagocytic cells engulf the mycobacteria, which helps the bacilli to replicate further. Next the bacilli are transported via local lymphatics to the draining peribronchial and surrounding lymph nodes where critical immunological defenses are coordinated. Hematogenous spread of the bacilli also occurs during this stage. Whether the bacilli spread intracellularly within the phagocytes or as free bacilli is still unknown.

Stage 3: Onset of Host Immune Response

During the primary tuberculosis infection, the host's immune response mainly consists of two cell lines namely macrophages and lymphocytes. The antibody-mediated immune responses which are crucial against extracellular bacteria are considered to play a minimal role against tuberculosis. Antigen presenting cells (APCs) such as macrophages and DCs process and present the mycobacterial antigens to T lymphocytes. The antigens are processed and presented either through MHC class II pathway or through the MHC class I pathway depending on whether *M. tuberculosis* antigens are within the phagosomes or have escaped into the cytosol respectively. The cytokines, released by CD4 or CD8 lymphocytes after antigen presentation, recruit and activate macrophages leading to controlling proliferation or killing the bacilli. But some hosts are not able to mount a sufficient immune response resulting in progressive invasion by bacilli and clinical disease. People who clear primary infection remain at risk of reactivation of the disease later in life.

Stage 4: Severe Disease and Retransmission

During the fight against primary infection, the release of cytokines such as tumor necrosis factor α (TNF α) and proteolytic enzymes released by the dying macrophages and neutrophils are hypothesized to contribute to tissue damaging liquifactive necrosis. Further inflammation at the site results in cavitation due to complete loss of local tissue. When bronchial structures are involved, liquefied material spills into the airways. This material is expectorated by the host during coughing resulting in retransmission of the bacilli. The individual components of these stages are described in detail below.

Entry of *M. tuberculosis* into Macrophages

Uptake of mycobacteria requires receptor mediated phagocytosis by macrophages. These consist of complement receptors via either opsonized or non-opsonized entry (Brown 1991; Schorey et al 1997), mannose receptors, Fc receptors that can internalize IgG-opsonized bacteria and scavenger receptors (Armstrong & Hart 1975; Zimmerli et al 1996). Among complement components, mycobacteria enter macrophages predominantly via the C3 dependent complement receptor pathway. Recent studies suggest that a complement cleavage product C2a binds selectively to *M. tuberculosis* and other pathogenic mycobacteria (but not nonpathogenic mycobacteria) and might play a role in uptake (Schorey et al 1997). Besides binding to one or more specific receptor molecules, mycobacteria can interact with the plasma membrane steroid, cholesterol. When macrophages are depleted of cholesterol by pharmacological treatment, mycobacteria could no longer enter the macrophage suggesting a possible role for cholesterol in *M. tuberculosis* entry (Gatfield & Pieters 2000).

Mycobacterial Evasion Mechanisms

Virulent mycobacteria have developed strategies to escape the major killing mechanisms used by macrophages and take advantage of the environment within the host cell to avoid the antibody and complement mediated humoral immune response.

Macrophages infected with *M. tuberculosis* show faulty antigen processing, reduced responsiveness to interferon- γ (IFN γ) and reduced production of cytokines as well as reactive oxygen and nitrogen intermediates. Several reports point out that the induction of apoptosis by *M. tuberculosis* is inversely proportional to bacterial virulence. Less virulent strains were unable to prevent host cell apoptosis, leaving them exposed to the antibacterial activity that accompanies apoptotic death. Virulent strain H37Rv induced expression of anti-apoptotic genes in infected macrophages, while avirulent strain H37Ra induced a pro-apoptotic profile (Balcewicz-Sablinska et al 1998; Danelishvili et al 2003; Keane et al 1997). Moreover, macrophages from resistant strains of mice were prone to undergo apoptotic death, while those from susceptible mice did not (Rojas et al 1997). These observations support the hypothesis that apoptosis is a defense mechanism of the host, since reduced apoptosis correlates with greater susceptibility to infection.

Gradual acidification of the phagosome, phagosome–lysosome fusion, induction of reactive oxygen/nitrogen intermediates and antigen processing are main mechanisms by which intracellular organisms are killed. *M. tuberculosis* is shown to interfere with all these mechanisms. The phagosomes containing *M. tuberculosis* are resistant to fusion with the lysosome. Phagosomes containing viable, virulent mycobacteria show the presence of early endosomal markers whereas the phagosomes containing non-pathogenic strain *M. smegmatis* express the late endosomal markers. Mycobacteria

interfere with the presence of phosphatidyl-inositol-3-phosphate (PI3P) in the phagosomes. Mycobacterial cell wall component lipoarabinomannan (LAM) inhibits PI3P synthesis whereas mycobacterial acid phosphatase SapM hydrolyses the accumulated PI3P (Vergne et al 2003; Vergne et al 2005). Two other mycobacterial proteins, PtpA and PknG have also been shown to interfere with phagosome maturation (Bach et al 2008; Walburger et al 2004).

Calcium plays a major role in phagosomal maturation. However, macrophages infected with live *M. tuberculosis* demonstrated a decrease in the elevation of calcium normally associated with phagocytic uptake. Virulent mycobacteria also interfere with the host cell's actin filament network which is necessary for the phagolysosomal fusion (Russell 2001).

Coronin 1, also known as TACO, is actively retained at the phagosome containing live bacilli but not killed bacilli and it has been shown to prevent fusion with lysosomes, allowing the mycobacteria to survive within the phagosomes. Interestingly, liver which lacks TACO is completely able to eradicate mycobacteria in phagolysosomes (Ferrari et al 1999).

Involvement of Toll-like Receptors

The TLR2, TLR4, TLR1/TLR6 or TLR2/TLR6 heterodimers and TLR9 have been reported to recognize mycobacterial antigens (Bafica et al 2005; Bulut et al 2001). The araLAM (arabinosylated lipoarabinomannan) from non-pathogenic, fast-growing bacteria, the piLAM from *M. smagmatis*, and lipomannan (LM), a biosynthetic precursor of LAMs, have been shown to bind TLR2 and mediate their role in an MyD88 dependent

pathway (Quesniaux et al 2004b; Vignal et al 2003). PiLAMs and LM are usually considered as pro-inflammatory molecules stimulating the production of TNF- α and IL-12 (Maeda et al 2003).

The manLAMs found in the slow-growing mycobacteria including *M. tuberculosis*, *M. bovis* BCG and *M. avium* do not bind to TLRs. They bind to the dendritic cell surface receptor DC-SIGN and stimulate anti-inflammatory IL-10 production and inhibit the production of IL-12 and TNF- α by DC or monocytic cell lines (Geijtenbeek et al 2001). Consequently, the balance between LM and LAM synthesis by pathogenic mycobacteria is thought to provide pro- or anti-inflammatory signals during primary infection and during latent infection (Quesniaux et al 2004a).

Mycobacterial 19kDa lipoprotein bound to TLR2, but not TLR4, activated murine and human macrophages to secrete TNF and nitric oxide (Aliprantis et al 1999). A component of a short-term culture filtrate of *M. tuberculosis*, called soluble tuberculosis factor (STF) also interacted with TLR2, but not TLR4 (Jones et al 2001). Viable and killed *M. tuberculosis* bacilli (virulent and attenuated) activated both CHO cells and murine macrophages that expressed either TLR2 or TLR4 (Means et al 1999). A study has shown that infection of TLR4-competent and TLR4-deficient mice had similar outcomes measured in terms of the course of the disease, cell accumulation patterns in the lungs, and lung histopathology and concluded that TLR4 did not play a role in protective immunity to tuberculosis in the mouse model (Shim et al 2003). In another study, *M. tuberculosis* infected TLR2^{-/-} mice, showed defective granuloma formation, displayed only a small elevation in pulmonary bacterial loads late in infection and survived for at least 150 days (Drennan et al 2004). *M. tuberculosis* infected TLR9^{-/-}

mice showed slightly increased susceptibility whereas TLR2/TLR9 double knock-out mice displayed markedly enhanced susceptibility (Bafica et al 2005).

Various T-lymphocytes in Tuberculosis Immunity

The importance of T lymphocytes in the immune defence against *M. tuberculosis* was first established by studies showing the increased susceptibility of mice that were devoid of T cells due to thymectomy and adoptive transfer of *M. tuberculosis* specific immunity using T cells in rats (Lefford et al 1973; North 1973).

CD4 Lymphocytes

The critical involvement of CD4 T cells in the protective immune response against tuberculosis has been well established. Several studies using a mice model have shown the importance of CD4 cells by antibody depletion, and adoptive transfer and CD4 gene-knockout (Caruso et al 1999; Muller et al 1987; Orme & Collins 1983; 1984a; Tascon et al 1998). Moreover, significantly CD4 depleted human HIV patients are highly susceptible to active infection and reactivation of tuberculosis. IFN γ and other cytokine release to activate the macrophages to kill the bacilli, is considered to be the main effector mechanism for CD4 cells. However, the reactivation of tuberculosis in CD4 deficient mice occurs despite normal levels of IFN- γ and iNOS suggesting a possible IFN γ -independent role for CD4 cells (Scanga et al 2000).

CD8 Lymphocytes

CD8 cells also contribute to the immune response against tuberculosis although not to the same extent as CD4 cells. Several gene mutation studies using mice that lack β 2-microglobulin (an essential part of MHC class Ia) (Flynn et al 1992), CD8 α (Cooper et al 1997a) and the transporter protein associated with MHC I antigen processing (Behar et al 1999) showed the importance of CD8 cells in the defense against tuberculosis. Access to the MHC I processing is achieved by either active transport or escape of *M. tuberculosis* antigens from phagosomes to the cytosol or by alternative MHC class I pathways which permit processing of phagosomal antigens, thus allowing CD8 T cell activation or by the formation of apoptotic vesicles which can be internalized by DCs and presented to CD8 cells. The primary effector mechanisms of CD8 cells include cytokine production, mainly IFN- γ and lysis of infected cells directly. Protection conferred by CD8 is considered to occur during chronic stages of the disease (Turner et al 2001a). Perforin-deficient mice are capable of controlling the *M. tuberculosis* infection similar to wild type controls (Cooper et al 1997a). However, mice do not have a homolog for granulysin, the executer molecule in perforin-mediated killing. As a result, the role of CD8 in protection against human tuberculosis is still not completely understood.

$\gamma\delta$ T Lymphocytes

The involvement of $\gamma\delta$ lymphocytes in tuberculosis immunity is vague. They accumulate in mycobacterial lesions and with specific recognition of mycobacterial antigens. Since they can produce IFN- γ , TNF- α , GM-CSF and IL-3, $\gamma\delta$ cells are considered to play a role in the early immune response against tuberculosis. The TCR $\gamma\delta$ -knock-out mice did not provide sufficient evidence for their protective role (D'Souza et al

1997). However $\gamma\delta$ lymphocytes are considered to play a role in efficient granuloma formation and prevention of local tissue damage (Saunders et al 1998).

Natural Killer Cells

Natural killer (NK) cells were thought to contribute to protective immunity in the lungs to tuberculosis infection; however recent studies proved to be inconclusive (Orme 2004). Comparable patterns of bacterial growth and pathology were seen in mice which were unable to generate NKT cell responses and wild-type controls (Sugawara et al 2002). Even though NK cell activation has also been observed organ wide, depletion of these cells *in vivo* did not change the course of the infection (Junqueira-Kipnis et al 2003).

CD1-restricted T cells

Lipids and glycolipid antigens are presented by non-conventional MHC class I-like molecules of CD1. They are presented to CD1-restricted T cells. Group 1 CD1 molecules (CD1a, b, and c) are found in professional APCs and present mycobacterial lipid and glycolipid antigens to specific T cells whereas group 2 CD1 (CD1d) is constitutively expressed in variety of cells and they interact with NKT cells (Cohen et al 2009). CD1-restricted T cells express a wide range of effector mechanisms including cytotoxic activity and IFN γ secretion suggesting possible involvement of these cells (Ulrichs & Porcelli 2000). Direct experimental data to support this role using an animal model is not available yet.

Cytokines and chemokines

Proinflammatory Cytokines

M. tuberculosis recognition by phagocytic cells leads to cell activation and production of cytokines, which further activates macrophages and produces more cytokines. This cytokine network plays an essential role in the outcome of mycobacterial infections.

Monocytes, macrophages, and DCs when infected with *M. tuberculosis* produce a prototype proinflammatory cytokine, TNF α (Henderson et al 1997; Valone et al 1988). TNF α is critical for granuloma formation, macrophage activation, and immunoregulation (Senaldi et al 1996; Tsenova et al 1999). However, excess TNF α can lead to undesirable inflammatory effects like wasting. In mice, TNF α is also important for containment of bacilli within granulomatous lesions (Mohan et al 2001). Mice deficient in TNF α , or the TNF α receptor p55, display an increased susceptibility to tuberculosis (Roach et al 2001; Senaldi et al 1996).

The protective role of IFN γ in tuberculosis has been very well known. CD4, CD8, CD1 and NK cells produce IFN γ as their effector cytokine. IFN γ -deficient mice undergo widely disseminated tuberculosis with mice dying rapidly upon *M. tuberculosis* infection (Cooper et al 1993). Mycobacterial antigen-specific IFN γ production *in-vitro* can be used as a surrogate marker of infection with *M. tuberculosis* (van Crevel et al 1999).

IL-1 β is mainly produced by monocytes, macrophages, and dendritic cells (Roach et al 1993). In patients with tuberculosis, IL-1 β is expressed in high levels (Schauf et al 1993). IL-1 α and -1 β double-KO mice and IL-1R type I-deficient mice displayed

increased mycobacterial outgrowth and defective granuloma formation (Juffermans et al 2000).

IL-6 has both pro- and anti-inflammatory properties and is produced early during mycobacterial infection (Law et al 1996). One study showed a negative role for IL-6 in mycobacterial infections, as it inhibited the production of TNF α and IL-1 β and promoted *in vitro* growth of *M. avium* (Law et al 1996). In contrast, another study supported a protective role for IL-6 as deficient mice displayed increased susceptibility to infection with *M. tuberculosis* (Ladel et al 1997a).

IL-12 is produced mainly by phagocytic cells, and phagocytosis of *M. tuberculosis* is necessary for its production (Ladel et al 1997b). IL-12 has a crucial role in the induction of IFN γ production. The protective role of IL-12 is demonstrated by high susceptibility of IL-12-deficient mice to *M. tuberculosis* infection (Cooper et al 1997b).

IL-18 is similar to IL-1 in a few aspects (Dinarello et al 1998). IL-18 deficient mice are highly susceptible to *M. bovis* BCG and *M. tuberculosis*, and in mice infected with *M. leprae*, resistance is correlated with a higher expression of IL-18 (Kobayashi et al 1998; Sugawara et al 1999).

Anti-inflammatory Cytokines

IL-10 downregulates the production of IFN γ , TNF α , and IL-12 and thereby interferes with host defense against *M. tuberculosis* (Gong et al 1996). Transgenic mice over-expressing IL-10, develop a higher bacterial burden during mycobacterial infection. Similarly, IL-10-deficient mice show a lower bacterial burden early after infection (Murray et al 1997; Murray & Young 1999).

TGF β is another cytokine that is likely to counteract protective immunity during tuberculosis. Mycobacterial products induce production of TGF β by monocytes and dendritic cells. Notably, LAM from virulent mycobacteria selectively induces TGF β production (Dahl et al 1996). TGF β is also produced in excess during tuberculosis and is expressed at the site of disease (Condos et al 1998). TGF β contributes significantly to fibrosis during tuberculosis (Sporn et al 1986).

The possible deleterious effects of IL-4 in intracellular infections such as *M. tuberculosis* is attributed to this cytokine's suppression of IFN γ production and inhibition of macrophage activation (Powrie & Coffman 1993). The deleterious role of IL-4 is questioned by a study as IL-4 deficient mice displayed normal instead of increased resistance to mycobacteria (North 1998).

Chemokines

Chemotactic cytokines, also known as chemokines, are primarily responsible for the recruitment of inflammatory cells to the site of infection. IL-8 is a chemokine which attracts neutrophils, T lymphocytes and monocytes. Macrophages produce IL-8 either after phagocytosis of *M. tuberculosis* or stimulation by LAM. The cytokines TNF α or IL-1 β are thought to control IL-8 production. Monocyte chemoattractant protein 1 (MCP-1) is a second major chemokine, which is produced by, and acts on, monocytes and macrophages (Rhoades et al 1995). Murine studies have demonstrated that deficiency of MCP-1 resulted in inhibition of granuloma formation without enhancing susceptibility of mice to *M. tuberculosis* (Lu et al 1998). Moreover, mice overexpressing MCP-1 were

more susceptible to the infection, suggesting that MCP-1 induced recruitment of macrophages favors the bacilli.

A third chemokine RANTES, which is produced by a wide variety of cells, is known to attract primarily eosinophils and it binds to multiple chemokine receptors. Expression of RANTES was associated with development of *M. bovis*-induced pulmonary granulomas in mice (Chensue et al 1999). In human patients with tuberculosis, RANTES has been detected in bronchoalveolar lavage (BAL) fluid (Kurashima et al 1997).

Th1 and Th2 immune responses in tuberculosis

Protective immune response against *M. tuberculosis* requires a Th1 mediated immune response. Studies of patients with familial susceptibility to tuberculosis have confirmed that Th1 vigor is essential for protection. In human population, mutations which impair IFN-gamma receptors, IL-12 production, or IL-12 receptors (all Th1-associated) also increase susceptibility to *M. tuberculosis* and in patients who cannot produce or respond to IFN γ , the disease is severe and often fatal (Lienhardt et al 2002; Newport et al 1996). Genetic defects in IL-12 or IFN γ pathways lead to increased susceptibility to tuberculosis in mice (Cooper et al 1997b; Flynn et al 1993). A large study performed in Europe and Africa demonstrated that tuberculosis patients had significantly lower plasma soluble lymphocyte activating gene-3 (sLAG3) levels (Th1 marker), while healthy household controls had higher sLAG-3 than the community controls. All the Th2 markers such as plasma IgE, soluble CD30, and MDC/CCL-22 (a chemokine derived from macrophages) were consistently higher in tuberculosis patients

than in the community controls suggesting that individuals with weaker Th1 and/or stronger Th2 were more at risk for tuberculosis (Lienhardt et al 2002).

Despite the induction of a potent Th1 response, most animals and certain humans go on to eventually develop progressive disease (Rook et al 2005a). Approximately 90% of individuals who are exposed to *M. tuberculosis* control it with minimal immunological activity (Opie & Aronson 1927). Infection of the susceptible C3H mice with *M. tuberculosis* mutant which lacked the transcription factor *sigH* only demonstrated less immunopathology and virulence despite growing to wild type levels (Kaushal et al 2002). The *sigH* mutant strain infected mice recruited only about 10% CD4 and CD8 cells and hence it was suggested that relatively few Th1 cells were sufficient to control the bacterial proliferation and the rest of recruited T cells were unnecessary and might instead cause immunopathology (Rook et al 2005b). Moreover, efforts to increase the efficacy of BCG by enhancing IFN γ production through DNA vaccine priming have been unsuccessful suggesting that the larger amount of Th1 response does not essentially provide better protection. Consequently a corrupted Th1 response rather than a failed Th1 induction has been hypothesized to be critical of the development of the disease (Rook et al 2005a).

Animal Models of Tuberculosis

The Granuloma

The distinct pathologic feature of mycobacterial infection is granuloma formation. Its role in tuberculosis pathogenesis is paradoxical. In earlier stages of infection, the granuloma controls the spread of the bacilli, however it also contributes to significant

immunopathology which undermines the ability of the granuloma to control the spread of the bacilli. The classical granuloma has a central core of acellular eosinophilic necrotic debris surrounded by few layers of epithelioid macrophages and a few concentric layers of lymphocytes and fibrosis. Scattered multinucleated giant cells and plasma cells are also present.

Based on the gene knockout mice studies, CD4 cells, CD8 cells, $\gamma\delta$ T cells were shown to play varying degrees of role in granuloma formation (D'Souza et al 1997; Saunders et al 2002; Turner et al 2001a). Granuloma development also involves elaborate interplay between various chemokines and cytokines as well as some of the adhesion and integrin molecules (Saunders & Cooper 2000). Animal models have been used since the time of Robert Koch to study tuberculosis. Currently mouse, guinea pig, rabbit, and non-human primate models are the ones that are widely used. The choice of the most appropriate model for a study depends mainly on the research question of the investigator. The comparative aspects of the granuloma are discussed under each animal model below.

Mouse Model

The inbred mouse has been the most popular model and has contributed immensely towards addressing basic immunological questions pertaining to *M. tuberculosis* infection (Orme 2003).

Acute Infection Models

The use of acute-infection models makes the study of the initial immune responses possible. Previously intravenous delivery of bacteria was used to achieve the

acute infection in the mouse; however, in this method the bacteria are deposited directly in the spleen, resulting in very rapid induction of the acquired T-cell response (Cardona et al 1999; Orme 1987). The compromised innate immunity due to the rapid induction of adaptive immunity makes the intravenous model less desirable (Flynn et al 2005). The aerosol route of infection most closely mimics natural infection. In this model, the outcome resembles human natural infection (Middlebrook 1952; Ratcliffe & Palladino 1953). A low dose inoculum with roughly 50 to 100 bacilli per mouse delivered to the lungs by special equipment results in steady bacterial growth, reaching a plateau of between 10^5 to 10^6 bacilli by 30 days after the induction in the lungs (Orme 2003).

Chronic or Latent Infection Models

In humans, latent infection is defined as *M. tuberculosis* infection with no signs of active disease. Two mice models are available to study chronic or latent tuberculosis (Flynn & Chan 2001b; Orme 2001). In the first, resistant strain of mice are infected by aerosol eventually resulting in a stable chronic disease in the lungs (Orme 1988). The drawback of this model is that the actual bacterial load is higher than that in true latent disease in humans.

In the second model, named the Cornell model, tuberculosis infection is established followed by drug treatment to reduce the bacterial burden to an undetectable level. After the withdrawal of chemotherapy the infection either spontaneously reactivates, or is induced by immuno-suppressive steroids (McCune et al 1966; McCune et al 1956; McCune & Tompsett 1956). The artificial intervention and too many variables that could influence the outcome are the limitations of this model (Lenaerts et al 2004).

Despite its distinct advantages, the murine model still has some drawbacks. The major limitation is the difference between disease progression in mice and human. Notably there are large number of bacteria and evidence of more dispersed information in the lungs following even a low-dose infection in mice (Rhoades et al 1997). Significant pathologic features of human disease such as caseous necrosis and cavitation do not occur in the mice model. Additionally, certain aspects of the human system, such as the non-classical antigen-presenting molecules CD1a, CB1b, and CD1c and the effector molecule of CD8 T cells, granulysin, are absent in mice (Orme 2003).

Variability among inbred mouse strains

In the low-dose aerosol model of infection, inbred mouse strains vary in their susceptibility to *M. tuberculosis* (Orme 2003). Whereas virtually all mice initially control infection in the lungs, strains such as DBA/2, A/J and CBA/J eventually develop a form of reactivation disease that is fatal to the animal. In contrast, mice on the C57 background maintain a chronic bacterial load and deteriorate more slowly. Inadequate expression of lymphocyte adhesion markers in the susceptible strains could explain such differences (Turner et al 2001b).

Rabbit Model

Rabbits have been used as a model of human tuberculosis since the early 20th century (Lurie et al 1952). Several groups have extensively studied the rabbit model (Allison et al 1962; Bishai et al 1999; Converse et al 1998; Dannenberg & Collins 2001; Tsenova et al 1999; Yamamura 1958). Similarities of rabbit tuberculosis to the human

disease include caseation, cavitation, and containment of bacilli in a potential latency-like state (Dannenberg 1993; Flynn et al 2005). An important characteristic of human tuberculosis is the development of the pulmonary cavitory lesion with accompanying local tissue destruction. Rabbits can also develop similar cavitory tuberculosis (Lurie 1932; Ratcliffe & Palladino 1953; Steenken et al 1953). Cavitation in rabbit lungs is observed roughly 8 to 12 weeks following *M. bovis* infection and is characterized by reinvasion of the lesions by mononuclear cells and neutrophils. This cellular infiltration leads to further local tissue destruction. Eventually this process evolves into liquefaction and production of a pulmonary cavity. In general, *M. tuberculosis* aerosol infections are contained whereas *M. bovis* infections progress to cavitory disease (Flynn et al 2005).

Due to the resistance of rabbits to fatal tuberculosis, traditional survival studies and colony forming units (CFU) enumeration are not particularly valuable parameters in comparing the virulence of different mycobacterial strains. Quantification of disease extent of the caseous tubercle has been used in the rabbit model (Dannenberg & Collins 2001). In the past decade, Kaplan and colleagues developed the rabbit as a model of tuberculous meningitis (Tsenova et al 1999).

Nonhuman Primate Model

The nonhuman primate model resembles human tuberculosis more closely than any other model. Active tuberculosis is highly fatal in monkeys. Like humans, an actively infected monkey can transmit infection to other animals, and this can lead to massive outbreaks of tuberculosis in animal housing facilities. For tuberculosis modeling both rhesus (*Macaca mulatta*) and cynomolgus (*M. fascicularis*) macaques have been used.

Aerosol infection can be difficult (Barclay et al 1973; Shen et al 2002). Usually the bacilli are delivered into the trachea or into the lungs using a bronchoscope. In a study, the cynomolgus monkeys infected with higher doses (10^3 to 10^5) showed signs of disease and succumbed to tuberculosis between 3 and 29 weeks post-inoculation, whereas those infected with 10 to 100 CFU controlled the infection up to the time of euthanasia and showed only minimal lung disease suggesting the possibility of latent infection modeling (Walsh et al 1996).

Cynomolgus macaques are comparatively less susceptible to tuberculosis than rhesus macaques. However, BCG immunization is much more protective in cynomolgus macaques than in rhesus macaques (Langermans et al 2001). The pathology of monkey tuberculosis is very similar to that in humans. The granulomas of monkeys have a similar cellular hierarchy. Caseation, liquefaction, calcification and cavity formation are also observed in the lungs of monkeys with advanced disease (Capuano et al 2003; Langermans et al 2001). Nonhuman primates are currently used as a final step in animal testing of vaccines and drugs against *M. tuberculosis* before they enter human clinical trials.

The nonhuman primate model also provides an excellent opportunity to study the interaction of SIV (a model for HIV) and *M. tuberculosis*. Monkeys with deficient immune systems (due to SIV) prior to *M. tuberculosis* infection do not control the infection and rapidly succumb to fulminant tuberculosis (Safi et al 2003). As coinfection with *M. tuberculosis* and HIV is a substantial problem worldwide and tuberculosis is a major killer of AIDS patients, this model facilitates the study of the interaction of these two important pathogens (Flynn et al 2005).

Guinea Pig Model

Guinea pigs are considered to be ideal model to study tuberculosis since the pathology resembles humans more closely than mice model and guinea pigs are not as expensive as nonhuman primate models (McMurray 2001b; Orme et al 2001). They have been used since historical times to study tuberculosis. Robert Koch used guinea pigs to establish Koch's postulates and to identify the tubercle bacillus as the etiologic agent of tuberculosis (Koch 1932). Guinea pigs are extremely susceptible to infection with *M. tuberculosis*, progress rapidly to active disease and were used for many of the seminal animal experiments in studies of tuberculosis. The guinea pig model is an important tool for testing of effective anti-tuberculosis chemotherapy and vaccines, as well as for the characterization of mycobacterial virulence factors (Dai et al 1998; McMurray 2001b; Orme 2005a; Orme et al 2001; Smith et al 1991a).

The cellular response to tuberculosis in the guinea pig is rapid, with discrete lesions primarily composed of macrophages present as early as 10 days after aerosol infection. These early lesions also contain numerous eosinophil-like heterophils. These heterophils can contribute to the development of the necrotic core. In contrast, there is only mild interstitial inflammation present at this time in the mouse. After another 10 days, the granuloma in the guinea pig begins to form, with an influx of mononuclear cells surrounding the core as it grows in size. In mice, perivascular cuffing by infiltrating lymphocytes is prominent at this stage. By day 30, the lesion in the guinea pig attains the characteristic of the "classical granuloma" with a large necrotic core, a layer of

vacuolated ("foamy") macrophages, a layer of lymphocytes, and evidence of collagen deposition beginning to form a rim (Orme 2005b).

The CD4 and CD8 cells appear to be relatively uniformly distributed across the guinea pig granuloma (Turner et al 2003). In contrast, in the mouse, CD4 cells predominate, with CD8 cells more scattered towards the periphery of the lesion (Gonzalez-Juarrero et al 2001). An additional feature of the guinea pig granuloma is the presence of Langhans' multinucleated giant cells. These giant cells form as macrophages fuse and are also present in human granulomas. Thus, the guinea pig granuloma exhibits many characteristics typical of its human counterpart (Orme 2005b). A recent study from this laboratory showed marked influx of CD4 cells expressing CD45 and minimal influx of CD8 cells within lungs over the first 30 days after the infection. After day 30 of infection, there is the dramatic decrease in CD4 response with corresponding increase in influx of B cells and granulocytes (heterophils and eosinophils) (Ordway et al 2007).

The major difference between guinea pig and human disease is the inherent susceptibility of guinea pigs to *M. tuberculosis* and it suggests that guinea pigs lack robust immune mechanisms similar to humans. Guinea pigs can be efficiently infected by aerosolizing very small number of bacilli (McMurray 2001b; Riley et al 1962). At the sites of primary infection in the lungs, the bacilli grow exponentially for the first 21 days. After such rapid growth, hematogenous spread from the primary foci of infection to other organs, as well as to the lungs, occurs. All animals eventually succumb to the infection, characterized by progressive pulmonary tuberculosis.

The invariably fatal disease progression in guinea pigs provides a reliable readout for evaluating the protection conferred by a vaccine candidate or efficacy of drugs.

Compared to the 1- log reduction in bacterial load achievable by BCG vaccination in the mouse, the 2- to 3-log-reduction in BCG-vaccinated guinea pigs provides a wider range for the assessment of vaccine efficacy (McMurray 2001b; Orme et al 2001). The limitations of the guinea pig model include the expense of maintaining guinea pig colonies in a bio-containment facility compared to the expense of housing mice. The limited availability of immunologic reagents for guinea pig studies is another limiting factor (Jeevan et al 2002; McMurray 2001b).

Guinea pig Primary Lesion Pathogenesis

Primary lesion denotes the foci of granulomatous inflammation and necrosis of the lung and draining lymph nodes that result from the initial aerosol exposure. In humans the site of primary pulmonary infection is called the Ghon lesion. The combination of Ghon lesions and chronic lesions in the hilar lymph nodes that are often calcified is referred to as Ranke's complex (Smith et al 1991b).

Primary Lesion Necrosis

Georges Canetti in his extensive study with nearly 1500 human tuberculosis cases classified caseating and cavitary lung lesions as unfavorable host responses. He concluded that highest numbers of bacilli were often associated with granulocytic inflammation or lesion necrosis (Canetti 1955). Necrosis represents a major change in host and pathogen interactions. The bacilli exit the intracellular milieu for the first time and replicate extracellularly within the necrotic core (Dannenberg 1994b). Necrosis not only allows the spread of the bacilli but also provides a conducive microenvironment rich

with nutrients to support mycobacterial growth. Moreover, we have demonstrated that the primary lesion necrosis can be an important correlate of virulence in the guinea pigs infected with *M. tuberculosis* (Palanisamy et al 2008). Necrosis was originally thought to be induced by a cell mediated immune response. Delayed type hypersensitivity (DTH) induced by such immune response was widely accepted to be the cause of lesion necrosis (Dannenberg 1994a; Dannenberg 1994b). Failure of macrophage activation was also hypothesized to be a contributing factor (Orme 1998). Recent literature however attributes innate immunity as a source of lesion necrosis (Turner et al 2003). This development of primary lesion necrosis in the guinea pig overlaps the influx of granulocytes that include eosinophils and neutrophils (called heterophils) (Ordway et al 2007). The influx of heterophils in the infected guinea pig is biphasic with the first influx occurring within hours or days of infection following infection and the second influx likely occurring between 3-4 weeks post-infection when lesion necrosis is most severe (Basaraba et al 2006a; Basaraba et al 2006b; Prabhakar et al 1987; Turner et al 2001a).

Multiple factors including host and pathogen factors play a role in the development of lesion necrosis. Host factors include strong proinflammatory milieu with high levels of TNF α , production of large amounts of tissue damaging free radicals and progressive, unregulated inflammation, among others. Bacterial factors such as early secretory proteins encoded by the RD1 region and cell wall lipid trehalose 6, 6'-dimycolate (TDM) have been shown to contribute to lesion necrosis (Hunter et al 2006; Junqueira-Kipnis et al 2006). Unlike the mouse model, the guinea pig granuloma microenvironment has been shown to be hypoxic similar to that of human disease (Aly et

al 2006; Tsai et al 2006). Low oxygen tension prevalent in these microenvironments can worsen lesion necrosis.

Interestingly, the rapid progression of primary lesion necrosis corresponded with the reduction in the number of viable bacilli in the infected guinea pig around 21 days after infection due likely to massive extra-cellular release of proteolytic enzymes from dead and dying cells (McMurray 2001b). Those that survive may represent the population of persistent extracellular bacilli that even survive anti-tuberculosis drug therapy (Lenaerts et al 2007).

Secondary Lesions

In humans, during the bacillemic phase, the resurgent inflammation in the apical and sub-apical lung lobes results in the fibro-nodular scarring (Simon's focus) and eventual cavitation leading to transmission of the bacilli (Balasubramanian et al 1994). The lesions in the lungs that occur due to hematogenous re-infection following dissemination and bacillemia are known as secondary lesions. Guinea pigs demonstrate distinctive morphologic differences between the primary lung lesions that occur due to aerosol seeding of the bacteria and the secondary lung lesions that occur due to hematogenous re-seeding. Lack of necrosis and calcification are the main features that differentiate secondary lesions from primary lesions. Moreover secondary lesions consist mostly of lymphocytes and fewer macrophages. The muffled nature of the secondary lesion is thought to be due to immunization effect conferred by previous exposure during primary infection (McMurray 2003). Close resemblance of secondary lesions in the naïve

guinea pigs to the primary lung lesions in the BCG vaccinated guinea pigs lends support to such theory (McMurray & Bloom 1994; Smith et al 1991b).

Beginning to develop at around day 20 after infection and very prominent around day 60, the secondary lesions can develop anywhere throughout the parenchyma unlike the primary lesions which have a more restrictive distribution pattern (sub-pleural, peribronchial and perivascular) (Basaraba et al 2006a). Secondary lesions often surround and encompass primary lesions. Unlike Simon's focus of human beings, there is minimal irreversible tissue damage within secondary lesions except in extremely chronic disease. Comprehensive pathogenesis of primary, secondary lesions and primary lesion necrosis are all still poorly understood despite their importance in the disease progression.

Another important feature of secondary lesions is the presence of granulocytes (intact and degenerate) in the associated airway lumens. The transmigration of senescent inflammatory cells as a normal process of resolving inflammation could explain this phenomenon (Erjefalt et al 2004). This is an important feature as the degenerate cells within the airways contain numerous acid-fast bacilli that can easily be aerosolized for re-transmission.

BCG Vaccination in Guinea pigs

BCG vaccination of guinea pigs prior to *M. tuberculosis* challenge results in improved clinical disease outcomes, decreased pulmonary and extra-pulmonary lesion burden, prolonged survival, delayed extra-pulmonary dissemination, and reduced numbers of viable bacilli in lungs and extra-pulmonary tissues (McMurray & Bloom 1994; Smith et al 1970; Takizawa et al 2006). BCG vaccination has also been shown to

prevent or decrease granulocytic inflammation, necrosis, dystrophic calcification and fibrosis in the primary lesion complex compared to naïve guinea pigs. The persistence of *M. tuberculosis* in primary lesions in naïve guinea pigs therefore is attributed at least in part to the distinctive morphologic feature namely necrosis (Canetti 1955). Similar to the lung, BCG vaccination of guinea pigs prior to aerosol challenge, prevents lymph node enlargement, primarily by preventing necrosis (Prabhakar et al 1987). The eventual loss of normal lymph node architecture with replacement fibrosis and inflammation can likely explain the ultimate fatal outcome even in the BCG-vaccinated guinea pigs (Prabhakar et al 1987). With novel flow cytometry reagents, it was shown that the bacterial control was associated with increased accumulation of both CD4 and CD8 T cells in BCG vaccinated guinea pigs lungs in the early phase whereas the numbers of T cells decreased as the infection progressed. The early influx of B cells and decreased influx of heterophils were also observed in the vaccinated animals. This decreased influx of heterophils may explain the lack of necrosis in the vaccinated guinea pig lung lesions (Ordway et al 2008).

Measure of Virulence

The ability of the pathogen to induce disease in the infected host is defined as its virulence. The parameters of virulence include severity of the disease and fatal outcome. Simply, virulence is the ability of the pathogen to impact the fitness of the host (Thomas & Elkinton 2004). In the mouse and experimental models of tuberculosis, the most direct correlate of virulence is the survival time of infected animals (North et al 1999a). In vaccine and drug evaluation studies, at least in the earlier stages of evaluation, the differences in the number of bacilli are used widely as a correlate of protection (Orme

2005a; Wiegeshaus et al 1970). However, the growth rate of *M. tuberculosis* can be an unreliable indicator of mycobacterial virulence (North et al 1999b). Clinical signs and weight loss are also considered to be correlates of virulence. The immunological correlates of mycobacterial virulence include delayed type hypersensitivity (DTH) and antigen specific IFN γ production by T cells, although the usage of DTH for such correlation is disputed (McMurray 2001a). Pathological characteristics such as extent of the lesions and extrapulmonary dissemination have also been tried as virulence correlates (Dormans et al 2004). In humans, the ability of the mycobacterial organisms to be readily transmitted via aerosol is a critical correlate. However, due to the lack of transmissibility of *M. tuberculosis* in the mouse and guinea pig models, this correlate is not useful.

Variability among Clinical and Drug-resistant Strains of *M. tuberculosis*

The clinical strains of *M. tuberculosis* isolated from various community outbreaks all over the world vary widely in their ability to grow and induce disease in experimental animal models (Kelley & Collins 1999; Orme 1999). Certain strains are more virulent than others. For example certain strains of W-Beijing strains of *M. tuberculosis* are associated with outbreaks characterized by rapid spread with high morbidity and mortality. The presence of a highly biologically active phenolic glycolipid, PGL-tb, in W-Beijing strains has originally been attributed to their increased virulence (Reed et al 2004). However, a later study showed that PGL-tb, by itself, did not confer hypervirulence (Sinsimer et al 2008). The drug resistance in several strains of *M. tuberculosis* is considered to be due to genetic mutations (Ellner et al 1993). This combined with the studies by Mitchison and colleagues that demonstrated low virulence

of isoniazid-resistant *M. tuberculosis* isolated from patients in India, led to the idea that drug-resistant strains of *M. tuberculosis* are of low virulence (Mitchison et al 1960). The subsequent study using a few drug-resistant *M. tuberculosis* strains in the mouse model did not show an association between low virulence and drug-resistance (Ordway et al 1995). This could reflect species differences between the mouse and guinea pig model. Most of the animal studies that evaluate drugs and vaccines, use laboratory strains such as H37Rv and Erdman and the results obtained using these laboratory strains may not reflect the “real world” situations (Ordway et al 1995).

Oxidative Stress

The clinical manifestations of tuberculosis in humans and animals are directly proportional to the severity and extent of pulmonary and extra-pulmonary inflammation. In the early stages of the infection, before the development of adaptive immunity, lesions are made up largely of macrophages and granulocytes (Canetti 1955). As bacilli replicate and disseminate, acquired immunity develops and the cellular infiltrates progress to include B and T lymphocytes (Chackerian et al 2002). Central to their function as professional phagocytes, macrophages and granulocytes generate reactive oxygen species and nitrogen intermediates (ROS/RNI). The cellular balance between the production of oxidants (i.e. ROS/RNI) and the host antioxidant scavenging system is important in cellular homeostasis. Oxidative stress develops when the generation of ROS exceeds the oxidant scavenging capacity of host antioxidants.

Oxygen is the terminal electron acceptor for respiration in cells that function in an aerobic environment. At the molecular level, the concurrent transfer of four electrons is

necessary for the total reduction of an O₂ molecule. However, the reduction of O₂ with less than four electrons leads to the production of ROS (Haddad 2004). The generation of ROS is important for normal cell functions by acting as signaling molecules (redox signaling) in immune regulation, blood circulation, and endocrine hormone control (Thannickal & Fanburg 2000). Various physiological factors (hormones and cytokines) as well as external factors (xenobiotics and UV-radiation) enhance the production of cellular ROS. When present in excess amounts, ROS can cause cell injury by oxidation of cell components such as proteins, lipids, and DNA, a process that is implicated in the pathogenesis of a variety of inflammatory diseases as well as atherosclerosis, pulmonary fibrosis, Alzheimer's disease, and Parkinson's disease (Cho & Kleeberger 2007; Kwiatkowska et al 1999).

Inflammatory Cell-derived Free Radicals

Macrophages and neutrophils are potent generators of ROS/RNI at sites of inflammation. The rapid release of ROS/RNI from these cells in response to phagocytic stimuli or proinflammatory cytokines is referred to as the respiratory or oxidative burst (Raha & Robinson 2000). During the respiratory burst, the enzyme complex, NADPH oxidase, assembles in the cell membrane, where it catalyzes the release of superoxide free radical and hydrogen peroxide. Superoxide can be produced outside the cell or within phagosomes where it serves as a critical effector arm for the bactericidal function of the cell. Superoxide is a particularly important ROS, as it can catalyze the generation of hydrogen peroxide as well as other tissue damaging ROS (Xia et al 2006).

RNI, specifically nitric oxide produced by IFN γ - or TNF α -activated macrophages is considered to be one of the major antibacterial mechanisms (Flynn & Chan 2001a). Studies indicate that inducible nitric oxide synthase (iNOS) generated free radicals in macrophages play a role in control of *M. tuberculosis* in mice model (Zahrt & Deretic 2002). The iNOS deficient mouse strain, demonstrated higher risk of dissemination and mortality compared with wild type mice when infected with *M. tuberculosis* (MacMicking et al 1997). However a similar study in this laboratory has shown that expression of iNOS is not essential for the early control of *M. tuberculosis* infection in deficient mice (Cooper et al 2000a). The role of nitric oxide (NO) in killing or limiting the growth of *M. tuberculosis* in humans is also similarly controversial. A recent report indicated that a polymorphism in the human NOS2A gene influenced the susceptibility to tuberculosis (Gomez et al 2007).

The role of phagocyte NADPH oxidase (phox) generated ROS in killing of *M. tuberculosis* has not been demonstrated convincingly. In *phox* deficient mice, *M. tuberculosis* infection only results in a transient loss of protection until the emergence of adaptive immunity (Cooper et al 2000b). Moreover, *M. tuberculosis* grew to similar levels in an ROS-deficient murine macrophage cell line compared to ROS-sufficient J774 macrophages (Chan et al 1992). *M. tuberculosis* seems to have evolved effective strategies to survive this hostile macrophage killing mechanism to establish long term infections inside these cells (Gupta & Chatterji 2005).

The role of neutrophils in the pathogenesis and resistance to *M. tuberculosis* infection remains controversial. Neutrophils have been shown to be one of the first cells to encounter *M. tuberculosis* early in infection and may be responsible for early extra-

pulmonary dissemination to draining lymph nodes (Abadie et al 2005; Canetti 1955). While neutrophils are not thought to be critical in the resistance to *M. tuberculosis* infection, neutrophil depletion or loss of function was associated with increased susceptibility (Cooper et al 2000b; Denis 1991; Kusuhara et al 2008; Lee et al 2008). While neutrophils have important bactericidal functions in other infections, it has been suggested that the antimicrobial activity of neutrophils for *M. tuberculosis* is through a non-phagocytic process (Pedrosa et al 2000). More importantly, neutrophils may contribute to disease pathogenesis through the generation of pro-inflammatory cytokines and tissue damaging ROS (Lyons et al 2004). By flow cytometry, this lab has shown that the major cell types in the lungs of *M. tuberculosis*-infected guinea pigs are macrophages and heterophils (Basaraba et al 2007; Ordway et al 2008; Ordway et al 2007). In these studies, the development of lesion necrosis correlated with the progressive influx and co-localization of heterophils and the release of their cytoplasmic contents including iron (Basaraba et al 2007). This process was blocked by BCG vaccination prior to aerosol challenge. Circulating neutrophils from human patients with active tuberculosis are in a state of heightened activation and a variety of *M. tuberculosis* products have been shown to activate neutrophils *in vitro* (Aleman et al 2001; Aleman et al 2002; Zhang et al 1991). Live bacilli as well as purified phenolic glycolipids, sulfolipids, and a secreted 19kDa lipoprotein have been shown to induce an oxidative response in neutrophils *in vitro* (May & Spagnuolo 1987; Neufert et al 2001; Zhang et al 1991). Neutrophils appear to have a role in innate resistance to *M. tuberculosis* infection, but there is also sufficient *in vivo* and *in vitro* evidence to suggest that activated neutrophils contribute to the adverse inflammatory response during *M. tuberculosis* infection.

Oxidative Stress in Human Tuberculosis

Tuberculosis is one of the chronic inflammatory diseases characterized by systemic oxidative stress. Markers of free radical damage are elevated in the peripheral circulation of patients with active tuberculosis (Kwiatkowska et al 1999; Lamsal et al 2007). Moreover, inflammation-related oxidative stress has been implicated in the pathogenesis of lung fibrosis and dysfunction in chronic pulmonary tuberculosis even following antimicrobial therapy (Jack et al 1994; Kwiatkowska et al 1999). Besides increased free radical generation, several studies have shown the depletion of critical antioxidants, including ascorbic acid and glutathione, in tuberculosis patients (Bakaev 1986; Madebo et al 2003; Vijayamalini & Manoharan 2004). Antioxidant depletion is thought to result from increased ROS generation in inflamed tissues and inadequate dietary replenishment of ascorbic acid (vitamin C) (Schorah 1992; Vijayamalini & Manoharan 2004).

Historically, the antioxidant, ascorbic acid, was of particular interest in clinical tuberculosis research because *M. tuberculosis* infected patients had approximately 50% less serum ascorbic acid compared to healthy individuals (Awotedu et al 1984). Moreover, because of its known role in the biosynthesis of collagen, it was predicted that ascorbic acid supplementation would promote the formation of fibrous connective tissue, which is essential in the healing of the necrotic or cavitory lesions. This approach was supported by studies that showed that daily subcutaneous injections of ascorbic acid improved appetite and overall clinical condition of tuberculosis patients (Albrecht 1938).

Ascorbic acid therapy in human tuberculosis patients decreased pulmonary hemorrhage and resulted in resumed bleeding when discontinued (Borsalino 1937).

Another factor contributing to the importance of oxidative stress during tuberculosis is that lung tissue is exceedingly sensitive to oxidative damage. This is because the lung parenchyma represents a large surface area with an extensive blood supply, which is continuously exposed to high levels of atmospheric oxygen. Other contributing factors include breathing ambient air, which exposes the lung to environmental oxidants that have been shown to be risk factors for tuberculosis (Gajalakshmi et al 2003; Lienhardt et al 2003). Because of continued oxidant exposure, the lungs are well equipped with potent antioxidant defense mechanisms. However, if inflammation-mediated oxidant generation exceeds the antioxidant capacity of the lung parenchyma, oxidative stress results in irreversible tissue damage even after completion of tuberculosis drug therapy (Guo & Ward 2007; Jack et al 1994).

Oxidative Stress in Pathogenesis of Lesion Necrosis

Few studies have implicated oxidative stress in the pathogenesis of lesion necrosis in either human or experimental *M. tuberculosis* infections in animals. In the 1930s, Birkhang showed that increasing supplemental ascorbic acid in guinea pigs infected with *M. tuberculosis* resulted in increased body weight and reduction of primary pulmonary lesions (Birkhang 1939). By microscopy, he demonstrated that lesions had less necrosis and more fibrous healing compared to non-supplemented controls. These data are among the few that have implicated ROS in tuberculosis lesion pathogenesis based on *in vivo* observations in human or animal tissues.

M. tuberculosis is a successful pathogen in part because it has the ability to resist host bactericidal mechanisms including the damaging effects of ROS (Braunstein et al 2003; Chan et al 1992; Gupta & Chatterji 2005; Timmins et al 2004). Most critical to the survival of *M. tuberculosis* during the host inflammatory response is its ability to resist intracellular ROS during the early stages of macrophage infection. *M. tuberculosis* possesses virulence genes that encode antioxidant proteins that are essential to resisting oxidative stress. They include but are not limited to superoxide dismutase (SOD) and catalase-peroxidase (CPx-encoded by *katG* gene) (Braunstein et al 2003; Garbe et al 1996; Timmins et al 2004). In aerobic organisms, iron levels and oxidative stress are directly related. IdeR is a transcriptional regulator of iron metabolism of *M. tuberculosis*. IdeR deficient strains of *M. tuberculosis* and *M. smegmatis* are more sensitive to hydrogen peroxide and superoxide, suggesting that IdeR protects against ROS (Rodriguez et al 2002).

Host Antioxidant Defenses

The antioxidant capacity of the host is maintained by numerous proteins and small molecules that prevent oxidative damage to lipids, proteins, and DNA. Antioxidants can be non-protein or protein (enzymatic or non-enzymatic) and are classified as direct or indirect. Direct antioxidants scavenge the pro-oxidant ROS and prevent ROS-initiated reactions. Examples of direct enzymatic protein antioxidants include superoxide dismutases (SODs), catalase, glutathione peroxidase (GPx) and peroxiredoxin (Prx). The antioxidant functional groups for GPx and Prx are small thiol-compounds that use glutathione (GSH) and thioredoxin (Trx) as substrates in the redox cycle. Some direct,

non-enzymatic antioxidants include vitamin C (ascorbate), vitamin E (α -tocopheral), beta-carotene, and lipoic acid (Cho & Kleeberger 2007; Hanta et al 2006).

Indirect antioxidants function by facilitating the excretion of oxidized, reactive, secondary metabolites such as aldehydes, quinines, and peroxides. They may also participate in the biosynthesis and recycling of thiol molecules that serve as substrates for direct antioxidant enzymes. Examples include glutathione-S-transferase (GST), NADPH:quinone oxidoreductase (NQO1), glutamate cysteine ligase (GCL), glutathione synthetase (GS), γ -glutamyl transpeptidase (GGT), UDP-glucuronyl transferase (UGT), Trx reductase, and heme oxygenase. Collectively, these protein antioxidants, that detoxify ROS through conjugation, are referred to as phase II detoxification enzymes (Mithen et al 2003).

Nrf2-mediated Antioxidant Defenses

The induction of phase II enzymes requires binding of specific inducers to the antioxidant response element (ARE) in the promoter regions of phase II genes. Nuclear erythroid 2 p45-related factor 2 (Nrf2) is a redox-sensitive transcription factor with a basic leucine zipper (bZIP) structure found in essentially all cells throughout the body. Nrf2 is directly involved in the transcriptional activation of ARE-driven phase II antioxidant enzymes. Under normal physiologic conditions, Nrf2 is sequestered in the cytoplasm by the binding protein, Kelch-like ECH-associated protein (Keap1). Under oxidative stress conditions, Nrf2 dissociates from Keap1 allowing it to translocate to the nucleus where it heterodimerizes with small Maf-family proteins. Binding of the Nrf2/Maf complex to ARE sequences activates the transcription of the various phase II

antioxidant enzymes (Juge et al 2007). Retention of Nrf2 in the cytoplasm by Keap1 is dependent upon binding to actin (Zhang & Hannink 2003). Under oxidative stress conditions the structure and function of proteins is altered by post-translational addition of carbonyl groups (Berlett & Stadtman 1997). The protein-protein interactions between Nrf2, Keap1 and actin may be altered either directly or indirectly. Oxidation of actin results in disruption of the actin cytoskeleton and thereby impairs the binding of Keap1 (Dalle-Donne et al 2001; O'Reilly et al 2003). Direct oxidation of Nrf2 or Keap1 also results in defective translocation of Nrf2 into the nucleus thus impairing antioxidant capacity of phase II detoxification enzymes (Kode et al 2008).

Glutathione and Nrf2 Regulation

Glutathione is a non-protein thiol tripeptide molecule that is present in the cytoplasm of all mammalian cells. It is essential for the detoxification of xenobiotics and reactive carbonyls that are formed during lipid peroxidation and oxidation of glycation end products. Glutathione is important in maintaining; 1) the redox status of the cells by directly scavenging free radicals, 2) the thiol status of proteins and 3) the levels of the critical but unstable amino acid cysteine. Glutathione deficiency has been implicated as a major pathogenic mechanism in several chronic diseases of humans and animals including diabetes mellitus, cholestasis, alcohol-induced liver disease and Alzheimer's (Lu 2009). Glutathione is essentially a tripeptide i.e. γ -glutamyl-cysteinyl-glycine. It is synthesized within the cytoplasm of cells in a two step process in an energy dependent manner. The first step, catalyzed by glutamate-cysteine-ligase (GCL) (also known as γ -

glutamylcysteine synthetase (GCS)), is the rate-limiting step and the second step is mediated by glutathione synthetase (GS).

GCL is a heterodimeric protein that comprises of a heavy catalytic unit (GCL-C) and a light modifier unit (GCL-M). Experimental evidence with Nrf2 deficient mice and *in-vitro* studies suggest that both GCL-C and GCL-M gene expressions are regulated by Nrf2 transcriptionally (Chan et al 2001; Wild et al 1999). Studies that characterized rat and human promoter elements of GS genes also demonstrate their transcriptional regulation by Nrf2 (Lee et al 2005; Yang et al 2002).

Nrf2 Regulation of Other Crucial Enzymes

NAD(P)H dehydrogenase, quinone 1 (NQO1) catalyzes the two-electron reduction of quinones and thereby prevents the one-electron reduction of quinones that result in free radical formation. As mentioned earlier glutathione conjugation is a major detoxification mechanism for xenobiotics and reactive carbonyls and this reaction is catalyzed by the enzyme glutathione-S-transferase (GST). The free radical hydrogen peroxide is converted to water molecule by glutathione-peroxidase (GPx) in the presence of selenium. These three well-studied enzymes are transcriptionally regulated by Nrf2 (McWalter et al 2004; Zhu et al 2008).

Nrf2 regulation of Host Immunity

While much is known about Nrf2 molecular mechanisms, there is relatively little known about its function *in vivo*, particularly in chronic inflammatory diseases like tuberculosis. Nrf2 may play an important role in the regulation of both innate and

adaptive immune responses. Nrf2 has been shown to be critical in the regulation of innate immunity in the mouse model of septic shock. In response to LPS challenge, Nrf2-deficient mice showed intense inflammation along with exaggerated activation of the pro-inflammatory transcription factor, NF- κ B which is also regulated by the oxidant-antioxidant system (Thimmulappa et al 2006a; Thimmulappa et al 2006b). Disruption of the Nrf2 gene in mice leads to severe, allergen-driven airway inflammation resulting in airway hyper-responsiveness and increased expression of the T helper type 2 cytokines, IL-4 and IL-13, in bronchoalveolar lavage fluid and splenocytes (Rangasamy et al 2004; Rangasamy et al 2005).

Therapeutic Nrf2 Induction

The Nrf2-Keap1 pathway has been targeted therapeutically to control inflammatory disease conditions related to oxidative stress, but it has not been investigated in the context of tuberculosis (Chen et al 2004; Li & Nel 2006). Several naturally occurring compounds such as isothiocyanates and thiols have been shown to induce the Nrf2-Keap1 pathway and increase anti-oxidant and anti-inflammatory responses. Sulforaphane is an isothiocyanate compound found in broccoli and other cruciferous vegetables. It increases Nrf2 translocation and ARE binding resulting in the induction of several phase II detoxification and antioxidant genes (Jeong et al 2005; Zhang & Hannink 2003). Synthetic triterpenoids are potent inducers of antioxidant and cytoprotective enzymes and inhibitors of inflammation. An imidazolide triterpenoid derivative, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), is a potent inducer of Nrf2 and antioxidant expression when administered orally to mice

(Yates et al 2007). CDDO-Im-mediated induction of Nrf2-dependent antioxidant response also confers protection against LPS-induced inflammatory response and survival in mice (Thimmulappa et al 2006a).

N-acetyl cysteine (NAC) is one of the most commonly used ROS scavenger/anti-oxidant drugs currently used clinically and in preclinical trials (Ritter et al 2004). NAC is an FDA approved drug that is used in aerosol form (Mucomyst®) as a mucolytic agent in patients with cystic fibrosis or chronic airway disease. NAC is also prescribed as an injectable formulation to counteract the oxidative liver damage associated with acetaminophen (Tylenol®) toxicity. The synthesis of glutathione is dependent on the availability of cysteine and the activity of the rate-limiting enzyme GCL (Lu 2009). NAC provides the cysteine as well as increases the GCL activity through the induction of Nrf2. As it pertains to human tuberculosis treatment, NAC is currently being tested in a human clinical trial to counteract oxidant-mediated liver toxicity associated with tuberculosis drug therapy (NCT00564642, ClinicalTrials.gov). NAC has also been shown to have anti-inflammatory and immune-stimulatory activity. NAC reduces *in vivo* lung inflammation and improves lung function by modulating pro-inflammatory cytokines and inhibiting neutrophilic inflammation thereby diminishing oxidant-mediated tissue damage. *In vitro*, NAC induces the translocation of Nrf2 and activation of anti-oxidant gene transcription in treated HepG2 cells (Chen et al 2004).

Host Lipids and Tuberculosis

It has been known nearly for a century that tuberculosis lesions are rich in a lipid and host lipids likely play a crucial role in tuberculosis pathogenesis (Virchow 1989).

Tuberculosis has even been called lipid pneumonia (Hunter et al 2007). Fatty acids induce respiration of mycobacteria isolated from infected mouse lung suggesting there possible role as nutrient source to the bacilli (Bloch & Segal 1956). The presence of foamy macrophages laden with lipid has been reported in numerous pathological conditions including atherosclerosis, various infectious disease conditions such as tuberculosis, chlamydiosis and toxoplasmosis (Galkina & Ley 2009; Kalayoglu & Byrne 1998; Portugal et al 2008). The accumulation of lipid vacuoles within macrophages during infection depends likely on TLR activation by pro-inflammatory $TNF\alpha$ and MCP1 (D'Avila et al 2008). The pathogenesis of formation of foam cells has been described in detail in the atherosclerosis literature. Altered equilibrium between influx and efflux of low-density lipoprotein (LDL) has been attributed to the conversion of macrophages into the foam cells. LDL is taken up by macrophages in a receptor-mediated process involving LDL receptors or in an oxidized form known as oxidized LDL (OxLDL) involving scavenger receptors such as SR-A, SR-B1, CD36 and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1).

CD36 deficient mice that had been backcrossed to apolipoprotein E deficient mice have increased circulatory cholesterol levels and are prone to atherosclerosis. These mice demonstrated nearly 77% decrease in aortic lesion area and 60% less copper-oxidized LDL intake compared to the apolipoprotein deficient mice (Febbraio et al 2000). LOX-1 deficient mice that were backcrossed to LDLR deficient mice also demonstrated significant reduction in atherosclerosis and foam cell formation (Mehta et al 2007). The individual lipid components of LDL include cholesterol, triacylglycerides and phospholipids. Cholesterol is either retained as an esterified form within in the

endoplasmic reticulum of the macrophages or released out of the cell by transporters ABC-A1 and G1. Genetic deficiency of these transporters can also lead to enhanced foam cell formation (Baldan et al 2008).

Isocitrate lyase 1 (Icl1) is a mycobacterial enzyme required for their growth in fatty acids as a main carbon source. The Icl1-deficient *M. tuberculosis* was impaired in its ability to maintain a chronic infection (McKinney et al 2000). *M. tuberculosis* can metabolize host cholesterol during bacterial persistence (Pandey & Sasseti 2008). Mce4, a cholesterol transporter and HsaC, an enzyme involved in cholesterol catabolism do not grow under cholesterol-as-carbon-source media suggesting that mycobacteria have well developed machinery in place to utilize host lipids (Pandey & Sasseti 2008).

Literatures discussed herein have led me to my hypothesis and design experiments to answer hypothesis-driven questions. The individual studies that address my hypothesis are discussed in detail in the following chapters.

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Chapter Two

Disseminated disease severity as a measure of virulence of

Mycobacterium tuberculosis in the guinea pig model

Abstract

Virulence is the measure of pathogenicity of a microorganism as determined by its ability to invade host tissues and to produce severe disease. In the low-dose aerosol guinea pig model, the virulence of multiple strains of *Mycobacterium tuberculosis* was determined by measuring time of survival, bacterial loads in target organs, and the severity of pulmonary and extra-pulmonary lesions. Erdman K01, CSU93/CDC1551 and HN878 had shorter survival times compared to the common laboratory strain H37Rv. After thirty days of the infection, bacilli had disseminated from the lungs resulting in microscopically visible lesions in peribronchial lymph nodes, peripancreatic lymph nodes, spleen, liver, pancreas, adrenal and heart. The extent of lesion necrosis paralleled virulence when survival times were used as a measure as Erdman K01 and the two clinical isolates caused more necrosis and resulted in sooner death in infected animals than H37Rv. The extent of extra-pulmonary lesion necrosis was a better predictor of virulence than the number of viable bacilli in the tissue. Overall, this study emphasizes the point that extra-pulmonary disease is a prominent feature of the guinea pig model and dissemination to organs not normally assayed such as the heart and adrenal glands should be taken into account in the assessment of the disease process.

Introduction

Tuberculosis is one of a small group of diseases that together cause 90% of infectious disease related deaths worldwide. *M. tuberculosis*, the causative agent of tuberculosis, infects approximately 8 million new individuals per year and the disease results in a death every ten seconds (WHO 2006). Despite the WHO declaring tuberculosis as a global health emergency nearly fifteen years ago, no significant progress in eradication of the disease has been made. Multiple factors such as poor socioeconomic conditions, limited access to diagnosis and treatment, lack of treatment compliance and the development of multidrug-resistant *M. tuberculosis* strains have contributed to this lack of progress. In addition, different clinical isolates of *M. tuberculosis* may undergo genetic changes and result in differences in pathogenicity and variations in the ability to cause disease in humans (Manca et al 2004).

Virulence is defined as the severity of disease symptoms resulting in death of the infected host. Virulence of *M. tuberculosis* strains is generally determined by the differences in survival in experimentally infected mice or guinea pigs (North et al 1999a). Since survival studies are lengthy and expensive, the number of cultivatable bacilli in lungs is alternatively used in short term experiments as a correlate of virulence (Orme 2005; Wiegeshaus et al 1970). Weight loss and clinical signs are considered the most common correlates of disease progression (Baldwin et al 1998). Antigen-specific delayed-type hypersensitivity (DTH) (Smith 1985) and antigen-induced production of IFN- γ by T cells (Martin et al 2000) have also been used as the determinants of protective immunity with IFN- γ production generally being considered a better correlate of resistance in mice (McMurray 2001). As tuberculosis in guinea pigs, like other animal

models, is a systemic disease with morbidity and mortality associated with pulmonary and extra-pulmonary pathology, we compared the virulence of clinical and laboratory isolates of *M. tuberculosis* based on lesion burden of the different body organs, the survival times and the number of culturable bacilli.

M. tuberculosis CSU93/CDC1551 is a clinical isolate that exhibited high levels of infectivity and virulence during an outbreak of tuberculosis from 1994 to 1996 in rural Tennessee and Kentucky (Valway et al 1998). It was characterized by an unusually high rate of tuberculin skin test conversions as well as cases of active disease. Many of these patients developed active disease following very brief exposures to the index case (Valway et al 1998). CSU93/ CDC1551 was originally shown to grow to very high numbers in mice lungs ($>10^7$ per lung) but later observations indicated that properly passaged laboratory strains can reach a similar level of growth (Manca et al 1999; Orme 1999). Survival studies carried out in different laboratories using mice did not show any increase in virulence of CSU93/ CDC1551 compared to H37Rv (Kelley & Collins 1999; Manca et al 1999; North et al 1999b).

M. tuberculosis HN878, a member of the W/Beijing family, caused 60 cases of TB during three outbreaks in Texas between 1995 and 1998 (Sreevatsan et al 1997). HN878 was demonstrated to be hyper-virulent in mice based on comparative survival studies (Manca et al 2001). The hyper-virulence of HN878 was suggested to be due to the failure of this strain to stimulate CD4 T cell mediated Th1 type immunity associated with increased induction of Type-I interferon (Manca et al 1999; Manca et al 2001). The same researchers later showed that a cell-wall phenolic glycolipid antigen expressed by HN878 was capable of inducing Th2 type immunity and its absence resulted in abrogation of the

strain's hyper-virulence (Manca et al 2004; Reed et al 2004). A recent study from our laboratory has confirmed the hyper-virulence of HN878, but instead showed that this organism induced a very substantial Th1 response and this response then declined in concert with the emergence of a CD4⁺ CD25⁺ FoxP3⁺ CD223⁺ IL10⁺ regulatory T cell population (Ordway et al 2007).

In the present study, we compared the above strains in the guinea pig model, taking into account the extensive extra-pulmonary dissemination that may cause premature death. Our data shows that the rate of dissemination and the extent of extra-pulmonary pathology explain, in part, the differences in the virulence of *M. tuberculosis* isolates in this important animal model.

Materials and Methods

Experimental infections

Female out-bred Hartley guinea pigs (approximately 500g in weight) were purchased from the Charles River Laboratories (North Wilmington, MA, USA) and held under barrier conditions in a Bio-safety Level III animal laboratory. All experimental protocols were approved by the Animal Care and Use Committee of Colorado State University and comply with NIH guidelines.

M. tuberculosis H37Rv was originally obtained from the Trudeau Institute collection, Saranac Lake NY; HN878 was kindly provided by Dr. Barry Kreiswirth; CDC1551/CSU93 was provided by Dr. T. Shinnick, CDC, Atlanta; and Erdman-K01 was obtained from Mycos Research, Loveland CO with permission from the Aeras Global Tuberculosis Vaccine Foundation. Cultures were aliquoted into 1 ml tubes and frozen at

-70 °C until used. Thawed aliquots were diluted in milli-Q sterile water to the desired inoculum concentrations. A Madison chamber aerosol generation device was used to expose the animals to *M. tuberculosis*. This device was calibrated to deliver approximately 20 bacilli into the lungs.

Bacterial counts in the lungs, spleen and peribronchial lymphnodes (n=5) on day 30 were determined by plating serial dilutions of organ homogenates on nutrient 7H11 agar and counting colony-forming units after 3 weeks incubation at 37°C. In survival studies animals showing substantial weight loss with no evidence of weight rebound were euthanized. The results shown in the survival studies are based upon 6 guinea pigs per group.

Tissue fixation and processing

At the time of euthanasia either on day 30 post-infection or at the end of survival study a necropsy was performed. Organs which show extra pulmonary tuberculosis infection in human were collected from these animals and fixed in 10% neutral buffered formalin (NBF) for 3 days. The tissues were then trimmed and processed for paraffin embedding and 4µ thick sections were made, stained with hematoxylin and eosin and assessed histopathologically. The photomicrographs presented here are representatives of their respective groups based on mean lesion scores and are taken using an Olympus BX41 microscope with Olympus DP70 camera and software.

Lesion analysis

To evaluate the concurrent progression of tuberculosis lesions in lungs, lymph nodes, spleen, liver, pancreas, adrenal and heart, different histological grading systems were developed. The method was a modification of previously described methods for grading granulomatous lesions based on inflammatory cell numbers and their infiltrative distribution pattern (Basaraba et al 2006). The guinea pig lungs were scored based on the following six criteria. (i) percent of lung affected were ranked at low magnification by an estimation of the percent of the lung affected as follows: 0-no lesions in lung, 1-up to 25% of lung involved, 2-up to 50 % of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. (ii) primary lesions: 0-no primary lesions present, 1-a single primary lesion, 2-two or more primary lesions, multi-focal, 3-two or more primary lesions, multifocal to coalescing, 4-multiple primary lesions, coalescing and extensive. (iii) secondary lesions: 0-no secondary lesions present, 1-up to 25% of lung involved, 2-up to 50% of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. Necrosis (iv), mineralization (v) and fibrosis (vi) were scored based on severity as follows: 0-none, 1-minimal, 2-mild, 3-moderate, 4-marked and 5-severe. All individual scores were added for the final total score for each organ. Maximum possible total score for lungs is 27.

The peribronchial and peripancreatic lymph nodes, liver and spleen were scored based on four categories; (i) percentage involvement (same scale as lungs), (ii) degree of necrosis, (iii) fibrosis and (iv) mineralization (0–5 scale). The maximum total score for each of these organs is 19. The lesions in other organs such as heart, kidney and adrenal were scored based on severity only with the 0–5 degree scale as shown above.

Statistical analysis

Data is presented as mean values (n=6). The parametric log rank test was used to assess statistical significance between survival curves. Statistical analysis to evaluate whether the total lesion scores and necrosis scores showed statistically significant difference was done using oneway ANOVA (GraphPad Prism software).

Results

Evaluation of virulence of *Mycobacterium tuberculosis* strains

Based on survival, as shown in Figure 2.1 A and 2.1 B, among the four strains tested, Erdman K01 was the most virulent strain in guinea pigs followed by CSU93/CDC1551. The survival rate of both Erdman K01 and CSU93/CDC1551 demonstrated statistically significant differences from that of the H37Rv infected animals ($p < 0.05$). The other clinical isolate, HN878, was the third most virulent strain behind Erdman K01 and CSU93/CDC1551.

The number of viable bacilli in lungs, spleen and peribronchial lymph nodes on day 30 of the infection is shown in Figures 2.1C, D and Figure E. In both the lungs and peribronchial lymph nodes, HN878 showed marginally higher number of bacilli compared to the other three strains. In terms of bacterial dissemination and growth in the spleen, the Erdman K01 strain grew slightly higher than the other strains that grew to similar levels.

Lung histopathology

The differences in the relative size and lesion progression on day 30 in the lungs of guinea pigs infected with H37Rv (Figure 2.2A), Erdman K01 (Figure 2.2B), CSU93 (Figure 2.2C) and HN878 (Figure 2.2D) on day 30 are shown. In addition, the mean total lesion scores for each group were calculated as described in the materials and methods section (Table 2.1).

On day 30 of the infection with all four bacterial strains, the lung lesions (Figure 2.2 A–D) consisted of well delineated foci of granulomatous inflammation characterized by sheets of epithelioid macrophages and occasional multinucleated giant cells mixed with fewer numbers of lymphocytes, plasma cells and occasional neutrophils and eosinophils with a moderate amount of necrosis. As the infection progressed and animals started to die, lesions contained equal numbers of macrophages and lymphocytes and extensive necrosis, fibrosis and mineralization of central necrotic cores. Erdman K01, CSU93 and HN878 caused more lymphatic-associated lesions around the airway and more necrosis (Figure 2.2 A–D; arrows) on day 30 resulting in higher lesion scores compared to H37Rv. At death, necrosis was comparatively more extensive in lungs of guinea pigs infected with Erdman K01, CSU93 and HN878 compared to H37Rv and the mineralization of the necrotic core was most predominant in the Erdman K01 and HN878 infected animals compared to the other two strains (Table 2).

Peribronchial lymph nodes histopathology

The peribronchial lymph nodes of the infected guinea pigs showed remarkable lesion development and gross enlargement regardless of which *M. tuberculosis* strain was used. A mixed inflammatory response involving lymphocytes, macrophages and

granulocytes (heterophils and eosinophils) was present in all four groups resulting in granulomatous lymphadenitis (Figure 2.2 E–H). On day 30, Erdman K01, CSU93 and HN878 caused moderately more necrosis in the peribronchial lymph nodes than H37Rv (Table 2.2). At the time of death, H37Rv infected lymph nodes showed less necrosis but extensive fibrosis compared to Erdman K01, CSU93 and HN878. In the peribronchial lymph nodes on day 30 of the infection, the necrosis scores (Table 2.2) paralleled the survival times (Figure 2.1). At the time of death, the peribronchial lymph nodes in the HN878 infected animals had the most severe necrosis followed by Erdman K01 and CSU93 (Table 2.2).

Peripancreatic lymph nodes histopathology

The peripancreatic lymph nodes (Figure 2.3 A-D) from all four groups showed lesion progression similar to that of the peribronchial lymph nodes and the degree of granulomatous and necrotizing lymphadenitis was comparable. On day 30, the animals infected with Erdman K01, CSU93 and HN878 showed moderately more necrosis in these lymph nodes than in H37Rv infected animals. At the time of death, H37Rv infected peripancreatic lymph nodes had less necrosis but extensive fibrosis compared to those infected with Erdman K01, CSU93 and HN878 similar to peribronchial lymph nodes. In contrast to the peribronchial lymph nodes, the peripancreatic lymph nodes from guinea pigs infected with Erdman K01, CSU93 and HN878 however showed marked mineralization (Table 2.2). Both at day 30 and at the time of death, the necrosis scores of the peripancreatic lymph nodes did not parallel the survival times. However, the necrosis scores of the peripancreatic lymph nodes from the groups infected with Erdman K01,

CSU93 and HN878 were still consistently higher compared to those from the group infected with H37Rv (Table 2.2).

Spleen histopathology

Figure 2.3 (E–H) shows the extent of spleen lesions in all four groups on day 30. The lesion scores in Table 2.1 reflected more spleen lesion development in Erdman K01 and HN878 infected guinea pigs on day 30 compared to H37Rv and CSU93 infected guinea pigs. At the time of death, the spleens from the three groups other than H37Rv had slightly more mineralization. The degree of necrosis was almost equal at death in all four groups (Table 2.2). The necrosis scores of the spleens, however, paralleled the survival times on day 30 of the infection, as was the case for the peribronchial lymph nodes.

Liver histopathology

On day 30, the livers of all four groups showed moderate involvement with few foci of granulomatous inflammation scattered randomly throughout the parenchyma. At this time, there were no differences in liver pathology among the four groups. But at the time of death, livers from the guinea pigs infected with Erdman K01, CSU93 and HN878 showed more extensive lesion development and necrosis compared to H37Rv (Figure 2.4 A–D and Table 2.2). In the liver, the necrosis scores were similar among the different strains at day 30. However, the scores seen in the H37Rv infected animals were moderately lower.

Differences in other organs and notable lesions

At the time of death, granulomatous pancreatitis was severe in CSU93 infected guinea pigs, moderate in both Erdman K01 and HN878 infected guinea pigs and mild in H37Rv infected guinea pigs (Figure 2.4 E–H). Heart (Figure 2.5 A–D) and adrenal glands (Figure 2.5 E–H) also had mixed inflammation at the time of death. Granulomatous myocarditis was noticeable even on day 30 with Erdman K01 infected group showing marked inflammation (Figure 2.5 B and Table 2.1). CSU93 and HN878 caused moderate and H37Rv caused very minimal inflammation (Figure 2.5 A–D and Table 2.1). Granulomatous adrenalitis was moderate both in Erdman K01 and HN878 infected groups; mild in the H37Rv infected group and very minimal in the CSU93 infected group (Figure 2.5 E–H and Table 2.1).

Focal granulomatous lesions in the colon and the uterus were seen occasionally. These were isolated findings. Intestinal inflammation was primarily localized to the gut-associated lymphoid tissue and was composed of predominantly macrophages and lymphocytes with occasional granulocytes in granulomatous lesions.

Statistical analysis to evaluate whether the total lesion scores and necrosis scores showed statistically significant difference revealed that among mean total lesion scores, PBLN scores on day 30, liver scores on day 30 and the pancreas scores at the time of death were the only ones that did not show statistically significant difference. Among necrosis scores, spleen scores at the time of death, liver scores on day 30 and at the time of death were the only ones that did not show statistically significant difference. All other scores showed statistically significant difference among different groups ($p < 0.05$). Acid-fast staining revealed the tuberculous bacilli to be present in highest concentration in the

necrotic core of the lesions followed by the macrophages immediately surrounding the necrotic core.

Discussion

The results of the survival study indicated that the Erdman K01 strain was the most virulent strain in guinea pigs and H37Rv was the least virulent strain of the four tested. The order of virulence was Erdman K01, CSU93/CDC1551, HN878 and finally H37Rv. Despite the slightly higher growth of bacilli in the lungs and the peribronchial lymph nodes in HN878 infected animals compared to the other strains, the HN878 strain was in fact less virulent than Erdman K01 and CSU93 in the survival studies. Interestingly, the number of culturable bacilli (CFU) in the lungs, spleen and peribronchial lymph nodes did not correlate with the virulence of the strains as determined by survival. This is an important observation since comparison of CFUs in the lungs is often used as a major readout in challenge experiments and as a measure of virulence and vaccine efficacy (McMurray 2001). Our data supports an earlier study in mice that showed that growth rate by itself was an unreliable indicator of mycobacterial virulence (North et al 1999b).

Our results differ from studies in mice in which HN878 was demonstrated to be more virulent than CSU93 or H37Rv (Manca et al 2004; Manca et al 2001). These previous studies demonstrated that the CSU93/CDC1551 infected mice survived significantly longer than HN878 and even H37Rv. Our earlier study in mice also confirmed the increased virulence of HN878 (Ordway et al 2007). In that study, even though HN878 grew faster reaching a high bacterial load of 10^7 as early as day 15 of the

infection, yet death was delayed. In that study HN878 induced a population of CD4+ CD25+ regulatory T cells that were shown to suppress the protective effects of Th1 dominant immunity. These findings demonstrate that the virulence as reflected by survival is not predictable by tissue bacterial load alone, which was supported in this study. The guinea pigs showed both HN878 and CSU93/CDC1551 to be more virulent than the laboratory strains and CSU93/CDC1551 to be more virulent in this animal than HN878. Perhaps one reason for the differences seen between mice and guinea pigs could be the development of severe necrosis in multiple organs and the extensive extra-pulmonary dissemination observed in guinea pigs.

Morbidity and mortality in animal models of tuberculosis is due to the combined effect of pulmonary and extra-pulmonary pathology. While the lung is the first organ affected by *M. tuberculosis*, the infection spreads rapidly to extra-pulmonary sites by lymphatic and hematogenous dissemination. In most studies to date in which the lung pathology is considered, other organs are not often evaluated. In models relating survival to virulence, death of the animal can be due to multi-organ failure, not merely pulmonary disease. We show here that tuberculosis is a true multi-organ disease that spread to intestine, heart, adrenal glands among other organs. We also evaluated the lesion necrosis as a possible indicator of host susceptibility or the challenge-strain virulence. Necrosis plays a role in aerosol transmission of infectious bacilli (Dannenberg 1982; Russell 2007) and resistance conferred in BCG-vaccination in guinea pigs is characterized by absence of necrosis in the primary granulomas of lungs and lymph nodes. The increased susceptibility of IFN- γ -deficient mice is also related to lesion necrosis (Junqueira-Kipnis

et al 2006). Acid-fast staining revealed the bacilli to be present in the highest concentration in the necrotic core.

Compared to H37Rv, the Erdman K01, CSU93 and HN878 infected animals consistently had more extensive necrosis in granulomas when the respective scores were compared in lungs, peribronchial lymph nodes, peripancreatic lymph nodes, spleen and liver (Table 1). Our results suggest that necrosis in the primary organs on day 30 of the infection, paralleled virulence of the different strains used in this study. Considering the importance of necrosis in tuberculosis pathogenesis, it is not surprising that degree of lesion necrosis paralleled strain virulence as determined by survival. H37Rv infected peribronchial lymph nodes showed less necrosis but extensive fibrosis compared to the other three strains indicating that while lymph node lesions in the H37Rv group began to somewhat resolve, the Erdman K01, CSU93 and HN878 groups had progressive necrosis with less evidence of lesion resolution.

Extra-pulmonary organ lesion scores of lymph nodes, spleen, liver, pancreas, heart, and to an extent the adrenal glands ranked consistently higher for the Erdman K01 and the two clinical strains compared to the laboratory strain H37Rv. Although the lesion scores did not accurately indicate the order of virulence, they were clearly higher for Erdman K01, CSU93 and HN878 groups compared to H37Rv group. The severity of disease in organs such as the pancreas, heart and to a lesser extent the adrenal glands indicated more extensive extra-pulmonary dissemination by Erdman K01, CSU93 and HN878 compared to H37Rv. The severity of lesions in the heart directly correlated with the *M. tuberculosis*-strain virulence. These sites, which are rarely, if ever, examined are of particular interest since extensive lesions in these organs can cause premature death

before significant lung lesions develop and could therefore represent an important measure of virulence.

Another interesting finding was the development of severe inflammation and necrosis by day 30 of the infection in abdominal peripancreatic lymph nodes similar to those that directly drain the lungs (peribronchial lymph nodes). This finding along with pancreatic involvement and the fact that peripancreatic lymph nodes drain the stomach and anterior small intestine suggest that the bacilli reach the intestines directly and thus induce inflammation. This also suggests that bacilli might also spread through the gastrointestinal route early in the course of the infection. This might occur either at the time of aerosol challenge or through swallowing the exudate cleared from the lung by mucocilliary action. It is generally thought that the lungs and their draining lymph nodes are the source of extra-pulmonary dissemination (Chackerian et al 2002). However, our findings suggest that abdominal lymph nodes might also play a role in dissemination of the bacilli. Tuberculosis of pancreas and peripancreatic lymph nodes in humans is a rare condition (Franco-Paredes et al 2002). Nevertheless, the frequency of reports of pancreatic tuberculosis has increased in recent years along with the increase in the incidence of tuberculosis worldwide (Woodfield et al 2004). As guinea pigs showed significant pathology in pancreas and peripancreatic lymph nodes, they may represent an appropriate animal model to study human pancreatic tuberculosis. The pancreatic involvement could be through retrograde spread of infection via pancreatic ducts or direct spread from peripancreatic lymph node due to their closer apposition.

We conclude that other than the survival studies, granuloma necrosis in pulmonary and extra-pulmonary sites paralleled the virulence of *M. tuberculosis* strains.

The incidence of heart lesions, which are indicative of more extensive extra-pulmonary dissemination, in fact paralleled well with virulence. In contrast, bacterial growth (CFUs) in the lungs, spleen or liver did not parallel with virulence as determined by survival suggesting that organ pathology is a better correlate of virulence than the bacterial growth. As pulmonary and extra-pulmonary organ pathology parallel well with virulence, the method described in detail in the “materials and methods” section can be used to evaluate the organ pathology of guinea pigs either on day 30 or day 60 of the infection in vaccine and drug evaluation studies as a measure of protection. Since Erdman K01 strain caused more severe disseminated disease and resulted in shorter survival times, it would be ideal to use this strain as a target strain rather than H37Rv for challenge in vaccine and drug evaluation studies in guinea pigs or at least as an alternative strain for these studies.

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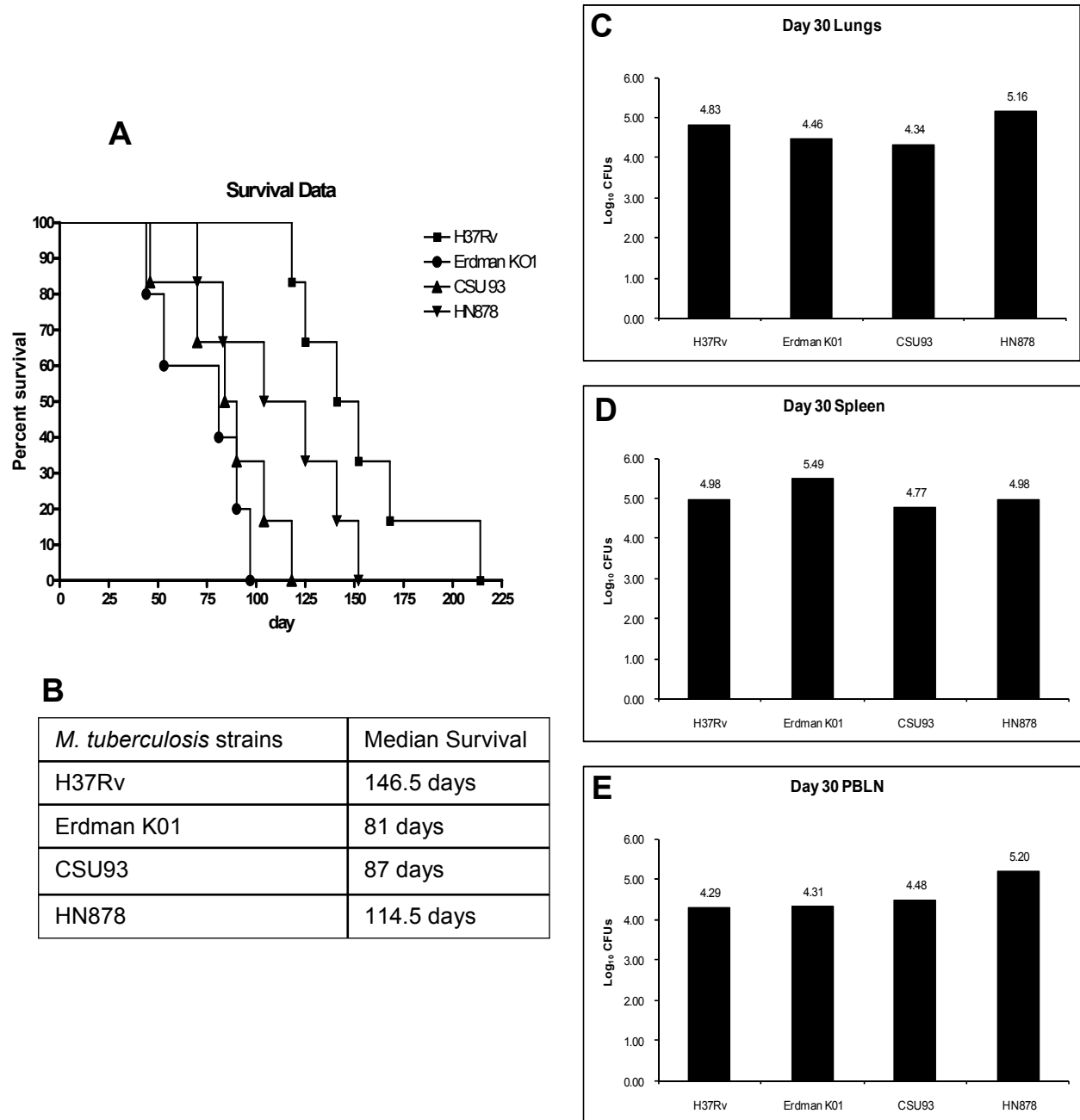


Figure 2.1 Survival of Hartley guinea pigs infected with *M. tuberculosis* H37Rv (solid squares), Erdman K01 (solid circle), CSU93 (solid triangle), and HN878 (solid inverted triangle) is compared. Median survival days are shown in Figure 2.1B. Survival results are from one experiment with 6 guinea pigs per group. Bacterial counts in the lungs (C), spleen (D) and liver (E) on day 30 from guinea pigs infected with a low dose of *M. tuberculosis* strain H37Rv, Erdman K01, CSU93 and HN878 were compared. Results are expressed as the average (n=5) of the bacterial load in each group expressed as Log₁₀CFU (\pm SE).

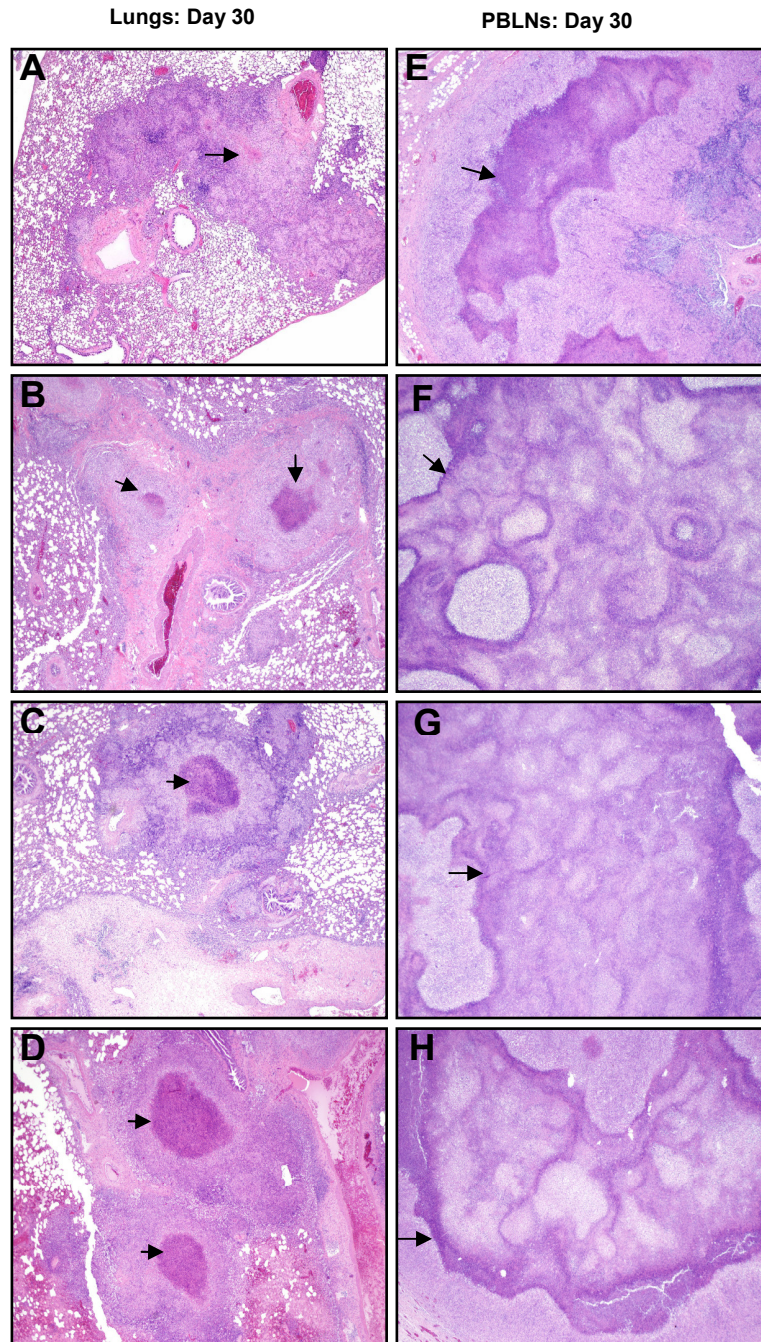


Figure 2.2 Light photomicrographs of lungs (2A–D) and peribronchial lymphnodes (2E–H) from representative guinea pigs that were aerosol challenged with H37Rv (Figure 2A, 2E), Erdman K01 (Figure 2B, 2F), CSU93 (Figure 2C, 2G) and HN878 (Figure 2D, 2H) on day 30 are shown. On day 30, all three clinical isolates show extensive peribronchial and peribronchiolar involvement and more necrosis (arrows) in lungs compared to standard H37Rv strain. Necrosis in peribronchial lymph nodes (arrows) is relatively more extensive in clinical isolate groups compared to H37Rv (magnification 20x).

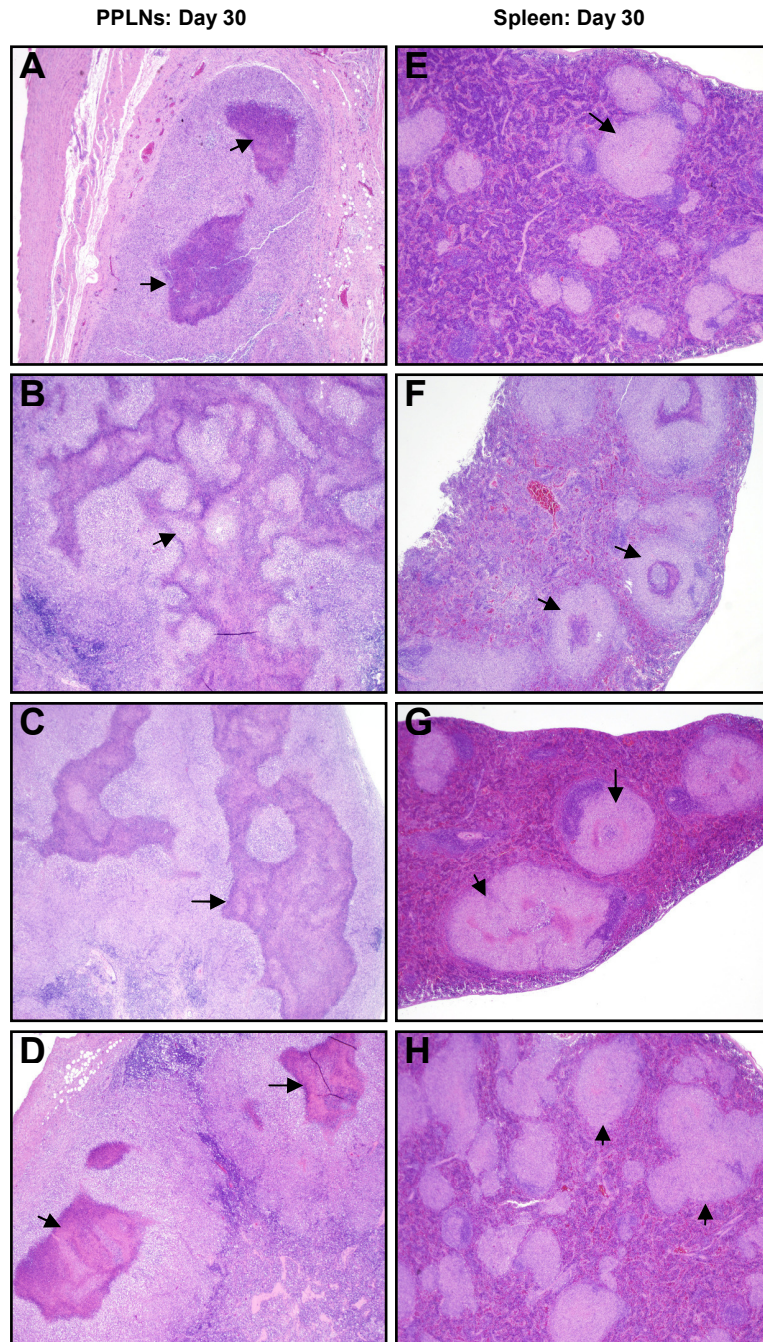


Figure 2.3 Light photomicrographs of peripancreatic lymph nodes (3A–D) and spleens (3E–H) from representative guinea pigs that were aerosol challenged with H37Rv (Figure 3A, 3E), Erdman K01 (Figure 3B, 3F), CSU93 (Figure 3C, 3G) and HN878 (Figure 3D, 3H) on day 30 are shown. Necrosis in peripancreatic lymph nodes (arrows) is relatively more extensive in clinical isolate groups compared to H37Rv on day 30. Erdman K01 and HN878 show more necrosis (arrows) in spleens on day 30 (magnification 20x).

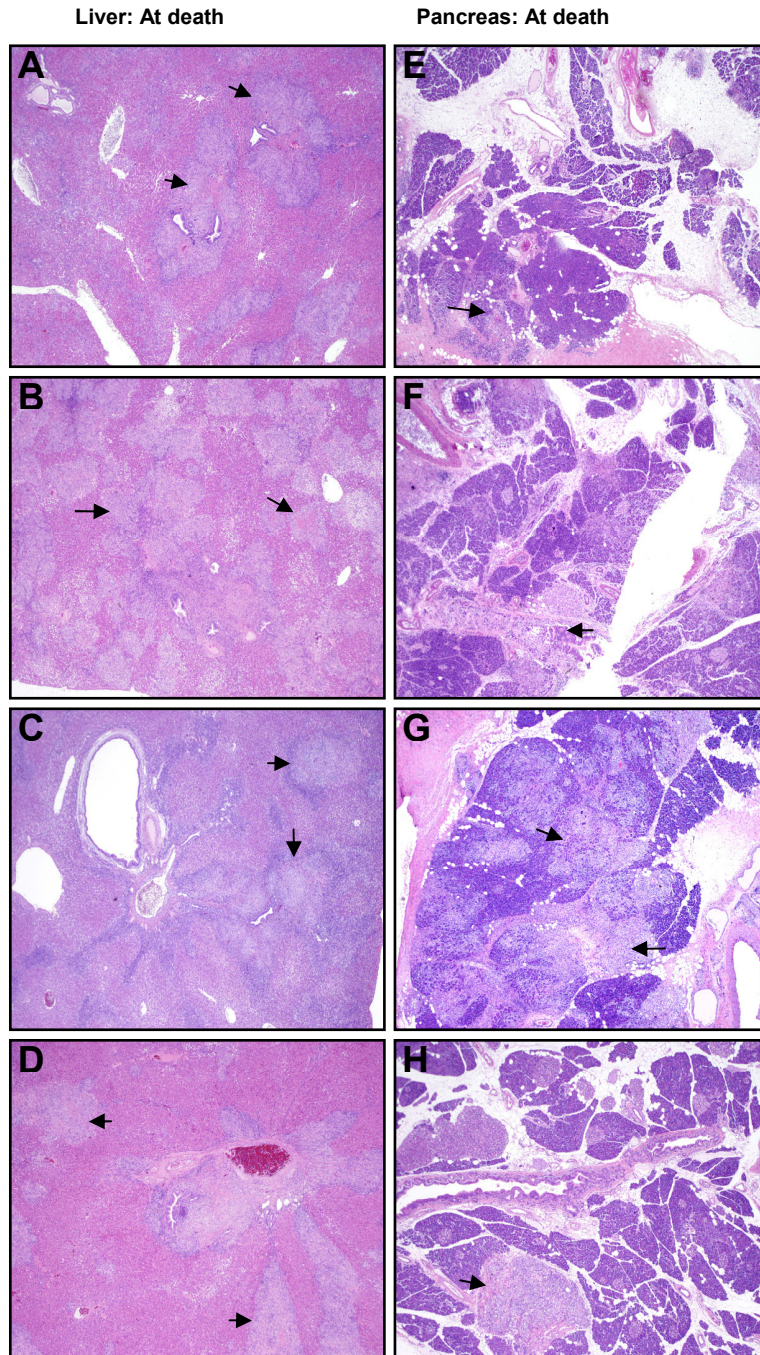


Figure 2.4 Light photomicrographs of livers (4A–D) and pancreas (4E–H) from representative guinea pigs that were aerosol challenged with *M. tuberculosis* strain H37Rv (Figure 4A, 4E), Erdman K01 (Figure 4B, 4F), CSU93 (Figure 4C, 4G) and HN878 (Figure 4D, 4H) are shown. In liver, Erdman K01, CSU93 and HN878 groups show more lesions and necrosis (arrows) at the time of death than H37Rv group. Among all four groups, CSU93 shows most severe pancreatitis (arrows) (magnification 20x).

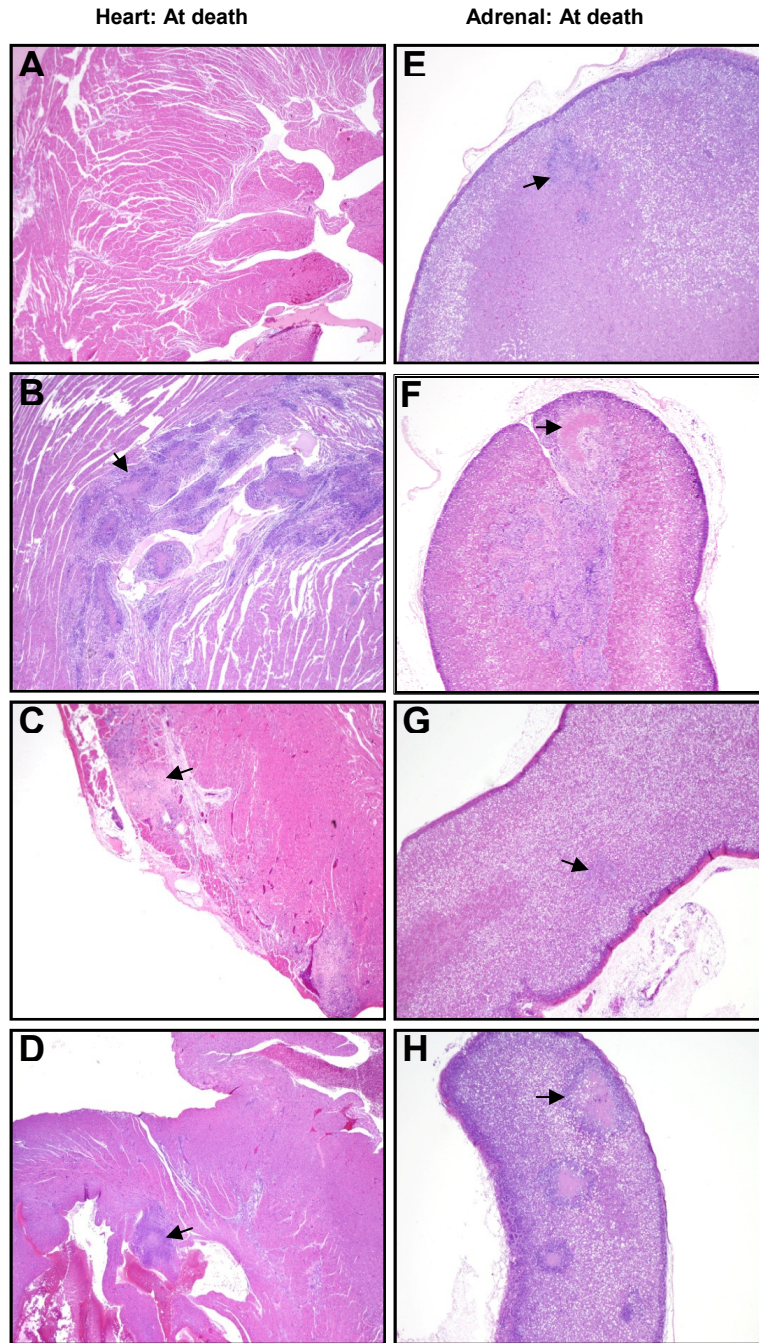


Figure 2.5 Light photomicrographs of hearts (5A–D) and adrenal (5E–H) collected from representative guinea pigs that were aerosol challenged with *M. tuberculosis* strain H37Rv (Figure 5A, 5E), Erdman K01 (Figure 5B, 5F), CSU93 (Figure 5C, 5G) and HN878 (Figure 5D, 5H) at the time of their death are shown. Among all four groups, Erdman K01 shows most severe myocarditis (arrows). Erdman K01 and HN878 show prominent adrenalitis (arrows) compared to the other two strains (magnification 20x).

Mean total lesion scores

Day 30	Lungs	PBLN	PPLN	Spleen	Liver	Pancreas	Adrenal	Heart
H37Rv	6.25	9.5	9	4	3.5	0	0	0.25
ErdK01	9	10.25	10	7.75	3.25	0	0	3
CSU93	8	10.5	11	4.75	3.5	0	0	1.25
HN878	9.25	10	10.75	6.25	4	0	0	2

At death	Lungs	PBLN	PPLN	Spleen	Liver	Pancreas	Adrenal	Heart
H37Rv	11	10	12.5	7.67	6.7	1.4	1.6	0.3
ErdK01	14.5	11.5	13.67	9.75	8.75	2.33	2.5	3.8
CSU93	12.3	10.75	12.67	10	7.8	3.8	1	2.8
HN878	13.1	14.4	15	8.6	9	2	3	2.8

Table 2.1 The lesion scores of lungs, peribronchial lymph nodes (PBLN), peripancreatic lymph nodes (PBLN), spleen, liver, pancreas, adrenal and heart were calculated as described on Materials and Methods for all the four groups on day 30 of the infection and at the time of death. The scores are presented as total mean lesion values.

Mean necrosis and mineralization scores

Organs	Lungs								PBLN							
	Day 30				At death				Day 30				At death			
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Necrosis	1.8	3.5	3.3	4.0	2.5	4.0	3.3	3.5	3.8	4.8	4.5	4.0	3.0	4.0	3.5	4.4
Mineralization	0	0	0	0	1.7	3.0	1.8	2.7	0	0	0	0	0	0	0	3.0

Organs	PPLN								Spleen							
	Day 30				At death				Day 30				At death			
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Necrosis	3.0	4.0	5.0	4.8	3.7	4.0	4.0	4.4	1.5	3.3	2.8	2.8	2.2	2.5	2.8	2.6
Mineralization	0	0	0	0	0.2	0.5	1.2	2.4	0	0	0	0	0	0	0.4	0.7

Organs	Liver							
	Day 30				At death			
	a	b	c	d	a	b	c	d
Necrosis	2.8	2.3	2.3	2.8	2.7	3.5	3.3	3.7
Mineralization	0.4	0	0	0	0	0.5	0	0

a- H37Rv
b- Erd K01
c- CSU 93
d- HN878

Table 2.2 The necrosis and mineralization scores of lungs, PBLNs, PPLNs, spleen and liver were calculated as described on Materials and Methods for all the four groups on day 30 of the infection and at the time of death and are presented as mean values.

Chapter Three

Clinical strains of *Mycobacterium tuberculosis* display a wide range of virulence in guinea pigs

Abstract

Virtually all new tuberculosis vaccine candidates are tested in animals using the laboratory strains H37Rv or Erdman. However, naturally occurring *M. tuberculosis* infections are caused by strains that are widely different in phenotype and genotype. Very little is known about the characteristics of these clinical isolates in terms of basic biology, virulence and *in vivo* pathogenicity. In this study, we have used a standardized aerosol infection of guinea pigs to compare *in vivo* differences between clinical strains of *M. tuberculosis*. Strains consisted of both drug sensitive and multi-drug resistant (MDR) strains of Beijing and non-Beijing varieties. Collectively, these clinical isolates tested in the guinea pig model exhibited a wide range of virulence. Infection with certain isolates caused severe and rapidly progressive pulmonary and extra-pulmonary lesion necrosis, some of which progressed to atypical cavitary lesions in draining mediastinal and tracheobronchial lymph nodes. The two MDR-TB strains used in this study exhibited low level virulence as determined by bacterial growth, lesion scores and survival. Since infections with clinical *M. tuberculosis* isolates produce such varied disease, it is unknown whether new tuberculosis vaccines being developed will provide the same level of protection as seen when tested using laboratory challenge strains. The use of appropriate animal models allows for this important question to be addressed.

Introduction

Most studies reporting *in vivo* virulence and pathogenicity of experimental *M. tuberculosis* infections in animals, challenge with the laboratory strains H37Rv or Erdman (Orme 2006). These strains are completely drug susceptible, and cause progressive pulmonary and extra-pulmonary disease in animals by low dose [~20 bacilli] aerosol infection (McMurray 2001). Accordingly, studies on the protective effects of *M. bovis* BCG, new tuberculosis vaccine candidates and a limited number of drug studies have used one of these two strains.

Worldwide, the incidence of tuberculosis continues unabated, driven in many places by co-infection with HIV (Dye et al 2002). Within this epidemic, the incidence of multi-drug resistant tuberculosis [MDR-TB] appears to be rising with previous estimates being a gross underestimate as revealed in a recent 2008 report (WHO 2008). Over 80 countries reported [33 previously unreported], covering about one-third of all new TB cases worldwide. The frequency of resistance to “any” drug ranged from 0% in certain developed countries to as high as 56% in Azerbaijan. As for defined MDR cases, the former Soviet Union and China had the highest rates of MDR- TB. In certain places, such as the Eastern Mediterranean, Jordan, and Yemen, the incidence was also higher than previously estimated. Overall, this report estimated that there were 489,139 new cases of MDR-TB in the year 2006, with China and India representing 50% of the global MDR-TB burden.

In terms of tracking these organisms, substantial advances have been made using newly developed molecular typing methods (Mathema et al 2006; Tsolaki et al 2005; Vitol et al 2006). These techniques have identified at least 36 TB clades according to a

2003 analysis (Filliol et al 2003). A large family referred to as the W-Beijing family is widespread among outbreaks with an incidence that is increasing and now also includes drug-resistant strains. The W-Beijing strains of *M. tuberculosis* are genetically related and are distinguishable from other strains by specific molecular markers that are linked to outbreaks characterized by rapid spread with high morbidity and mortality. In certain areas of the world, such as Asia and Russia, as well as North America, W-Beijing strains have become highly prevalent. Given the current-day molecular techniques, the epidemiology of W-Beijing and non-W-Beijing tuberculosis is better understood and can be linked to particular cases or outbreaks. Unfortunately, the underlying host-pathogen interactions that account for these distinct clinical characteristics are unknown.

In this regard, an early hypothesis was that MDR-TB strains lost virulence as a trade-off for acquiring drug resistance. *In vivo* studies in mice however, did not support this hypothesis (Ordway et al 1995; Orme 1999) and in fact showed faster growth in lungs over the first 20 days of infection. In the present study we revisit this issue using the guinea pig model that differs significantly from rodent species in response to *M. tuberculosis* infection. Our results with two MDR-TB strains support the earlier hypothesis as these isolates produce less severe disease *in vivo* when compared to laboratory or drug-sensitive W-Beijing strains. Clinical *M. tuberculosis* isolates including drug susceptible W-Beijing genotypes, produced pulmonary and extra-pulmonary disease in guinea pigs that differed significantly in severity. While the H37Rv and Erdman strains may be useful in the initial screening for efficacy of vaccine candidates, protective responses may be less favorable when challenged with the more relevant and highly pathogenic clinical isolates such as those described here.

Methods

Experimental infections

Female out-bred Hartley guinea pigs (approximately 500g in weight) were purchased from the Charles River Laboratories (North Wilmington, MA, USA) and held under barrier conditions in a Bio-safety Level III animal laboratory. All experimental protocols were approved by the Animal Care and Use Committee of Colorado State University and comply with NIH guidelines.

The isolates used in this study were chosen specifically because they were all associated with outbreaks in United States. *M. tuberculosis* H37Rv was originally obtained from the Trudeau Institute collection, Saranac Lake NY. Three strains from the Public Health research institute, Newark, NJ (PHRI) TB Center collection were selected in regard to their genetic backgrounds and their resistance phenotype. Strains TN7642 [“W” DNA fingerprint] and TN14149 [“W10” DNA fingerprint] are both members of the W-Beijing strain family. The W strain is resistant to streptomycin, isoniazid, rifampin, ethambutol and kanamycin. Strain TN14149 is a pan-susceptible strain with the identical fingerprint to the sequenced strain 210. Strain TN5904 is a non-W-Beijing family MDR with resistance to streptomycin, isoniazid, rifampin, and PAS. It was recently identified as the strain causing exogenous re-infections among HIV-positive patients (Small et al 1993). W-Beijing strains SA161, SA627, and TB282, as well as non-Beijing strains SA310 and TB284. W-Beijing strains SA161 and SA627 are case isolates from Arkansas. These strains were chosen on the basis of the Beijing spoligotype, an IS6110 insertion in the dnaA-dnaN region and the region of deletion (RD) 105. SA161 is an isolate from a cluster of cases, while SA627 isolate was from a unique case. TB282 and TB284 were

selected from an epidemiological study in central Los Angeles. TB282 isolate is of the Beijing spoligotype and was associated with an outbreak. TB284 was isolated from a single patient and is not of the Beijing spoligotype, whereas SA310 is a non-Beijing isolate from the Arkansas collection. All of the strains used in this study were grown in 7H9/ADC + 0.05% Tween-80. Thawed aliquots of frozen cultures were diluted in milli-Q sterile water to the desired inoculum concentrations. A Madison chamber aerosol generation device was used to expose the animals to *M. tuberculosis*. This device was calibrated to deliver approximately 20 bacilli into the lungs. The numbers of bacilli grown from the inoculum prepared for the aerosol infection from each of the strains were identical. However the actual implanted bacterial numbers were undetermined.

Lung bacterial counts on days 30 and day 90 were determined by plating serial dilutions of tissue homogenates on nutrient 7H11 agar and counting colony-forming units after 3 weeks incubation at 37°C. In survival studies, animals showing substantial weight loss with no evidence of weight rebound were euthanized. The results shown in the survival studies are based upon 10 guinea pigs per group.

Tissue fixation and processing

Lungs, spleen and intra-thoracic draining lymph nodes were collected at the time of necropsy and fixed in 4% paraformaldehyde for 3 days. The tissues were embedded in paraffin wax and 4µ thick sections were cut and stained with hematoxylin and eosin. Microscopic lesions were scored while the reviewer was blinded to the treatment groups. Photomicrographs representative of their respective groups were taken using an Olympus BX41 microscope with Olympus DP70 camera and software.

Lesion analysis

To evaluate the progression of lesions over time, a histological grading system was used, as previously described (Palanisamy et al 2008). Four animals/time point were used for the data reported in the figure 3. The lung sections were scored based on the following six criteria: (i) percent of lung affected were ranked at low magnification by an estimation of the percent of the lung affected as follows: 0-no lesions in lung, 1-up to 25% of lung involved, 2-up to 50 % of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. (ii) primary lesions: 0-no primary lesions present, 1-a single primary lesion, 2-two or more primary lesions, multi-focal, 3-two or more primary lesions, multifocal to coalescing, 4-multiple primary lesions, coalescing and extensive. (iii) secondary lesions: 0-no secondary lesions present, 1-up to 25% of lung involved, 2-up to 50% of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. Necrosis (iv), mineralization (v) and fibrosis (vi) are scored based on severity as follows: 0-none, 1-minimal, 2-mild, 3-moderate, 4-marked and 5-severe. All individual scores were added for the final total score for each organ. The lymph node and spleen lesions were scored based on four categories; (i) percentage involvement (same scale as lungs), (ii) degree of necrosis, (iii) fibrosis and (iv) mineralization (0-5 scale).

Statistical analysis

One-way ANOVA is used to compare the statistical differences in numbers of bacilli, mean lesion scores and necrosis scores in different groups.

Results

Growth in the lungs of each isolate

The course of each infection is shown in Fig 3.1, in comparison to the *M. tuberculosis* laboratory strain H37Rv. In all cases the initial growth of each isolate was similar at day 30, but whereas the growth of H37Rv plateaued, six of the isolates, other than MDR ones, continued to grow in the lungs reaching bacterial loads approximately a log higher by day 90. The two MDR strains TN5904 and TN7642 grew to lesser levels than H37Rv on day 90 both lungs and spleen. The numbers of bacilli between different groups demonstrate statistically significant difference in all time points and organs except for spleen at day 30 after infection.

Animal survival patterns

Starting from as early as day 40 of infection, animals from various groups began to die or were euthanized due to advanced clinical disease (Fig 3.2). By day 125, all of the animals infected with the isolate SA161 had died or were euthanized, as had the majority of animals infected with SA627. As the study continued approximately half of the animals infected with TN14149, TB284, SA310, and TN5904 had died or were euthanized when the study was halted on day 150. Fewer deaths were seen in animals infected with TN7642, TB282, or H37Rv.

Lung pathology

The different clinical isolates produced an unexpectedly wide range in severity of pulmonary and extra-pulmonary lesions compared to laboratory challenge strains. In

terms of total lesion scores (Fig 3.3), TN7642 and TN5904 had the lowest while SA627 and SA161 had the highest scores. All the other strains had lesion scores that were comparable to the H37Rv infected animals on day 30 with values increasing in various groups by day 90. A similar trend was seen in other parameters except that necrosis scores were significantly higher in animals infected with SA161, and SA627.

Based on lesion scores at the different time points, isolates could be categorized into three distinct groups (Fig 3.4). In the first, characterized by TN5904, TN7642 and TB282, the granulomatous inflammation was minimal with no/minimal necrosis. In a second group, characterized by TN14149, SA310, TB284 and H37Rv, the lung involvement was moderate and the lesions had the expected appearance with multiple foci of granulomatous inflammation with areas of central lesion necrosis. In a third group, characterized by SA161 and SA627, day 30 primary lesions were more expansive with extensive necrosis compared to other isolates.

On day 90, lung lesions in animals infected with H37Rv and TB282 showed evidence of necrosis, dystrophic calcification and progressive coalescing of post-primary lesions which was less in the TB282 group. In the case of the two MDR strains TN5904 and TN7642, since primary lesions never progressed to necrosis, mineralization was minimal as was percent lung involvement by post-primary inflammation. This was in contrast to strains SA310 and TB284, in which post-primary lesions accounted for more substantial lung consolidation. In the case of TN14149, SA161, and SA627, lung inflammation continued to progress to necrosis and dystrophic calcification that involved extensive areas of parenchyma.

In general, the patterns and extent of inflammation and necrosis in the intra-thoracic draining lymph nodes was similar to those in the lung (Figure 3.5). By day 30 of infection, marked lymph node enlargement was due to extensive granulomatous inflammation and necrosis that is a characteristic of the course of the H37Rv infection. In contrast, animals infected with the TN5904 and TN7642 strains had multifocal to coalescing foci of inflammation with no or minimal necrosis and retained moderate amounts of normal lymphoid tissue architecture. By day 90, the lymph nodes lesions in TN5904 and TN7642 infected groups, had progressed slowly to involve the majority of the parenchyma but failed to develop necrosis compared to other isolates. With the remaining isolates, by day 90, lymph node involvement was 100% with the normal architecture effaced by inflammation and extensive necrosis. Besides the marked necrosis and mineralization, unusual cavitory lesions are present in animals infected with strains SA161, SA627, and TB284 (Figure 3.5B).

The lung, spleen and lymph node histopathology results of SA161 and SA627 are from the fewer animals that did not die by day 90, and yet they show remarkable pathology. The statistical analysis showed all mean score values between groups to be significantly different in all groups ($p < 0.05$) except lymph node mean lesion and necrosis scores on day 90 and spleen necrosis scores on day 30.

Discussion

There were a number of unexpected results of this study. The most notable one was the wide range in lesion severity and virulence differences between the various clinical isolates compared to laboratory strains of *M. tuberculosis*. With at least two clinical isolates, the rapid and extensive development of primary lesion necrosis was

significantly more severe than what we have previous seen using this model. Moreover, unlike our experience with laboratory challenge strains, we saw examples (TN5904 and TN7642) in which primary lesions developed slowly and failed to progress to necrosis or dystrophic calcification which is typical of even laboratory strains. Similarly, during the stage of post-primary lesion development, these strains failed to progress to involve significant regions of lung parenchyma. In contrast, several W-Beijing strains showed rapid and progressive disease with extensive pulmonary and extra-pulmonary lesion necrosis including lymph node lesion cavitation, a rare event in animals infected with H37Rv or Erdman.

It was also apparent that not all W-Beijing strains were intrinsically more virulent than non-W-Beijing isolates as reflected by rate of bacterial growth, lesion scores and survival. Whereas the W-Beijing strains SA627, SA161, and TN14149 were highly pathogenic, a W-Beijing MDR-TB strain associated with recent outbreaks (TN7642) and a non-MDR-TB Beijing spoligotype strain (TB282) were of lower virulence in this model. We also noted that TN7642 as well as the “P” strain TN5904 caused less lung involvement compared to other strains in the chronic stages of disease. Progressive post-primary inflammation which is a reflection of dissemination of bacilli through intra-pulmonary and hematogenous routes was also minimal with these isolates. The lack of post-primary inflammation may also be an indicator of decreased virulence in the post-immune phase of infection.

Whereas rapid destruction of lymphoid tissue is feature of experimental tuberculosis in the guinea pig model, cavitation is not, as it is rare in animals infected with H37Rv or Erdman. However, cavitory lesions in lymph nodes were observed among

SA161, SA627 and TB284 groups. In the present study like previous studies, cavitation was not a feature of guinea pig lung lesions but may reflect the dose or method of aerosol exposure. In an ongoing study in which guinea pigs were infected by exposure to room air containing MDR-TB from a South African hospital ward, cavitation of guinea pig lung lesions were seen at a higher incidence than our standard aerosol infection model. (A. Dharmadhikari, R.Basaraba, I.Orme, E.Nardell; unpublished studies).

It is difficult to definitively conclude, given the small sample size, whether the genetic changes that render certain strains drug resistant also result in a loss of virulence *in vivo*. However, the two MDR-TB strains tested here, the TN7642 W-Beijing strain and the TN5904 “P” family strain, were not as virulent based on pathology and survival compared to other strains including the conventional challenge strain H37Rv. These results do support the notion that MDR-TB strains may have lost virulence, the pathogenesis of which can be further investigated in the guinea pig model. These results further support the classical studies done by Mitchison et al that showed that majority of the isoniazid-resistant *M. tuberculosis* strains had low virulence in the guinea pig infection model (Mitchison et al 1960). Moreover, our results of reduced numbers of bacilli in the spleen among low virulence strain groups TN7642, TN5904 and TB282 also support the conclusions of Balasubramanian et al that indicated that the low virulence strains had significantly reduced ability to disseminate via blood stream (Balasubramanian et al 1992).

The strain TN14149 is a New York isolate that is identical to the sequenced strain 210 and to the Texas strain HN878. This is highly virulent in mice, where it causes severe inflammatory lesions similar to these seen in our guinea pig study (Manca et al 2001;

Ordway et al 2007). It also subverts protective immunity by inducing a population of CD4 T cells that have a regulatory T cell Foxp3+ phenotype (Ordway et al 2007). It is not known if this is unique to TN14149 or whether other strains that induce severe inflammation and necrosis such as SA161 and SA627 studied here also have this property. Indeed, as shown above, the extreme virulence of these strains cannot simply be explained just in terms of accelerated rates of growth in the lungs, and thus reflects the complexity of the host-pathogen interaction. Another corollary to our study is that it further establishes our earlier report that pulmonary and extra-pulmonary lesion scores can be used as a measure of virulence which better correlates with survival than the tissue viable counts (Palanisamy et al 2008).

Vaccines and now experimental tuberculosis drugs are routinely tested in the guinea pig model using the laboratory challenge strains H37Rv and Erdman (Lenaerts et al 2008; Orme 2006). However, we conclude from these results that laboratory strains may not represent the virulence of naturally occurring tuberculosis in people and hence activity of a given vaccine or treatment cannot be guaranteed from assays using these laboratory strains for challenge. This is an important consideration when testing new vaccine candidates or drugs. Indeed, the failure of BCG to protect mice from a W-Beijing strain has already been demonstrated (Grobe et al 2005).

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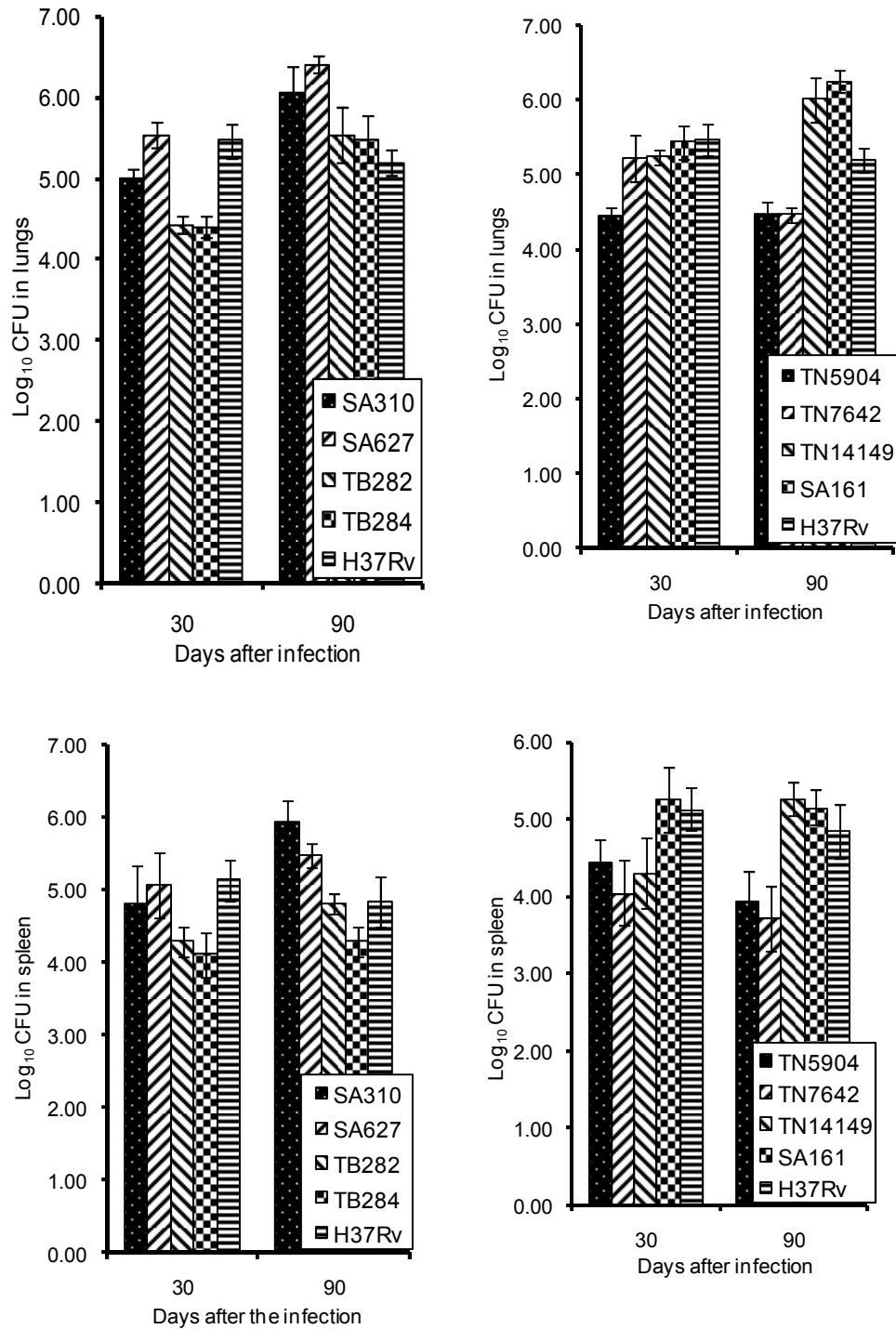


Figure 3.1 Bacterial growth graphs of the clinical isolates in the lungs and spleen of guinea pigs after low dose aerosol exposure. Data shown as mean values [n=5].

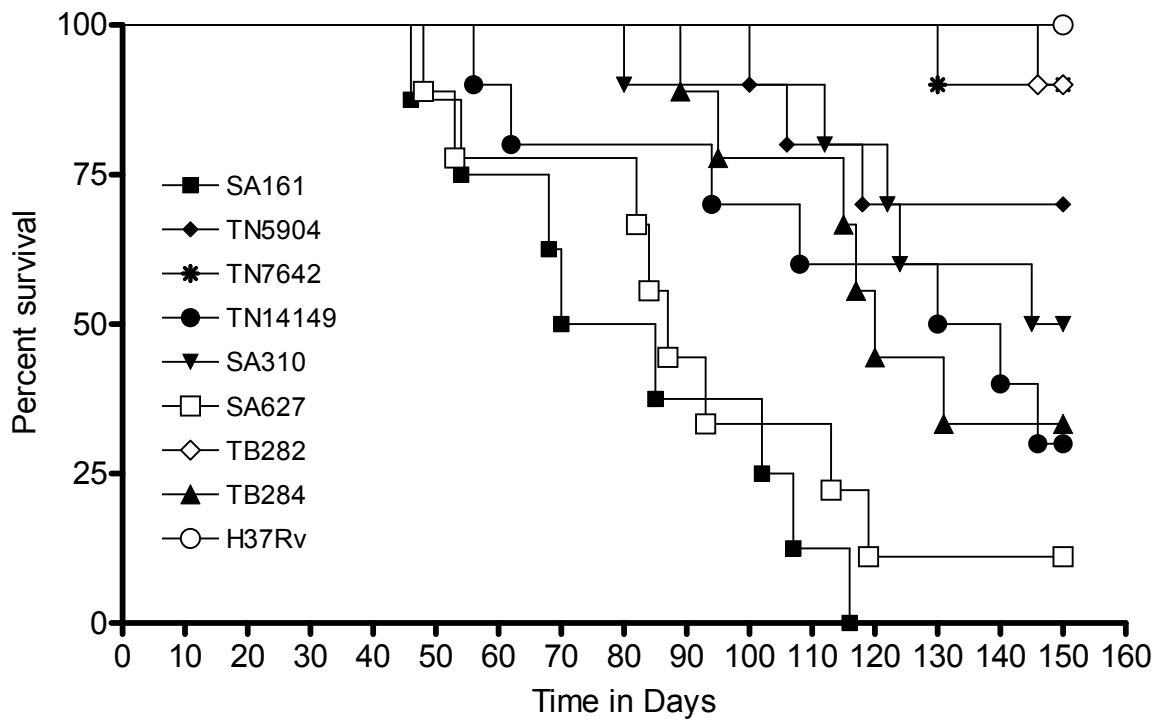


Figure 3.2 Kaplan Meier analysis of the survival of guinea pigs infected with the clinical isolates by low dose aerosol exposure [n=9-10 animals].

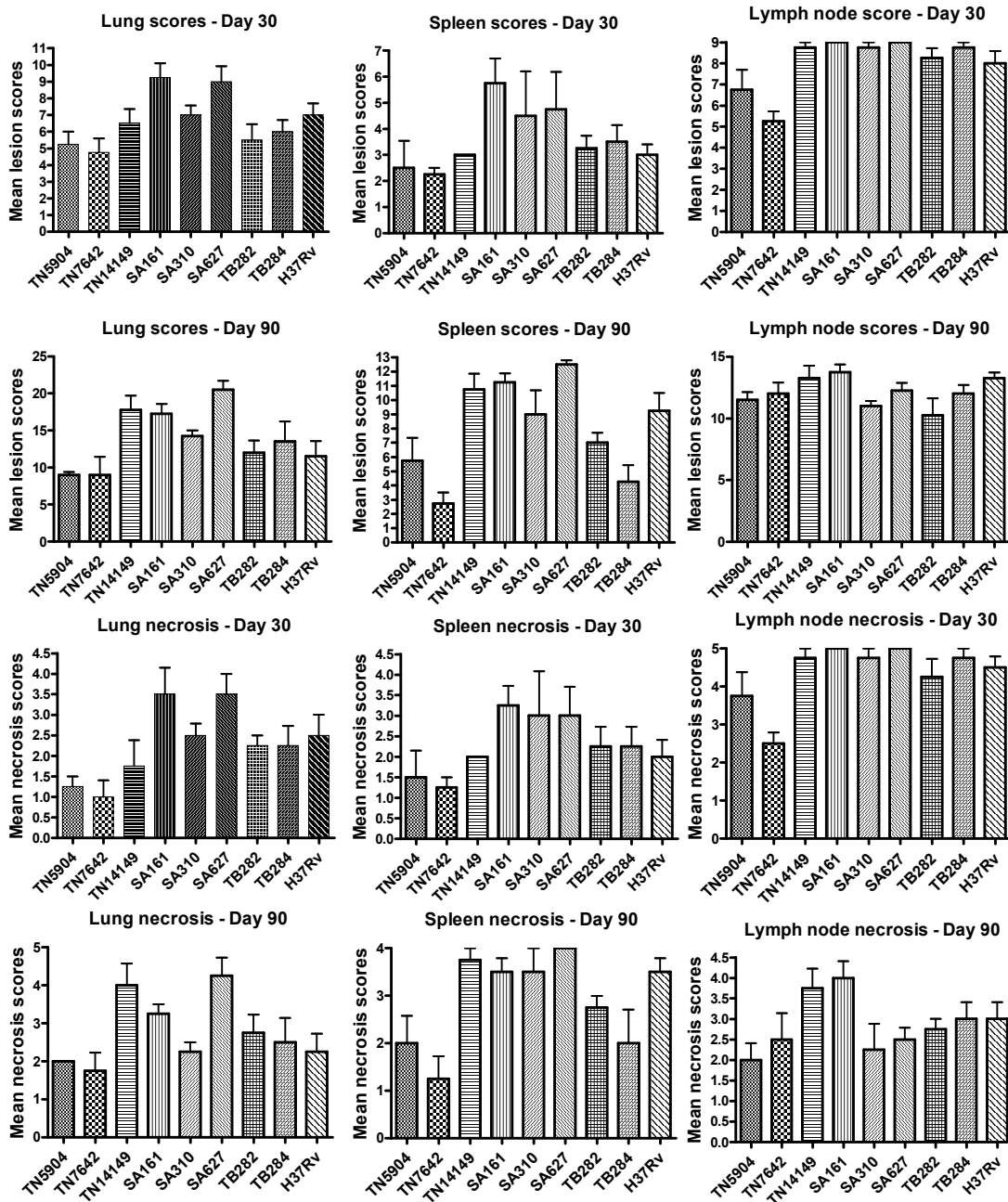


Figure 3.3 Mean organ lesion scores and lesion necrosis scores of infected guinea pig lungs, spleen and draining lymph nodes 30 and 90 days after low dose aerosol infection with each of the clinical isolates.

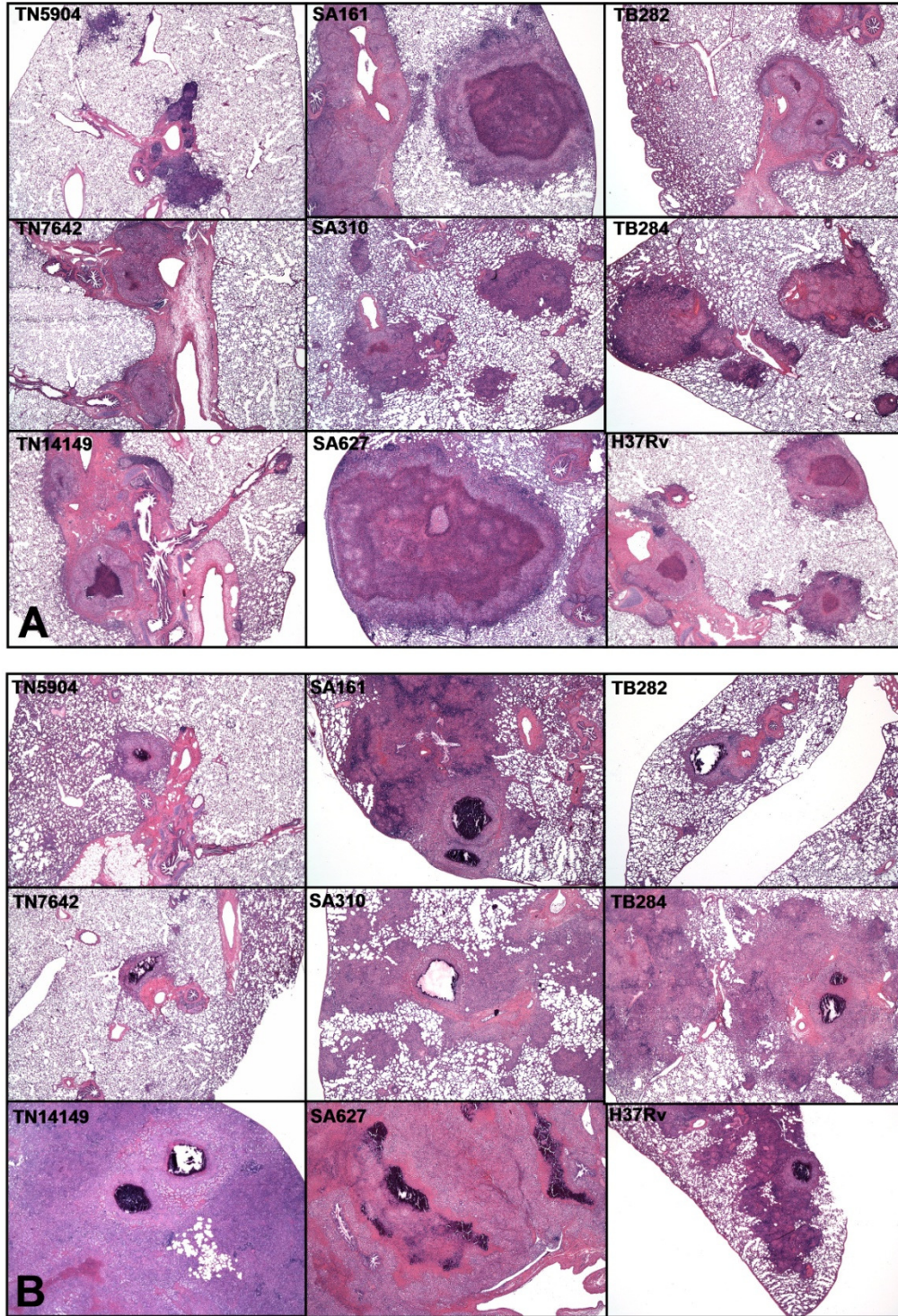


Figure 3.4 Representative histopathology images of lungs (H&E staining; 20x magnifications) from animals infected with the clinical isolates and harvested on day 30 (A) and day 90 (B) of the infection.

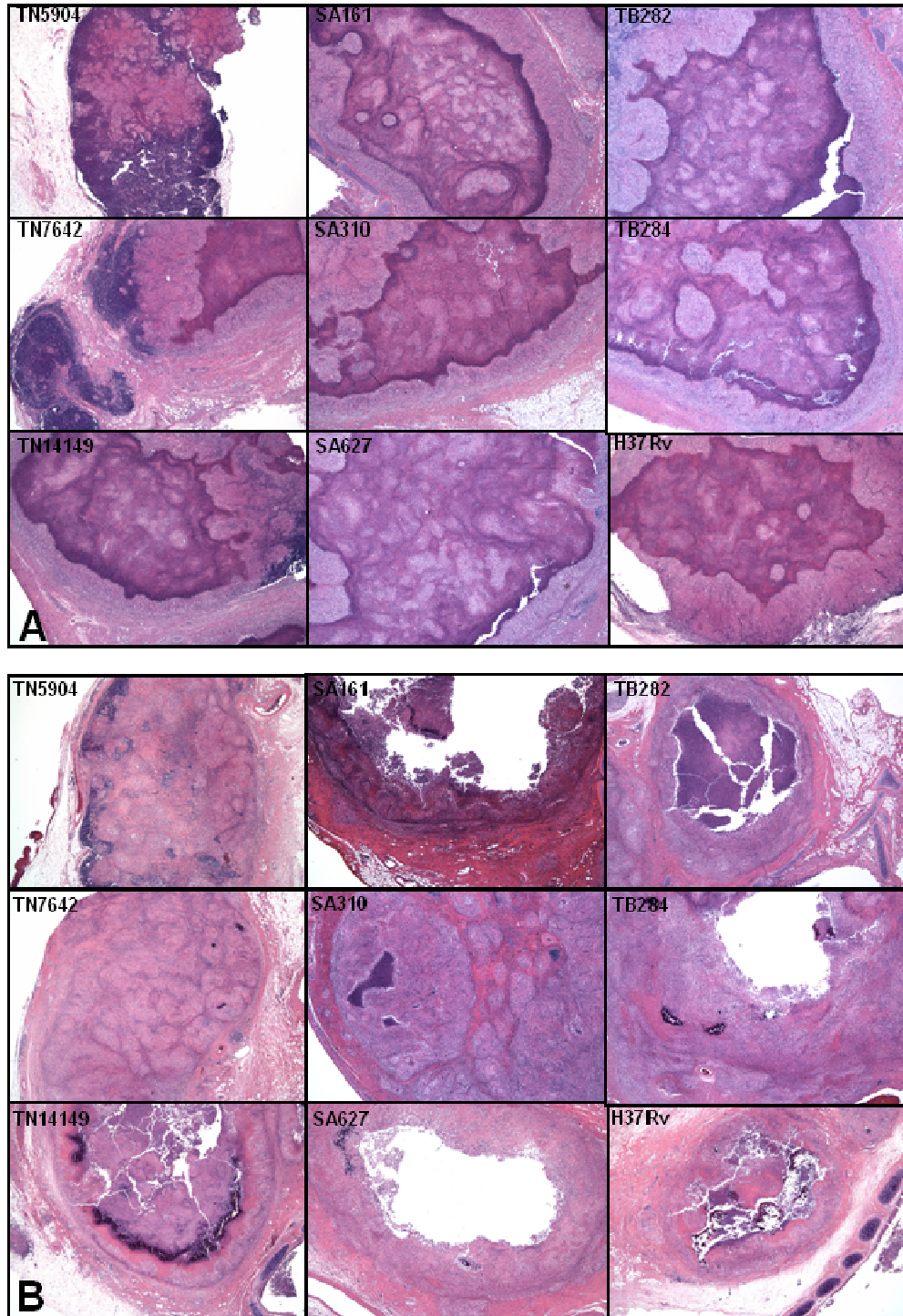


Figure 3.5 Representatives histopathology images of peribronchial lymph nodes (PBLNs, H&E, 20x magnifications) from each group on day 30 (3.5A) and day 90 (3.5B) of the infection.

Chapter Four

Evidence for oxidative stress and a defective antioxidant response during tuberculosis in guinea pig

Abstract

Caseous necrosis is an important feature of granulomatous inflammatory lesions in humans and some animals infected with *Mycobacterium tuberculosis*, however the pathogenesis is poorly understood. In this study we examined the role oxidative tissue damage has in the pathogenesis of primary lesion necrosis using the guinea pig model of the naturally occurring disease in humans. Oxidative tissue damage was evident within 2 weeks of experimental *M. tuberculosis* infection and was characterized by increased expression of tissue malondialdehyde, a byproduct of lipid peroxidation, within primary lung lesions as well as a marked decrease in serum total antioxidant capacity. The host response to oxidative tissue damage was also evident by an increased expression of the host transcription factor Nrf2 that regulates the expression of a number of antioxidant proteins. Despite the increase in cytoplasmic Nrf2 expression in primary granulomas, a defect in Nrf2-mediated antioxidant defense mechanisms was suggested by a lack of nuclear expression and a decrease in primary granuloma staining of NQO1, an Nrf2-regulated antioxidant enzyme. An Nrf2-mediated defect in antioxidant defenses was also supported by decreased expression of the rate-limiting enzyme involved in the synthesis of the critical host tripeptide antioxidant glutathione. The defect in Nrf2-mediated antioxidant defense was partially reversed by oral treatment of *M. tuberculosis*-infected guinea pigs with the antioxidant N-acetylcysteine (NAC) which is known to

promote nuclear translocation of Nrf2 during oxidative stress conditions. NAC treatment resulted in a decrease in lung and spleen lesion burden as well as extent of lesion necrosis. Compared to mock-treated animals, NAC partially restored total and reduced glutathione levels as well as the total antioxidant capacity of the serum by day 60 of infection. These data suggest that lesion and systemic oxidative stress is progressive during experimental tuberculosis in guinea pigs and leads to a marked depletion of host antioxidant capacity, in particular 'reduced glutathione'. The depletion of host antioxidant defenses may be due in part to a defect in the *in vivo* function of the redox sensitive transcription factor Nrf2.

Introduction

Human infections with *Mycobacterium tuberculosis* are the one of the most common causes of death from an infectious disease worldwide (Frieden et al 2003). It is estimated that in 2007, there were 9.27 million new cases of active tuberculosis reported. The spread of tuberculosis, particularly in developing countries, has been made more challenging by the emergence of multidrug-(MDR) and extensively drug-resistant (XDR) strains of bacilli that are refractory to first and second line anti-tuberculosis drugs respectively (Espinal & Raviglione 2003). While several promising new drugs are in clinical trials, no new FDA approved anti-tuberculosis drugs have been introduced in the last 25 years. Moreover, the effective treatment of patients for 6 to 9 months with combinations of existing drugs is limited by the complications associated with toxic side-effects of drug therapy. Development of new anti-tuberculosis drugs or drugs that could

potentiate the therapeutic value of currently available drugs is urgently needed for global tuberculosis control.

The difficulty in effectively treating human tuberculosis is due to the chronic nature of the disease and the long-term persistence of drug-tolerant bacilli. There is increasing evidence from human and animal studies that drug-tolerant bacilli can persist in an extracellular microenvironment in lesions with necrosis or cavitation (Canetti 1955; Lenaerts et al 2007). A better understanding of the pathogenesis of lesion necrosis may lead to better therapeutic approaches that specifically target bacilli that persist in this unique *in vivo* microenvironment. Tuberculosis, like other chronic inflammatory diseases, is characterized by the generation of oxygen and nitrogen free radicals. Oxidative stress is defined as the generation of free radicals in excess of the antioxidant capacity of the host. Markers of systemic oxidative stress are elevated in the peripheral circulation of patients with active tuberculosis (Kwiatkowska et al 1999; Lamsal et al 2007). Moreover, inflammation-related oxidative stress had been implicated in the pathogenesis of lung fibrosis and dysfunction in tuberculosis patients even following antimicrobial therapy (Jack et al 1994; Kwiatkowska et al 1999). Besides an increase in the byproducts of free radical generation, several studies have demonstrated that critical serum antioxidants such as ascorbic acid and glutathione are depleted in tuberculosis patients (Madebo et al 2003; Vijayamalini & Manoharan 2004). Depletion of host antioxidants are thought to result from consumption by the excess free radicals generated in inflamed tissues that is further complicated by inadequate dietary replenishment (Schorah 1992; Vijayamalini & Manoharan 2004).

Previous studies have demonstrated that in guinea pigs, the progression of pulmonary and extra-pulmonary lesions can be modulated by BCG vaccination prior to experimental aerosol infection with virulent *M. tuberculosis* (McMurray & Bloom 1994; Smith et al 1970). Specifically, BCG vaccination delays the progressive infiltration of lesions by free radical generating phagocytes including macrophages and granulocytes (Ordway et al 2008).

The antioxidant capacity of the host is maintained by numerous proteins and small molecules that prevent oxidative damage to lipids, proteins, and nucleic acids. Collectively, the protein antioxidants that detoxify free radicals through conjugation are referred to as phase II detoxification enzymes (Mithen et al 2003). The induction of phase II enzyme expression requires binding of specific inducers to the antioxidant response element (ARE) in the promoter regions of phase II enzyme genes. Nuclear erythroid 2 p45-related factor 2 (Nrf2), a redox-sensitive transcription factor, is directly involved in the transcriptional activation of ARE-driven phase II antioxidant enzymes. Under normal physiologic conditions, Nrf2 is sequestered in the cytoplasm by the binding protein, Kelch-like ECH-associated protein (Keap1). Under oxidative stress conditions, Nrf2 dissociates from Keap1 allowing translocation of Nrf2 to the nucleus where it heterodimerizes with small Maf-family proteins which bind to ARE sequences leading to transcriptional activation of the various phase II antioxidant enzymes (Juge et al 2007).

NAD(P)H dehydrogenase, quinone 1 (NQO1) is one such protein that prevents free radical formation from quinones. Another Nrf2-regulated antioxidant is glutathione, an essential intracellular antioxidant tripeptide which is synthesized within cells in a two step, energy dependent reaction. The first and rate-limiting step is catalyzed by

glutamate-cysteine-ligase (GCL; also known as γ -glutamylcysteine synthetase-GCS). Among other antioxidant proteins, NQO1 and GCS are both transcriptionally regulated by Nrf2 (McWalter et al 2004; Wild et al 1999).

Here, we demonstrate that oxidative stress conditions deplete the antioxidant capacity of guinea pigs infected with *M. tuberculosis*. We further show that oxidative stress is due in part, to depletion of serum antioxidant capacity and a defective Nrf2-mediated antioxidant response. In keeping with the modulation of the pro-inflammatory response, BCG vaccination of guinea pigs prior to aerosol infection, maintained the total antioxidant capacity of the serum to near normal levels compared to mock-vaccinated animals. Finally, we demonstrate that the progression of lesion necrosis could be reversed by restoring the host antioxidant mechanisms through therapeutic administration of the antioxidant drug N-acetylcysteine. These data suggest that antioxidant therapy may prove to be a beneficial adjunct therapy to modulate host tissue damage when used in combination with conventional antimicrobial drugs.

Methods

Aerosol infection of guinea pigs with *M. tuberculosis*

Mycobacterium tuberculosis H37Rv strain (TMC#102; Trudeau Institute, Saranac Lake, NY) was grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase, aliquoted, and frozen at -80°C until used for infection. A thawed aliquot of *M. tuberculosis* was diluted in sterile water to 10^6 CFU/ml for a total working stock volume of 20 ml. Guinea pigs (approximately 9 months of age) from each treatment

group were aerosolized using the Madison infection chamber (University of Wisconsin Machine Shop, Madison, WI) with a starting volume of 15 ml of working stock.

BCG vaccination

Guinea pigs were vaccinated with 1×10^4 *Mycobacterium bovis* (BCG, strain Pasteur) or mock vaccinated with equal volumes of saline, intra-dermally 4 weeks prior to aerosol exposure to *M. tuberculosis*. *M. bovis* BCG was grown in Proskauer-Beck similar to *M. tuberculosis* and frozen at -80°C until used for infection.

NAC administration

Guinea pigs were treated or mock-treated orally from day 0 to the experimental end-point (day 30 or 60 of infection) with either NAC (400mg/kg, Calbiochem, San Diego, CA) dissolved in 2 ml water containing 20% sucrose or carrier alone.

Euthanasia and sample collection

At intervals of 5, 15, 20, 30 and 60 days post-infection, guinea pigs were euthanized humanely by an overdose (1 ml per 0.75 kg body weight) of sodium pentobarbital (Sleepaway; Fort Dodge Laboratories Inc.) by intraperitoneal injection. Following euthanasia, at each time point, the left pulmonary lobes were infused *in situ* and fixed for 48 hours in 4% paraformaldehyde and thereafter stored in 70% ethanol. At the time of processing, all tissues were embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin (H&E). At the same time points, bacterial load of lungs, spleen and peribronchial lymph nodes were determined by plating respective organ

homogenates onto nutrient 7H11 agar plates supplemented with OADC. Colonies are counted after 21 days of incubation at 37°C.

Measurement of total antioxidant status, glutathione levels and GSH/GSSG ratio

Serum or whole blood collected from guinea pigs were assayed for total antioxidant status (Sigma, St. Louis, MO), oxidized/reduced glutathione levels and GSH/GSSG ratio (GSH/GSSG Ratio Assay kit; Calbiochem, San Diego, CA) using calorimetric assay kits following respective manufacturer's protocols.

Immunohistochemistry

Paraffin embedded sections of lung from the experimentally infected guinea pigs, collected on positively charged glass slides, deparaffinized, rehydrated and subjected to antigen retrieval by incubation in Target Retrieval solution, pH 6.0 (DAKO, Carpinteria, CA) for 25 min at 90° C, followed by a 20 min cooling period at room temperature. The sections were then treated with 0.3% hydrogen peroxide in water for 15 min to quench endogenous peroxidase activity. Following a rinse in Tris buffered saline with 1% Tween-20 (TTBS), the slides were subjected to two blocking steps: (i) 15 min incubation with 0.15 mM glycine in PBS, and (ii) 30 min incubation with 1% normal horse serum with a rinse in TTBS in between. The slides were then incubated with rabbit polyclonal antibody to human Nrf2, GCS (Santa Cruz Biotechnology, Santa Cruz, CA), MDA, NQO1 (Abcam, Cambridge, MA) or mouse monoclonal antibody against glutathione (Virogen, Watertown, MA) or respective isotype IgG controls at a 1:100 dilution in blocking buffer followed by several rinses in TTBS. This was followed by 30 min

incubation with biotinylated goat-anti rabbit-IgG (Vector Laboratories) and visualization of bound antibody by the Avidin-Biotin system (Vectastain; Vector Laboratories) and diaminobenzidine substrate (Dako; Carpinteria, CA). The sections were counterstained with Meyer's hematoxylin (Scytek Laboratories; Logan, Utah), mounted with coverslips, and examined on an Olympus BX41 light microscope.

The specificity of anti-human antibodies on guinea pig tissues was validated by blocking the primary antibodies with purified antigens (Nrf2 - Santa Cruz Biotechnology; NQO1 - Abcam; GCS - Santa Cruz Biotechnology) at a concentration ratio of 1:5 before adding them to the respective sections in lieu of the unblocked primary antibodies. Antibody specificity was confirmed by the effective blocking of anti-human Nrf2 and NQO1 antibodies binding to guinea pig tissues with the respective purified proteins. Photomicrographs were acquired with an Olympus DP70 camera and the associated computer software. Microscopic lesions stained with H&E and sections stained by immunohistochemistry were scored by the reviewer blinded to the treatment groups.

Immunohistochemical scoring

The lung lesions were classified as either primary lesions or primary lesion free (PLF) lung for immunohistochemical scoring purposes. Primary lung lesions are the initial foci of inflammation that develop following aerosol infection of immunologically naïve animals and are characterized by central necrosis. Secondary lesions are the foci of inflammation that develop in the late stage of infection as a consequence of hematogenous dissemination. Primary lesion free lung constitutes non-necrotic secondary lesions and residual normal lung parenchyma (McMurray 2003). Within these regions the

overall immunohistochemical scoring was determined based on extent of staining (0-none; 1-less than 25%; 2-26-50%; 3-51-75%; 4-more than 75%) and staining intensity (0-none; 1-mild; 2-moderate; 3-marked; 4-extensive). The resultant total overall scores were converted to a four-point scale. Since the *in vivo* function of Nrf2 as a transcription factor is dependent on cytoplasmic to nuclear translocation, the relative nuclear and cytoplasmic expression of Nrf2 in lesions and PLF lung was also scored using the following scale (0-none; 1-less than 25%; 2-26-50%; 3-51-75%; 4-more than 75%).

Lesion analysis

To evaluate the progression of lesions over time, a histological grading system was applied, as previously described (Palanisamy et al 2008). The lung sections were scored based on the following six criteria: (i) percent of lung affected: 0-no lesions in lung, 1-up to 25% of lung involved, 2-up to 50 % of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. (ii) primary lesions: 0-no primary lesions present, 1-a single primary lesion, 2-two or more primary lesions, multi-focal, 3-two or more primary lesions, multifocal to coalescing, 4-multiple primary lesions, coalescing and extensive. (iii) secondary lesions: 0-no secondary lesions present, 1-up to 25% of lung involved, 2-up to 50% of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. Necrosis (iv), mineralization (v) and fibrosis (vi) are scored based on severity as follows: 0-none, 1-minimal, 2-mild, 3-moderate, 4-marked. The subcategory scores were added for the final total score for each organ (Range: 0-24). The spleen lesions were scored based on four categories; (i) percentage involvement (same scale as

lungs), (ii) degree of necrosis, (iii) fibrosis and (iv) mineralization. The total spleen score range is 0 to 16.

Statistical analysis

The statistical differences of lesion/necrosis scores or immunostaining scores among different time points were analyzed using non-parametric Kruskal-Wallis test. The specific statistical differences between two individual group scores were analyzed using non-parametric Mann-Whitney test. The statistical differences between numbers of bacilli, antioxidant levels, total glutathione levels and GSH/GSSG ratio were analyzed using two-way ANOVA and non-paired student t test. Statistical analysis of correlation was performed for the groups discussed in the results. All of the statistical tests were done using the Prism software (version 4.03, Graph Pad, La Jolla, CA).

Results

Presence of malondialdehyde (MDA) in infected guinea pig lungs

MDA is one of the major aldehyde byproducts of lipid peroxidation during oxidative stress conditions. During free radical generation, MDA forms numerous protein adducts that can be specifically detected by immunohistochemistry. There was a statistically significant ($p < 0.05$) increase in MDA expression in *M. tuberculosis*-infected guinea pig lungs beginning on day 20 of infection that progressed between days 30 and 60 (Figure 4.1A). Lungs from *M. tuberculosis*-infected guinea pigs on day 5 of infection and non-infected animals showed minimal or no immunostaining for MDA. The progressive increase in the MDA immunostaining occurred predominantly within the

primary granulomas (Figure 4.1B). Overall immunostaining scores represent the sum total scores for both primary lesion and primary lesion-free (PLF) lung. The staining was comparatively unchanged within the PLF lung at different time points (Figure 4.1C). The cell types that showed intense MDA staining included macrophages, vascular endothelial cells (Figures 4.1D and E) and granulocytes.

Systemic antioxidant capacity

The total antioxidant capacity of the serum was determined using an assay based on the ability of serum antioxidants to quench a radical cation (soluble chromogen) production when ferryl myoglobin reacts with an enzyme substrate. Total antioxidant capacity of the serum samples collected from mock-vaccinated and BCG vaccinated-*M. tuberculosis*-infected guinea pigs on days 15, 30, 60 and 90 of infection are illustrated in Figure 4.2. The serum total antioxidant capacity in *M. tuberculosis*-infected guinea pigs mock-vaccinated with saline ranged from undetectable levels as early as 15 days of infection to 0.06 ± 0.06 mM whereas the total antioxidant levels in the BCG vaccinated-*M. tuberculosis* infected animals were four to five folds higher ranging from 0.20 ± 0.06 mM to 0.27 ± 0.04 mM. The antioxidant capacity of naïve un-infected guinea pigs were closer to BCG vaccinated guinea pig values at 0.211 ± 0.042 mM (n=7).

Serum glutathione levels

Glutathione is a thiol tripeptide antioxidant that is among the most critical mammalian antioxidant molecules present in a reduced form (GSH) and oxidized form (GSSG) within all mammalian cells (Biswas & Rahman 2009). *M. tuberculosis*-infection

in guinea pigs resulted in a significant reduction in the whole blood “reduced glutathione (GSH)” levels on days 30 and 60 after infection compared to the naive animals (Figure 4.3A). The “oxidized glutathione” levels did not change significantly throughout the course of infection (Figure 4.3A). The progressive decrease in the antioxidant status of whole blood due to glutathione between days 30 and 60 of infection was expressed as a ratio between reduced and oxidized glutathione (GSH/GSSG) (Figure 4.3B).

Glutathione expression in lung lesions

To estimate the total glutathione expression in lung lesions from *M. tuberculosis*-infected guinea pigs by immunohistochemistry using an antibody against glutathione. There was a statistically significant ($p < 0.05$) increase in expression of glutathione in lung lesions from *M. tuberculosis*-infected guinea pig on days 30 and 60 (Figure 4.4A). Similar to MDA expression, the increase in glutathione immunostaining occurred predominantly within the primary granulomas (Figure 4.4B) and less so in the PLF lung at different time points (Figure 4.4C). The cellular expression pattern of glutathione was similar to that of MDA (Figures 4.4D and E).

Nrf2 expression in lungs

Nrf2 is a crucial redox sensitive transcription factor that controls expression of several antioxidant enzymes including NQO1 and GCS. Nrf2 immunostaining demonstrated an increase in overall expression in *M. tuberculosis*-infected guinea pig lung lesions as the infection progressed (Figures 4.5A through 4.5E). Predominant Nrf2 staining was present within the primary granulomas on days 30 and 60 (Figures 4.5C and

4.5D). Closer examination using higher magnification revealed roughly equal cytoplasmic and nuclear expression of Nrf2 throughout the lungs early in the infection (days 5 and 15) and in less-affected PLF regions of lungs late in the infection (days 30 and 60) (Figures 4.5G and 4.5H). In contrast, within epithelioid macrophages associated with primary lesions, cytoplasmic expression of Nrf2 was predominate with minimal nuclear staining ($p < 0.05$) as early as day 20 which was pronounced by day 30 and 60 (Figures 4.5F and 4.5I).

Nrf2-regulated antioxidant protein expression

The lack of Nrf2 nuclear translocation suggesting a defect in Nrf2-mediated antioxidant defenses was confirmed by evaluating the expression of the Nrf2-regulated expression of NQO1 and GCS. NQO1 expression decreased as the infection progressed in guinea pig lungs (Figure 4.6A) which inversely correlated with increased Nrf2 cytoplasmic expression over time ($r = -0.96$). Throughout the infection, predominant staining of NQO1 was localized within the PLF lungs where Nrf2 expression was highest compared to the primary granulomas (Figures 4.6C). The primary granulomas on day 20, 30 and 60 showed minimal NQO1 expression (Figures 4.6B and 4.6E). Airway epithelium (Figure 4.6D), vascular endothelium and alveolar macrophages were the cells that consistently expressed NQO1 in the early stages of infection (days 5, 15 and 20). As the disease progressed, only minimal immunostaining of these cells was seen (Figure 4.6E).

The expression of GCS was less pronounced than NQO1 in the lungs of infected guinea pigs compared to non-infected in naïve guinea pig lungs. However, no difference

in GCS immunostaining was seen among days 5 through 60. Also, no significant difference in expression levels of GCS was observed between primary granuloma and PLF lungs (data not shown).

Effect of NAC administration during tuberculosis

As NAC has been shown to induce Nrf2-mediated antioxidant defenses, *M. tuberculosis*-infected guinea pigs were treated with NAC in an attempt to reverse the adverse effects of oxidative stress (Chan et al 2001). The daily administration of NAC resulted in nearly one log reduction in the number of bacilli in the spleen on day 30 which correlated with a significant decrease in lesion burden (4.7C) which was less but still statically significant by day 60 (Figures 4.7A and 4.7B). No significant differences in the numbers of bacilli were observed between control and NAC-treated groups on day 30 and 60 in lungs and peribronchial lymph node (PBLN) (Figures 4.7A and 4.7B).

A statistically significant reduction in overall spleen lesion scores ($p < 0.01$) in NAC-treated animals was observed on day 30 of infection (Figure 4.7C). Moreover, statistically significant reduction in lesion necrosis scores was seen in NAC-treated guinea pig lungs ($p < 0.05$) and spleen ($p < 0.01$) compared to the control groups on day 30 of the infection (Figures 4.7D). The decrease in lesion burden and extent of necrosis in the lungs and spleen of NAC-treated animals are depicted in Figure 4.8.

There was a statistically significant increase in nuclear expression of Nrf2 ($p < 0.05$) within lung primary granulomas on days 30 and 60 in the NAC-treated group compared to mock-treated control groups (Figures 4.9A and 4.9B). Moreover there was a statistically significant increase in NQO1 expression ($p < 0.05$) within primary granulomas

on days 30 and 60 in NAC-treated guinea pig compared to the mock-treated control groups (Figures 4.9C).

An increase, albeit not-statistically significant, in serum reduced glutathione (GSH) was seen in NAC-treated animals compared to the mock-treated control group on day 30 which increased significantly on day 60 of infection ($p < 0.05$) (Figures 4.9D and 4.9E). No significant differences were noted between oxidized glutathione (GSSG) levels in control and NAC-treated animals throughout the course of infection. Similarly increases in the GSH/GSSG ratio was not statistically significant in NAC-treated animals compared to the control group on day 30 and but increased significantly by day 60 of infection ($p < 0.005$) in NAC-treated animals (Figure 4.9F). The total antioxidant capacity of the serum was statistically significant increase ($p < 0.05$) in NAC-treated guinea pigs compared to mock-treated controls by day 60 of infection (Figure 4.9G).

Discussion

The clinical manifestations of tuberculosis in humans and animals are directly proportional to the severity and extent of pulmonary and extra-pulmonary inflammation (Basaraba 2008). In the early stages of *M. tuberculosis* infection in humans lesions are composed primarily of macrophages and granulocytes with fewer lymphocytes (Canetti 1955). Similar cell phenotypes are seen in the early stages of experimental *M. tuberculosis* infection in guinea pigs. The significance of these findings is that like in other species, macrophages and heterophils in guinea pigs are among the most potent generators of ROS during an inflammatory response (Basaraba et al 2007; Ordway et al 2008; Ordway et al 2007). In these studies, the development of lesion necrosis correlated

with an increased influx of heterophils in lesions that progressed to necrosis (Basaraba et al 2007). Besides co-localizing to sites of necrosis, we showed that inflammatory cells released their cytoplasmic contents including extracellular iron which also catalyzes free radical generation.

Our primary objective in this study was to establish the presence of oxidative stress conditions during tuberculosis and the association of oxidative stress with the progression of primary lesions to necrosis. As free radicals have extremely short half-lives and are difficult to measure directly, we evaluated the byproducts of ROS generation *in vivo* and measured the serum and tissue antioxidant capacity of *M. tuberculosis* infected guinea pigs over time. The oxidative stress marker, MDA, increased in lung lesions as *M. tuberculosis* infection in guinea pigs progressed. The increased MDA expression correlated significantly with the increase in the mean lesion scores over time (data not shown). Interestingly besides macrophages and granulocytes, vascular endothelial cells associated with lesions also showed prominent MDA staining as the infection progressed. The significance of this finding is that free radical mediated endothelial damage may contribute to the pathogenesis of lesion necrosis by promoting microvascular thrombosis leading to lesion ischemia.

The serum total antioxidant capacity was significantly depleted as early as day 15 of infection suggesting that oxidative stress conditions are prevalent even before lesion progression is advanced. BCG vaccination prior to aerosol challenge maintained the serum antioxidant capacity near the levels of un-infected control animals. However, the serum antioxidant capacity fell even in BCG-vaccinated animals by day 90 of the infection.

As glutathione is among the most important intracellular and extracellular antioxidants, we compared the total, reduced and oxidized glutathione levels in serum of *M. tuberculosis*-infected guinea pigs. Reduced glutathione (GSH) levels were markedly decreased as the infection progressed which was reflected by a significant decrease in the GSH/GSSG ratio. These data are consistent with the depletion of total antioxidant which is due in part to total and more specifically reduced serum glutathione levels. Despite the decrease in serum glutathione levels, overall glutathione expression (both GSH and GSSG) in lung lesions reflected the host response to oxidative stress as a consequence of *M. tuberculosis* infection. Since the anti-glutathione antibody used in this study does not distinguish between the reduced and oxidized forms of glutathione, the relative antioxidant capacity in lesions was not determined. The MDA expression in lung lesions combined with the decrease in serum total antioxidant capacity, and GSH/GSSG ratio, confirms the presence of oxidative stress conditions in experimental tuberculosis in the guinea pigs similar to what is seen in the naturally occurring human disease (Jack et al 1994; Kwiatkowska et al 1999).

Our second objective was to identify potential mechanisms to explain the failure to maintain adequate antioxidant defense mechanisms in *M. tuberculosis* infected guinea pigs. Since the redox sensitive transcription factor Nrf2 regulates the expression of several important antioxidant proteins and nuclear translocation is necessary for normal Nrf2 function, we evaluated the relative nuclear to cytoplasmic expression in lung lesions and PLF lung. Overall Nrf2 expression increased in primary lesions as the infection progressed in non-treated and non-vaccinated animals. This increased expression correlated significantly with the progression of disease that was reflected in the increased

lesion pathology scores. Despite the increase in cytoplasmic Nrf2 expression, there was a significant lack of nuclear staining in primary lesions compared to naïve or non-affected regions of lung parenchyma. The lack of nuclear expression of Nrf2 within primary granulomas also correlated with the lack of expression of the Nrf2-regulated antioxidant protein NQO1 within the primary granulomas. NQO1 expression was limited to the areas with Nrf2 nuclear expression (non-affected lung regions), and is deficient within primary granulomas. Another known Nrf2-regulated enzyme GCS did not follow a similar pattern. In fact, GCS had moderately high level of expression throughout the course of the infection with minimal change in expression levels between different time points and between primary granulomas and PLF lungs. This is consistent with the data that showed that GCS is also likely regulated by Nrf2-independent mechanisms (Cai et al 1997; Galloway & McLellan 1998).

A similar lack of nuclear translocation of Nrf2 has been previously reported in an *in-vitro* model of cigarette smoke-mediated oxidative stress condition. In the same study, treatment with an Nrf2-inducing drug, resveratrol, induced nuclear translocation of Nrf2 and subsequent quenching of cigarette smoke-induced release of free radicals (Kode et al 2008).

Even though BCG vaccination maintained the antioxidant capacity of the serum early in the infection, it failed to reverse the defective Nrf2 translocation and the deficient NQO1 expression (data not shown). These data support the notion that despite conferring systemic protection against the disseminated disease, BCG vaccination fails to control lung infection in the guinea pig which is consistent with the eventual progressive disease resulting in death in BCG-vaccinated guinea pigs.

Therapeutic intervention with NAC aimed at reversing defective Nrf2-mediated antioxidant defense mechanism resulted in a decrease in pulmonary and extra-pulmonary lesion burden and more significantly decreased lesion necrosis. The improved disease outcome in NAC-treated animals was associated with increased Nrf2 nuclear staining, granuloma NQO1 expression, increased glutathione (reduced form) levels and improved serum antioxidant capacity compared to the mock-treated controls. NAC, being a carrier of cysteine molecule that is needed for glutathione synthesis along with its ability to induce Nrf2 activation, is likely to play both roles in combating oxidative stress conditions during tuberculosis (Chen et al 2004).

In summary, we have provided evidence to implicate oxidative stress during the progression of experimental tuberculosis in guinea pigs and that the oxidative stress contributes to the progression of primary lesion necrosis. Considering the importance of lesion necrosis in tuberculosis pathogenesis, supplementing with antioxidant drugs like NAC that reduce the severity of lesion necrosis could be beneficial as an adjunct to conventional anti-tuberculosis drug therapy.

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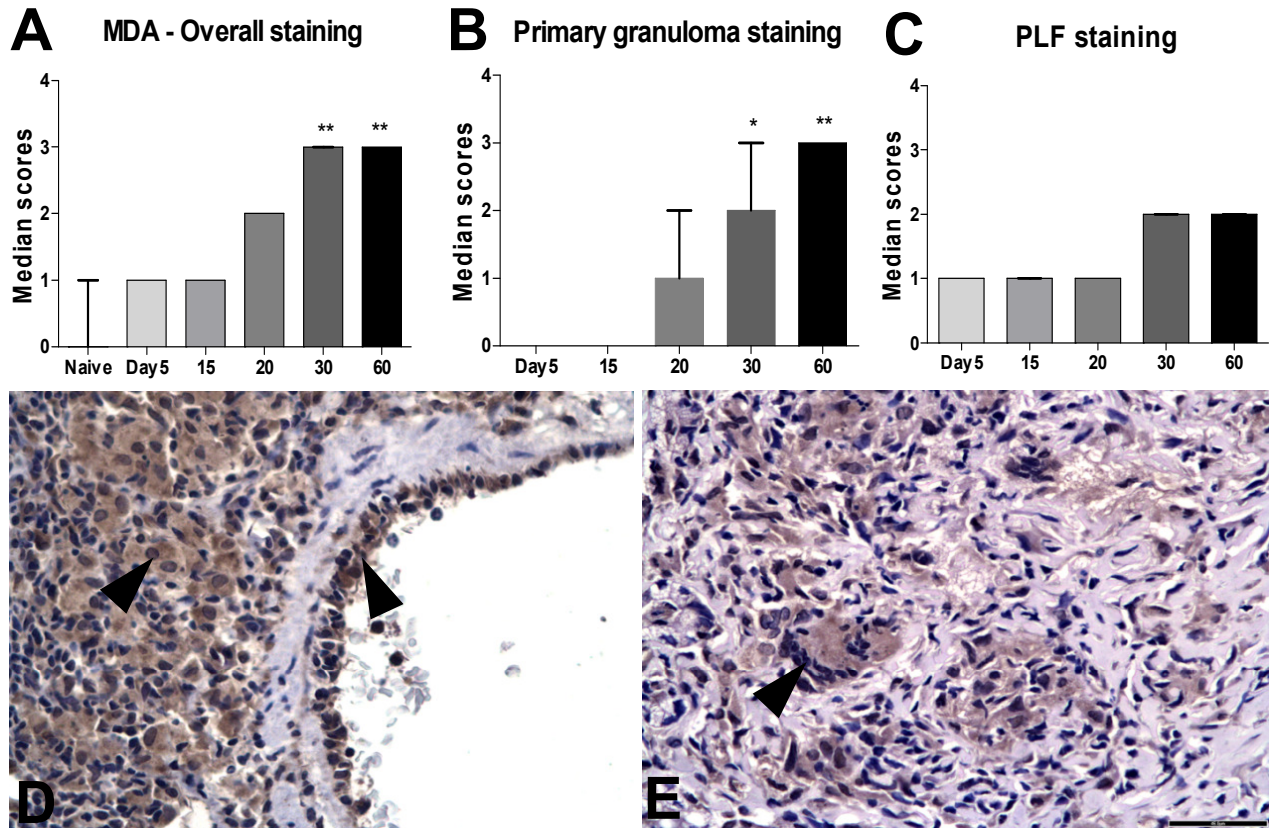


Figure 4.1 The graphs A, B and C represent MDA immunostaining scores within overall, primary granuloma and primary lesion free (PLF) lung areas respectively at different time points of Mtb H37Rv infection (median + range, n=5). The stars denote statistically significant increase compared to the naive animals (* = $p < 0.05$ and ** = $p < 0.01$). The photomicrographs D and E represent immunostaining of MDA in Mtb-infected guinea pig lungs from day 30 and day 60 of infection respectively (arrowheads show intracellular staining within macrophages and vascular endothelial cells).

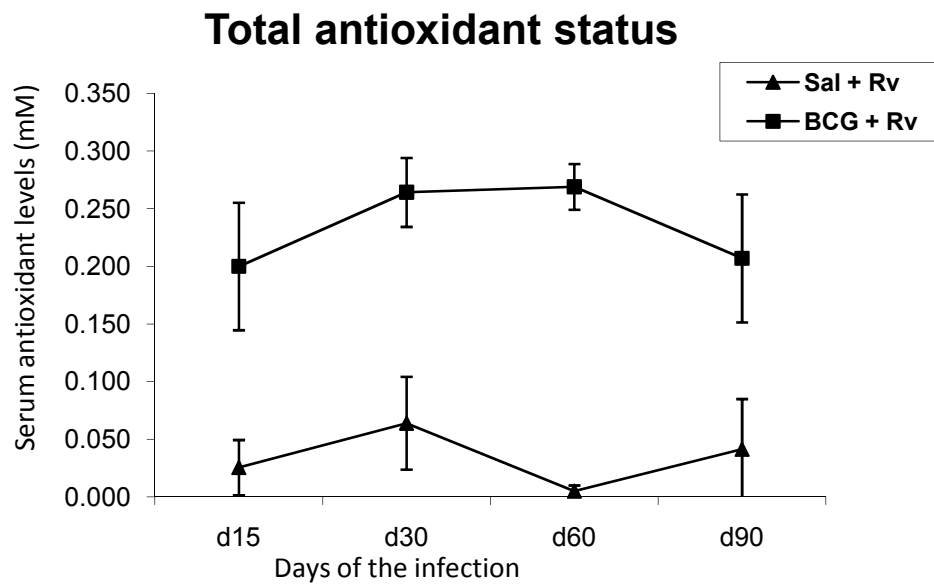


Figure 4.2 The graph illustrates systemic mean that total antioxidant levels in serum of Mtb-infected guinea pigs that are either saline vaccinated (control) or BCG vaccinated (n=5). The mean total antioxidant levels in serum of naïve in guinea pigs is 0.211 ± 0.042 mM (n=5).

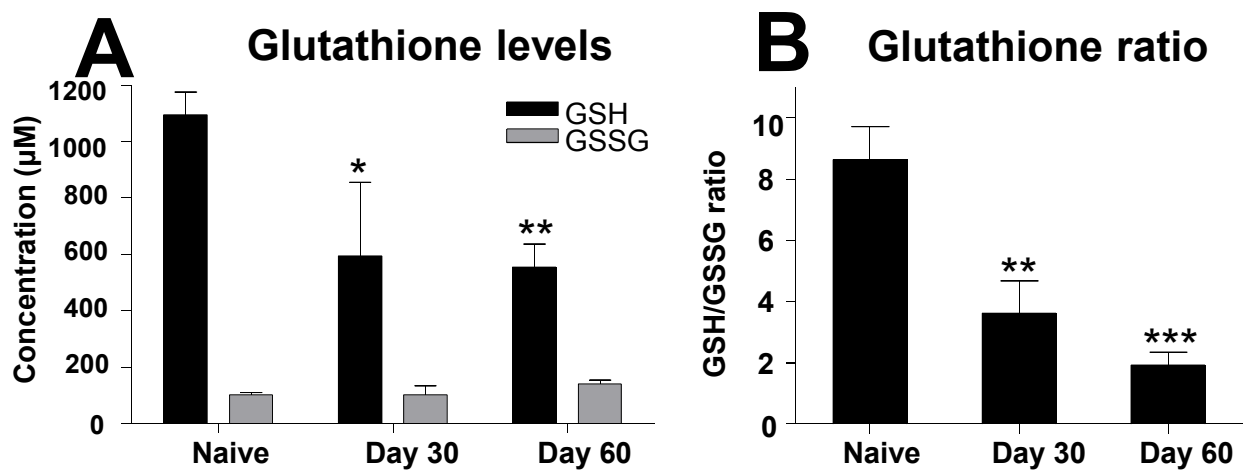


Figure 4.3 The graph A denotes the systemic levels of reduced and oxidized glutathione (GSH and GSSG respectively) in the blood of naive and Mtb-infected guinea pigs on days 30 and 60 after the infection. The graph B illustrates the ratio between reduced and oxidized glutathione in serum the same animals. Stars represent statistically significant decrease compared to the naive animals (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)

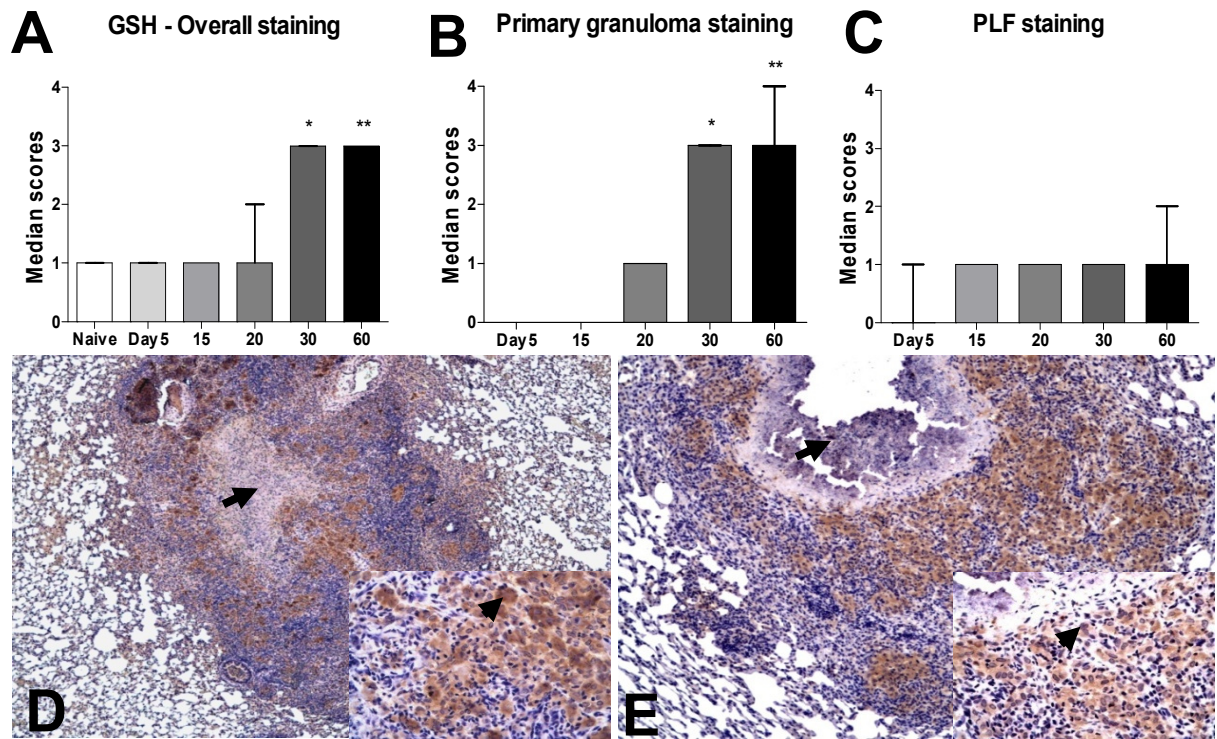


Figure 4.4 The graphs A, B and C represent glutathione immunostaining scores (median + range, n=5) within overall, primary granuloma and primary lesion free (PLF) lung areas respectively at different time points (* = $p < 0.05$ and ** = $p < 0.01$ when compared to the naive animals). Figures D and E illustrate predominant glutathione staining in the primary granulomas (arrows) with necrotic core on day 30 (non-mineralized core) and on day 60 (mineralized core). Inserts show the intracellular staining within the macrophages (200x, arrowheads).

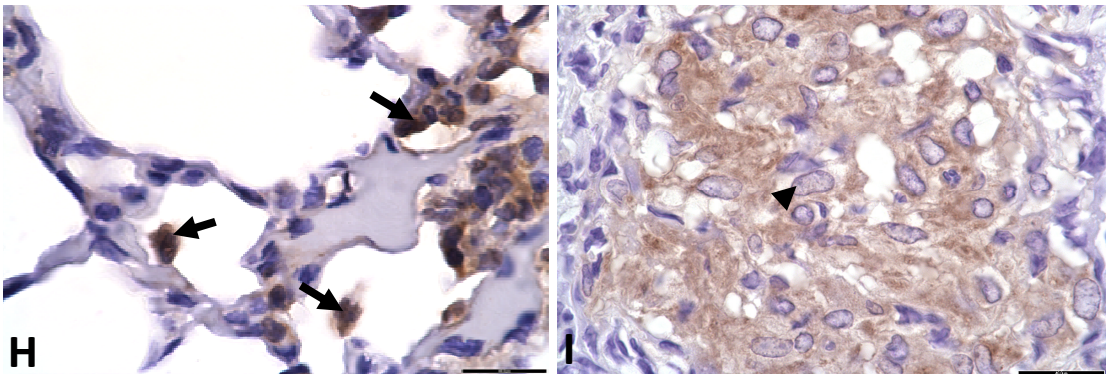
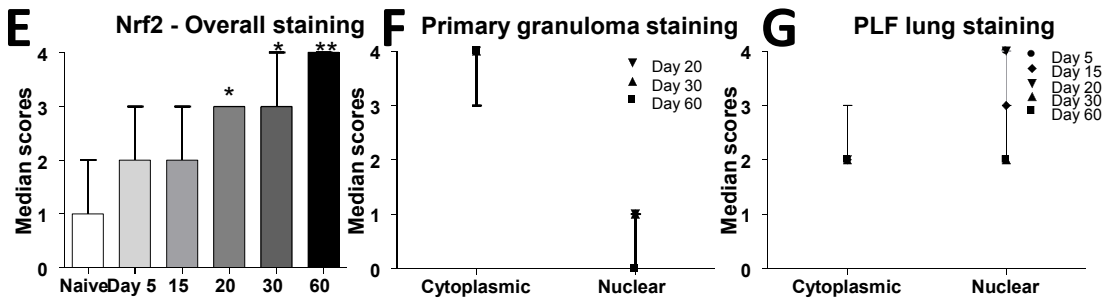
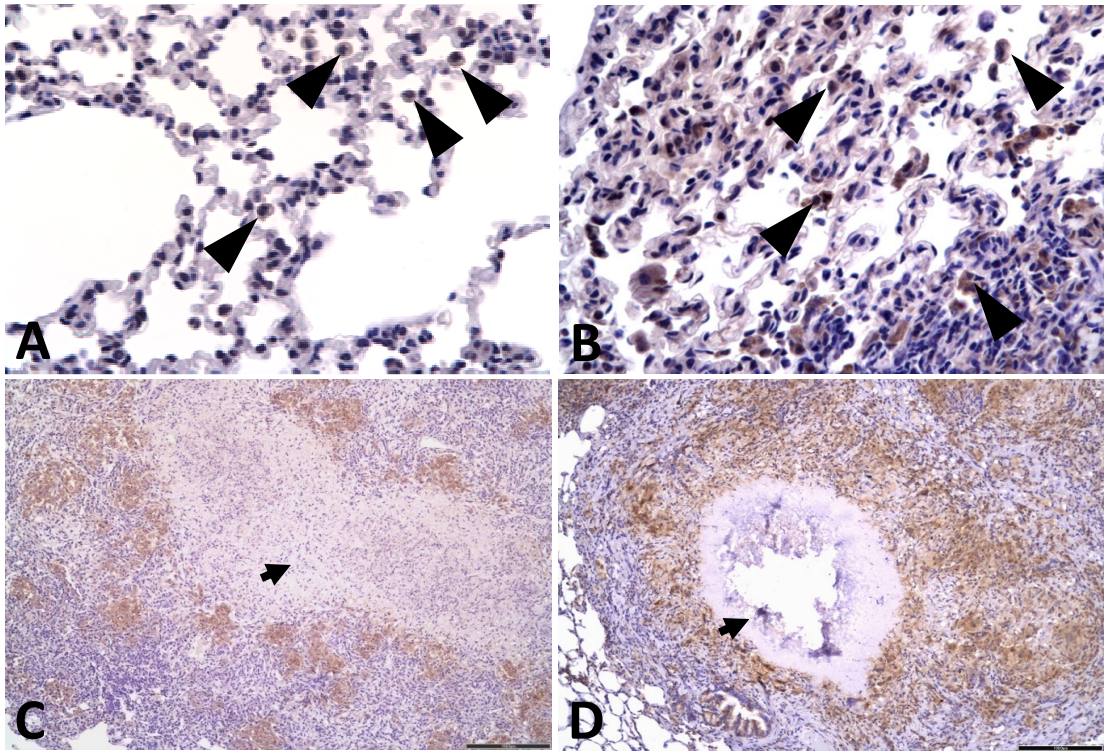


Figure 4.5 The photomicrographs A, B, C and D represent Nrf2 immunostaining within Mtb-infected lungs from day 0, 20, 30 and 60 respectively. Arrowheads (in A&B) show intracellular staining within macrophages. Arrows (in C & D) show primary granulomas with necrotic core demonstrating Nrf2 staining. The graph E represents Nrf2 overall lung immunostaining scores at different time points of infection. The bars represent median values (+ range) for each group (n=5). The stars denote statistically significant increase compared to the naive animals (* = $p < 0.05$ and ** = $p < 0.01$). The graphs F and G represent median cytoplasmic and nuclear staining scores (+ range) at different time points within primary granuloma and PLF lung areas respectively. The photomicrographs H and I represent immunostaining of Nrf2 in PLF lung (arrows show both intracytoplasmic and nuclear staining within macrophages) and primary granuloma (arrowhead shows predominantly intracytoplasmic but no nuclear staining of Nrf2 in macrophages) of a same animal on day 30 respectively.

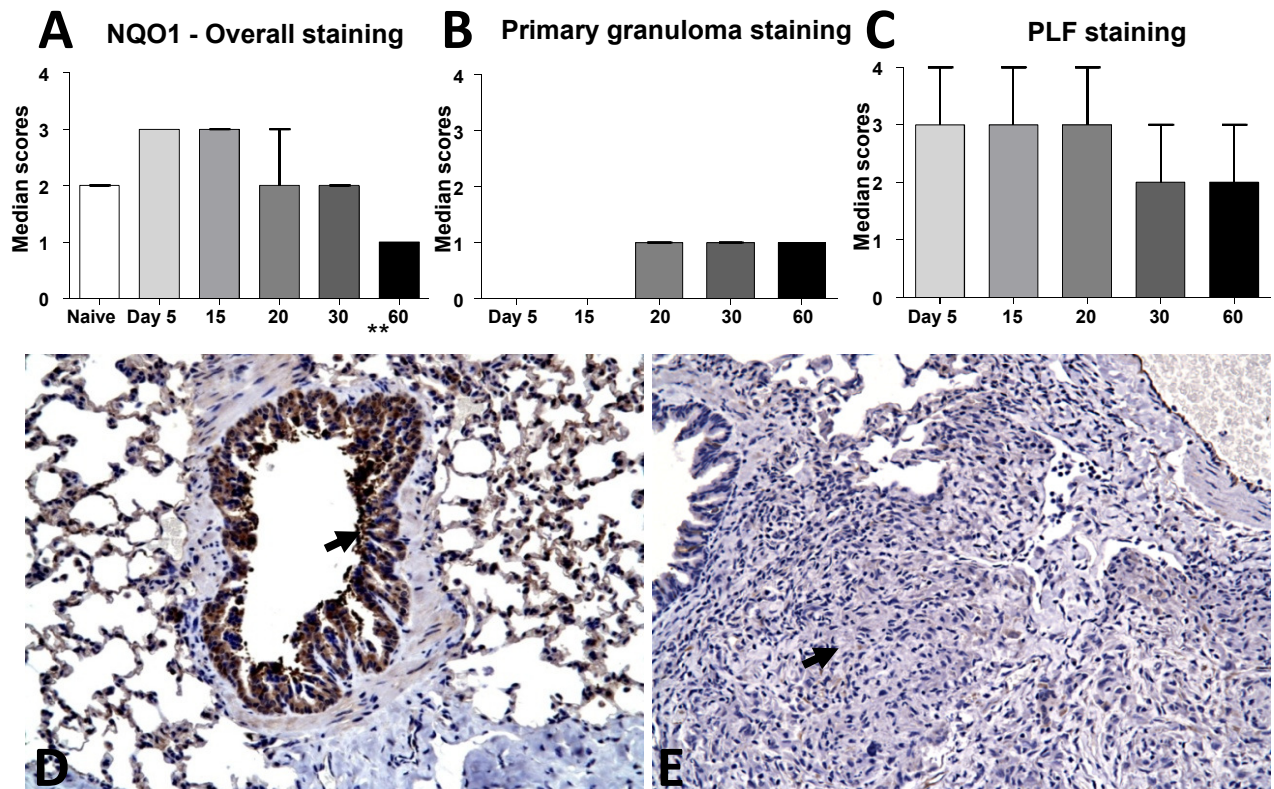


Figure 4.6 The graphs A, B and C represent NQO1 immunostaining scores within overall, primary granuloma and primary lesion free (PLF) lung areas respectively at different time points. The bars represent median values (+ range) for each group (n=5). The photomicrographs D and E represent immunostaining of NQO1 in Mtb-infected guinea pig lungs on day 5 (arrow shows strong immunoreactivity within airway epithelial cells) and day 30 (arrow points to a granuloma with no immunoreactivity) respectively.

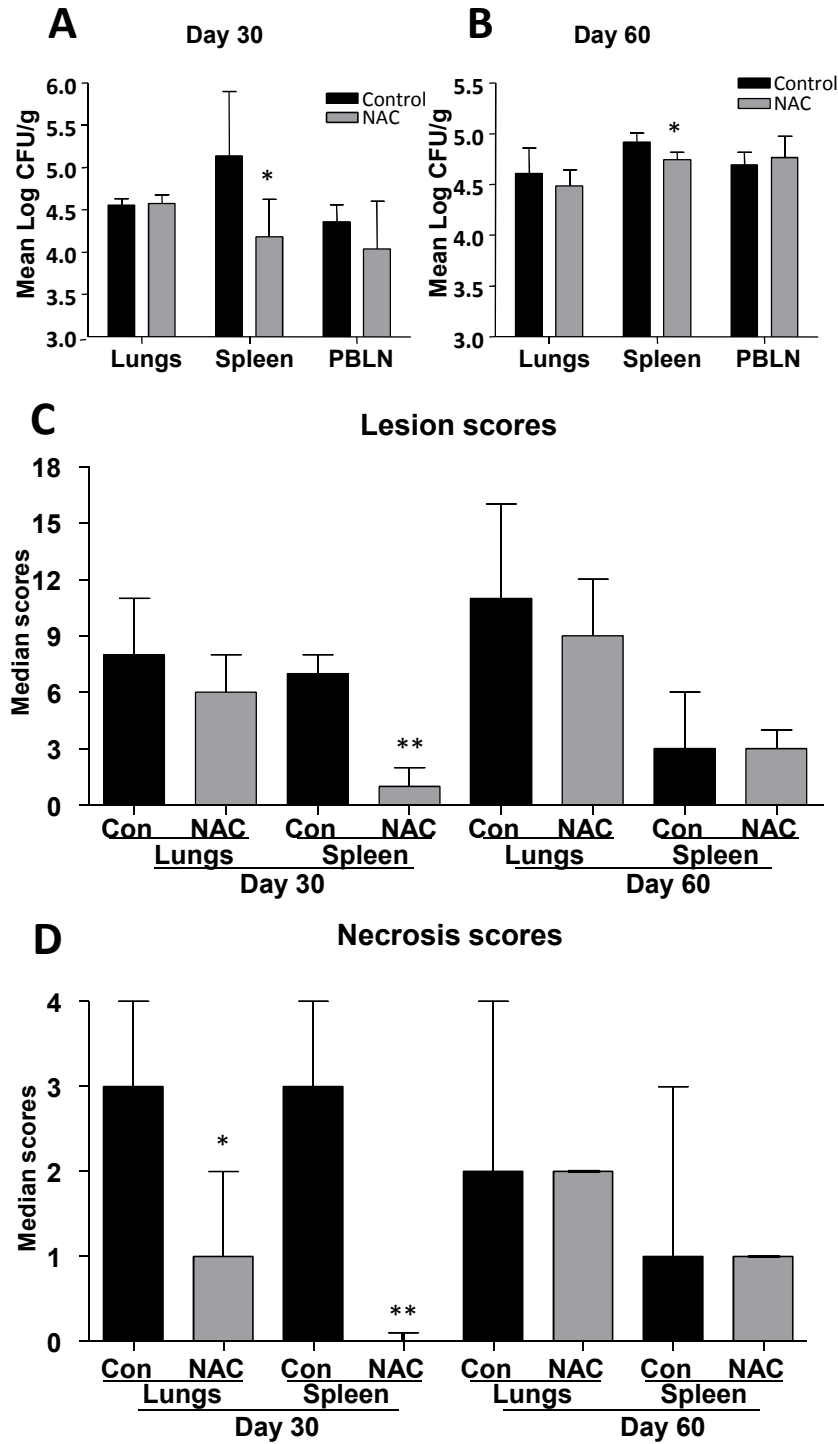


Figure 4.7 The graphs A and B represent mean numbers of bacilli in different organs from control and NAC treated guinea pigs on day 30 and 60 respectively (mean + SD, n=5). The graphs C and D represent overall lesion and necrosis scores (median + range) respectively of lungs and spleen from control and NAC treated animals on days 30 and 60 (n=5). Statistically significant change, if present, is indicated with stars (** = p<0.01).

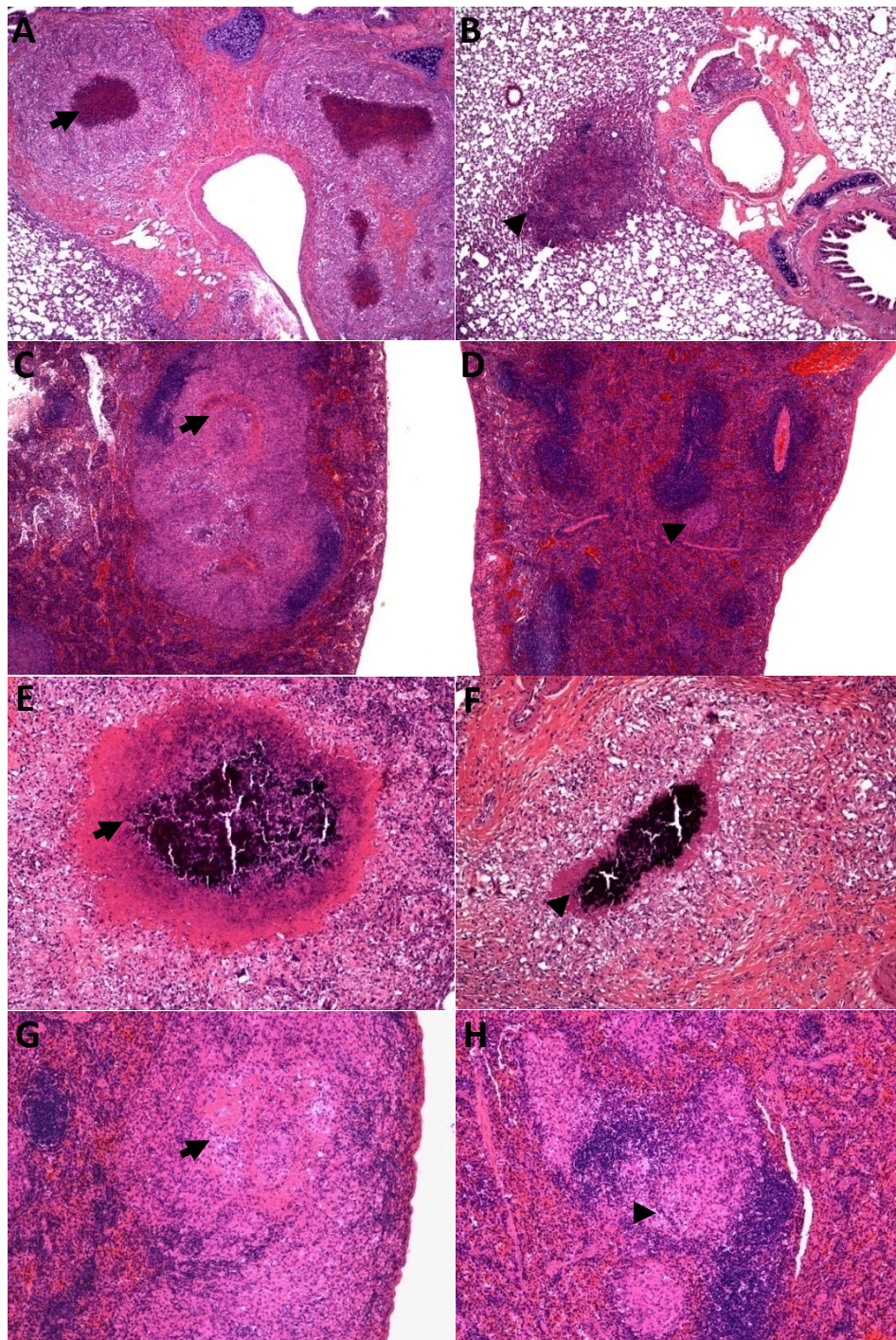


Figure 4.8 The photomicrographs A, B, C and D represent lesion pathology in the lungs (A & B) and spleen (C & D) on day 30 in control (A & C) and NAC (B & D) treated animals. The arrows in A and C (controls) show a large core of necrosis and the arrowheads in B and C (NAC treated) point to granulomas with minimal to no necrosis. The photomicrographs E, F, G and H represent the lesion burden in the lungs (E & F) and spleen (G & H) on day 60 in control (E & G) and NAC (F & H) treated animals. The arrows in E and G (controls) show a large area of necrosis and the arrowheads in F and H (NAC treated) show the granulomas with smaller area of necrosis.

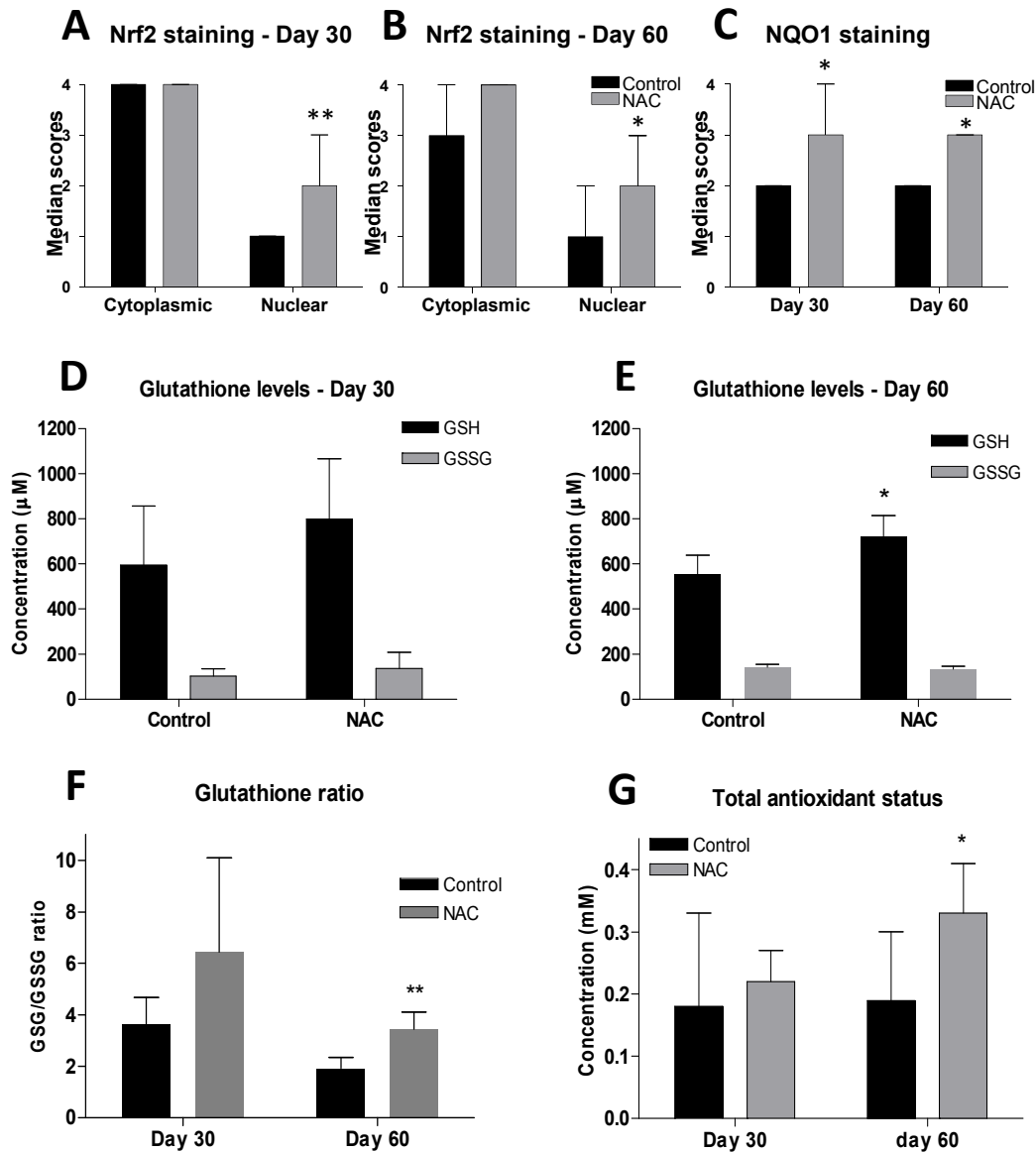


Figure 4.9 The graphs A and B represent the cytoplasmic and nuclear Nrf2 staining scores within primary granulomas of animals treated with control or NAC on days 30 and 60 respectively (median + range, n=5). The graph C represents NQO1 staining within primary granulomas of animals treated with control or NAC on days 30 and 60 (median + range, n=5). The graphs D and E represent reduced and oxidized glutathione levels (GSH and GSSG) in control and NAC treated animals on days 30 and 60 respectively (mean+SD, n=5). The graph F represents GSH/GSSG ratio in the blood of control or NAC-treated guinea pigs on days 30 and 60. The graph G represents the systemic total antioxidant levels of serum from control and NAC treated guinea pigs on days 30 and 60 (mean+SD, n=5). The stars denote statistically significant increase in NAC-treated animals compared to the control animals (* = p<0.05 and ** = p<0.01).

Chapter Five

Accumulation of oxidized low-density lipoprotein during *Mycobacterium tuberculosis* infection in guinea pigs; potential role in lesion pathogenesis and bacilli replication

Abstract

Tuberculosis is a chronic infectious disease characterized by the progressive accumulation of mixed inflammatory cells including macrophages and granulocytes that are capable of reactive oxygen species (ROS) generation. We have shown recently that the progression to necrosis of primary lesions in *Mycobacterium tuberculosis* (*M. tuberculosis*)-infected guinea pigs, coincides with oxidative stress and depletion of host antioxidant defenses. Moreover, treatment of guinea pigs with the antioxidant N-acetylcysteine partially restores host antioxidant capacity and lessens the severity of lesion necrosis. In this study, we hypothesized that during *M. tuberculosis* infection, lipids are among the host macromolecules that are oxidized and as a consequence oxidized low-density lipoprotein (OxLDL) are taken up and stored in macrophages. Lipid-laden macrophages are a distinct morphological feature in lesions from experimental *M. tuberculosis* infections in animals and in lesions from naturally occurring disease in humans. Here we show that OxLDL levels are elevated in the serum and within primary lung lesions in *M. tuberculosis* infected guinea pigs. Elevated OxLDL levels in the serum and primary lung lesions correlated with the progression of disease and were coincident with increased expression of the major OxLDL uptake receptors

CD36 and LOX1 by macrophages. Vaccination of guinea pigs with *M. bovis* BCG (BCG) prior to aerosol infection abrogated the increase of OxLDL in serum and partially decreased the lesion expression of CD36, LOX1 and OxLDL by macrophages within lesions. The functional significance of elevated OxLDL levels was illustrated by the enhanced replication of bacilli in guinea pig alveolar macrophages treated with OxLDL *in vitro*. Overall, this study provides additional evidence for *in vivo* oxidative stress in *M. tuberculosis*-infected guinea pigs and a potential role of OxLDL in the pathogenesis of tuberculosis.

Introduction

It is estimated that one out of every third person worldwide is infected with *M. tuberculosis* the causative agent of human tuberculosis (WHO 2009). Because tuberculosis is a chronic inflammatory disease, generation of excessive reactive oxygen species (ROS) leading to a depletion of host antioxidant defenses, has been implicated in the disease pathogenesis. Markers of free radical damage such as malondialdehyde were shown to be elevated in the peripheral circulation of the human patients with active tuberculosis along with decreased serum levels of the antioxidants, vitamin C and E (Kwiatkowska et al 1999; Lamsal et al 2007). In a previous study, we provided evidence for *in vivo* oxidative tissue damage with a concurrent depletion of host antioxidant defenses in guinea pigs experimentally infected with virulent *M. tuberculosis* (see Chapter IV). We also showed that treatment of *M. tuberculosis* infected guinea pigs with the antioxidant drug N-acetylcysteine reduced systemic oxidative stress by restoring the serum total antioxidant capacity and reduced the severity of primary lesion necrosis.

These data lend further support that lesion necrosis is in part mediated by ROS which can be reversed therapeutically.

It has been known nearly for a century that tuberculosis lesions are rich in lipids (Virchow 1989) and more recently that host lipids may serve as an energy source for bacilli *in vivo*. Several studies have shown the importance of cholesterol and fatty acids in mycobacterial entry, growth and persistence (Gatfield & Pieters 2000; McKinney et al 2000; Pandey & Sasseti 2008; Yam et al 2009). Macrophages with numerous clear cytoplasmic vacuoles are a prominent feature of tuberculosis lesions in experimentally infected animals and in humans with naturally occurring disease (Ordway et al 2005; Rhoades et al 1997; Russell et al 2009). Recently lipid-laden macrophages have been suggested to be one potential site for the persistence of non-replicating bacilli. Infected lipid-laden macrophages had functional defects including decreased phagocytic and bactericidal activities which favor *M. tuberculosis* persistence (Peyron et al 2008). This further demonstrates the ability of *M. tuberculosis* to exploit the intracellular microenvironment for survival within macrophages.

Lipid-laden macrophages occur in a variety of inflammatory conditions including infections with *Chlamydia pneumoniae* among others and atherosclerosis (Galkina & Ley 2009; Kalayoglu & Byrne 1998). Vacuolated macrophages also referred to as “foam cells” are central to the pathogenesis of atherosclerosis where oxidative stress plays a major role. This led us to hypothesize that low-density lipoprotein (LDL) is among the host macromolecules that is oxidized during *M. tuberculosis* infections in guinea pigs and subsequently accumulates within macrophages that make up the pulmonary and extra-pulmonary inflammatory lesions. Unlike LDL which is taken up by specific macrophage

LDL receptors (LDL-R), the oxidized form known as oxidized LDL (OxLDL) is taken up by the scavenger receptors SR-A, SR-B1, CD36 and lectin-like oxidized low-density lipoprotein receptor-1 (LOX1) during foam cell formation in atherosclerotic lesions (Esterbauer & Ramos 1996). Scavenger receptors recognize and facilitate the uptake of macromolecules with negative charges including modified LDL. Among the multiple OxLDL receptors that have been characterized, CD36 and LOX1 are the most dominant with both being involved in the pathogenesis of cardiovascular disease and atherosclerosis (Febbraio et al 2000; Mehta et al 2006; Mehta et al 2007; Podrez et al 2000). Moreover, CD36 has been shown to be involved in the uptake of *M. tuberculosis* by macrophages and non-phagocytic cells (Philips et al 2005).

In this study, we demonstrate an elevation of OxLDL levels in serum and within macrophages that comprise granulomatous inflammatory lesions in *M. tuberculosis*-infected guinea pigs, a model that develops lesions similar to what is seen in naturally occurring tuberculosis in humans. We also show by immunohistochemistry that the expression of the OxLDL receptors, CD36 and LOX1 are increased in the lung lesions during experimental *M. tuberculosis* infection. Finally, we show that vaccination of guinea pigs with *M. bovis* BCG prior to infection alleviates oxidative stress conditions including the accumulation of OxLDL and OxLDL receptor expression. Moreover, we show the accumulation of OxLDL by guinea pig alveolar macrophages enhances the intracellular growth of *M. tuberculosis in vitro*. These data suggest that oxidative stress during *M. tuberculosis* infection favors bacilli through the accumulation of host lipids in macrophages that harbor intracellular bacilli.

Methods

Aerosol infection

M. tuberculosis H37Rv strain (TMC#102; Trudeau Institute, Saranac Lake, NY) collected and frozen at mid-log phase of growth in Proskauer-Beck liquid medium containing 0.05% Tween 80 was used for infection. A thawed aliquot of *M. tuberculosis* was diluted in sterile water to 10^6 CFU/ml for a total working stock volume of 20 ml. Guinea pigs, approximately 9 months old, were purchased from Charles River Laboratories (North Wilmington, MA, USA) were divided into multiple groups and aerosolized using a Madison infection chamber (University of Wisconsin Machine Shop, Madison, WI) with a starting volume of 15 ml of working stock.

BCG vaccination

Guinea pigs were vaccinated with 1×10^4 *Mycobacterium bovis* (BCG, strain Pasteur) or mock vaccinated with saline, intra-dermally 4 weeks prior to aerosol exposure to *M. tuberculosis*. *M. bovis* BCG was grown in Proskauer-Beck similar to *M. tuberculosis* and frozen at -80°C until used for infection.

Euthanasia and sample collection

At intervals of 5, 15, 20, 30, 60 or 90 days post-infection, guinea pigs were euthanized by an overdose (1 ml per 0.75 kg body weight) of sodium pentobarbital (Sleepaway; Fort Dodge Laboratories Inc.) by intraperitoneal injection. Following euthanasia, at each time point, the left pulmonary lobes were infused *in situ* with 5 ml of 4% paraformaldehyde for 48 hours and stored in 70% ethanol. At the time of processing,

all tissues were embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin (H&E).

Evaluation of serum OxLDL levels

Systemic oxidized LDL levels were measured in serum collected at days 15, 30, 60 and 90 days after infection from guinea pigs that are either saline or BCG-vaccinated four weeks prior to *M. tuberculosis* infection using a commercially available mouse anti-human copper oxidized LDL (mAb-4E6)-based competitive ELISA (Merckodia, Winston Salem NC). The mAb-4E6 (kindly provided by Merckodia) was validated by using the antibody for immunohistochemical staining on guinea pig lung tissues and antigen excess blocking (with human copper-oxidized LDL) to confirm antibody specificity in guinea pig tissues.

Immunohistochemistry

Approximately 5 μm paraffin lung sections from infected guinea pigs were collected on positively charged glass slides, deparaffinized, rehydrated and antigen retrieved by incubating in Target Retrieval solution, pH 6.0 (DAKO, Carpinteria, CA) for 25 min at 90° C, followed by a 20 min cooling period at room temperature. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide treatment for 15 min. The slides were subjected to two blocking steps after rinsing once in Tris buffered saline with 1% Tween-20 (TTBS): (i) 15 min incubation with 0.15 mM glycine in PBS, and (ii) 30 min incubation with 1% normal horse serum with a rinse in TTBS in between. The slides were then incubated with rabbit polyclonal antibody to human CD36,

LOX1 (Santa Cruz Biotechnology, Santa Cruz, CA) and copper-oxidized LDL (Abcam, Cambridge, MA), or respective non-immune rabbit serum at a 1:100 dilution in blocking buffer followed by several rinses in TTBS followed by 30 min incubation with biotinylated goat-anti rabbit-IgG (Vector Laboratories). Bound antibody was visualized using the Avidin-Biotin system (Vectastain; Vector Laboratories) and diaminobenzidine substrate (Dako; Carpinteria, CA). Sections were counterstained with Meyer's hematoxylin (Scytek Laboratories; Logan, Utah), mounted with coverslips, and examined on an Olympus BX41 light microscope. For OxLDL immunocytochemistry, alveolar macrophages were collected at necropsy, from *M. tuberculosis*-infected guinea pigs at different time points by bronchioalveolar lavage as described later and placed onto charged glass slides by centrifugation cytopsin (Thermo Scientific, Waltham, MA). Slides were air dried and fixed with 100% ice cold methanol and processed as described earlier starting with the primary antibody incubation.

The use of anti-human antibodies on guinea pig tissues was validated by blocking the primary antibodies with the respective purified antigens (CD36 - Affinity Bioreagents, Golden CO; LOX1 - Santa Cruz Biotechnology, Santa Cruz, CA; copper oxidized LDL - Biomedical Technologies, Stoughton, MA) at a concentration ratio of 1:5 before incubation in lieu of the primary antibodies. Antibody specificity was confirmed by the effective blocking of anti-human CD36, LOX1 and OxLDL antibody binding to guinea pig tissues with the respective purified proteins. Photomicrographs were acquired with an Olympus DP70 camera and associated computer software. Microscopic lesions stained with H & E and sections stained by immunohistochemistry were scored by the reviewer blinded to the treatment groups.

Lesion analysis

To evaluate the progression of disease over time, a previously described histological grading system was used (Palanisamy et al 2008). Lung sections were scored based on the following six criteria: (i) percent of lung affected were ranked at low magnification by an estimation of the percent of the lung affected as follows: 0-no lesions in lung, 1-up to 25% of lung involved, 2-up to 50 % of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. (ii) primary lesions: 0-no primary lesions present, 1-a single primary lesion, 2-two or more primary lesions, multi-focal, 3-two or more primary lesions, multifocal to coalescing, 4-multiple primary lesions, coalescing and extensive. (iii) secondary lesions: 0-no secondary lesions present, 1-up to 25% of lung involved, 2-up to 50% of lung involved, 3-up to 75% of lung involved, 4-above 75% of lung involved. Necrosis (iv), mineralization (v) and fibrosis (vi) were scored based on severity as follows: 0-none, 1-minimal, 2-mild, 3-moderate, 4-marked. Subcategory scores were added for the final total score for each organ (Range: 0-24).

Immunohistochemical scoring

For immunohistochemical scoring purposes, the total lung area was divided into primary lesions (lesions with a central core of necrosis) and primary lesion free (PLF) lungs. Within these regions the overall immunohistochemical scoring was determined based on extent of staining (0-none; 1-less than 25%; 2-26-50%; 3-51-75%; 4-more than 75%) and staining intensity (0-none: 1-mild; 2-moderate; 3-marked; 4-extensive). The resultant total overall scores were converted to a four-point scale and reported here.

Isolation of alveolar macrophages

Guinea pig alveolar macrophages were collected from naive, non-infected guinea pigs by repeated infusion and aspiration of 10 ml of 1x Hank's balanced salt solution (MP Biomedicals, Solon, OH) three times. Cells were pelleted by centrifugation and washed twice with 1x PBS and the cell numbers and viability determined using a hemocytometer in combination with Trypan blue dye exclusion (Gibco Life Technologies, Grand Island, NY). The numbers of viable macrophages were adjusted to 2×10^5 cells/ml in a serum free media (Aim-V; Gibco, Grand Island, NY) containing bovine serum albumin, L-glutamine, streptomycin sulfate, and gentamicin sulfate.

Cell culture

Cells (2×10^5 / well) were allowed to adhere for 1 hour to each well of a 24 well flat-bottom plate (Becton Dickinson Labware, Franklin Lakes, NJ). Media and non-adherent cells were removed and the monolayers washed with 1x PBS. Representative wells were stained with Diff-Quik staining (Dade Behring, Newark, DE) and the staining revealed over 95% cells to have the morphologic characteristics of alveolar macrophages. Adherent alveolar macrophages that were treated with either non-oxidized low density lipoprotein or copper-oxidized low density lipoprotein (Biomedical Technologies, Stoughton, MA) at a dose of 25 $\mu\text{g/ml}$ then infected or mock-infected with *M. tuberculosis* H37Rv (MOI 5:1). At 4 hrs, 24 hrs, 3 days and 7 days after the infection, the adherent macrophages were washed and gently scraped from the surface and resuspended 1x PBS. The macrophages were then homogenized, plated on nutrient 7H11 agar plates

after serial dilution and *M. tuberculosis* numbers determine by three weeks of culture at 37°C and data expressed as colony-forming units.

Statistical analysis

The statistical differences of lesion or immunostaining scores among different time points were analyzed using non-parametric Kruskal-Wallis test. The specific statistical differences between two individual group scores were analyzed using non-parametric Mann-Whitney test. The statistical differences between serum OxLDL levels were analyzed using two-way ANOVA and non-paired student t test. Statistical analysis of correlation was performed for the groups discussed in the results. All of the statistical tests were done using the Prism software (version 4.03, Graph Pad, La Jolla, CA).

Results

Lung lesion burden during *M. tuberculosis* infection

The histopathologic changes within the lungs of *M. tuberculosis*-infected guinea pigs were quantified using a scoring system that correlates disease progression/lesion burden and immunohistochemical scores. Figure 5.1 shows data obtained from five animals each on days 5, 15, 20, 30 and 60 after infection. Statistically significant increase in lung lesion scores was observed as the infection progressed ($p < 0.001$).

Serum OxLDL levels

Serum OxLDL levels were determined in naïve guinea pigs or animals that were mock-vaccinated with saline or BCG-vaccinated prior to infection from 30 to 90 days.

Starting at day 30 of the infection, serum OxLDL levels increased in the mock-vaccinated animals (Figure 5.2.) and increased significantly on days 60 and 90 ($p < 0.05$) compared to the BCG-vaccinated animals. The increase in the serum OxLDL levels correlated with the progression of disease expressed by lesion scores ($r = 0.96$). The highest serum OxLDL levels were observed on day 60 of the infection. Although there was a decline in OxLDL levels from day 60 to day 90, the levels on day 90 were still higher than the basal levels observed in the naïve, non-infected animals. BCG vaccination enables animals to maintain OxLDL levels near immunologically naïve, non-infected animals throughout the infection.

OxLDL expression in lungs

Oxidized LDL, formed during oxidative stress conditions, is taken up and accumulates within the cytoplasm of macrophages (Febbraio et al 2001). Overall immunostaining for OxLDL which includes primary lesions and primary lesion-free (PLF) lung in *M. tuberculosis*-infected guinea pigs increased gradually (Figure 5.3A) as the infection progressed and like serum levels, correlated with disease progression ($r = 0.99$). The lungs from non-infected guinea pigs demonstrated minimal to no OxLDL expression in the normal lung parenchyma. Statistically significant increases in OxLDL staining was seen on days 30 and 60 of the infection ($p < 0.01$ for both) with most of the OxLDL staining being restricted to the primary granulomas (Figure 5.3B). There were no significant changes in the expression of OxLDL within the PLF lung regions which includes secondary lesions (without necrosis) and normal lung parenchyma. OxLDL expression was most prominent in lesion associated macrophages including those with

vacuolated cytoplasm (foam cells), airway epithelial cells, vascular endothelial cells and granulocytes.

In addition, the bronchioalveolar lavage cells (BAL) collected from *M. tuberculosis*-infected guinea pigs on days 30 and 60 after infection also expressed cytoplasmic OxLDL (Figures 5.4A and B respectively). The BAL cells comprised of predominantly alveolar macrophages intermixed with fewer numbers of heterophils (neutrophil equivalent) both of which demonstrated equal OxLDL staining pattern.

CD36 protein expression in lungs

Unlike other macrophage scavenger receptor types A-I and II (SR-AI/II) that recognize copper-oxidized and acetylated-LDL, CD36 can also recognize LDL modified by myeloperoxidase-hydrogen peroxide-nitrite system (MPO-OxLDL) which has more physiological relevance than A-I and II as a consequence of chronic inflammation (Podrez et al 1999). Overall CD36 expression in the lungs from *M. tuberculosis*-infected guinea pigs increased gradually as the infection progressed (Figure 5.5A). The increase in protein expression levels correlated ($r=0.99$) with the increase in the lung lesion scores. Increases in expression were statistically significant on days 30 and 60 after infection ($p<0.05$ and 0.01 respectively). Non-infected guinea pigs expressed minimal to no CD36 staining. The increase in the CD36 immunostaining occurred predominantly within the primary granulomas (Figure 5.5B). The staining was comparatively unchanged as reflected in the scores of the PLF lung at different time points (Figure 5.5C). The cell types that showed the most intense CD36 expression were macrophages including those with vacuolated cytoplasm, airway epithelial cells, endothelial cells and granulocytes.

LOX1 protein expression in lungs

LOX1 is another major receptor for oxidized LDL and is known to be transcriptionally up-regulated during various oxidative stress disease conditions such as atherosclerosis, diabetes mellitus and ischemia-reperfusion injury (Chen et al 2002). As tuberculosis is characterized by increased oxidative stress, we sought to evaluate the protein expression levels of LOX1 during *M. tuberculosis* infection in guinea pigs. Overall LOX1 protein levels in lungs during *M. tuberculosis* infection in guinea pigs increased significantly as the infection progressed (Figure 5.6A) and the increase in the protein staining levels correlated ($r=0.99$) with the increase in the lung lesion scores. Lungs from non-infected guinea pigs had minimal to no LOX1 expression by immunohistochemistry. This increase in LOX1 protein levels were statistically significant on days 30 and 60 after infection ($p<0.05$ and 0.01 respectively). Similar to CD36 expression, the increase in the LOX1 immunostaining occurred predominantly within the primary granulomas (Figure 5.6B). The staining was comparatively unchanged within the PLF lung areas at different time points (Figure 5.6C). The cellular expression pattern of LOX1 was similar to that of CD36.

Effects of BCG vaccination

As BCG vaccination delays the development and progression of the disease and prevents primary lesion necrosis (McMurray & Bloom 1994; Smith et al 1970) in *M. tuberculosis*-infected guinea pigs, we compared the protein expression characteristics of CD36, LOX1 and OxLDL in animals that were BCG-vaccinated prior to infection. All

three proteins demonstrated a similar trend of increasing protein levels, albeit less robust, compared to saline-vaccinated animals as the infection progressed (Figures 5.7A, B and C). Consistent with the delayed effect of BCG vaccination on disease progression, statistically significant increases in CD36, LOX1 and OxLDL expression were seen only on day 60 in all three groups compared to the naive non-infected animals ($p < 0.05$). On days 30 and 60, the staining of all three proteins was predominantly within primary granulomas compared to the PLF lung regions.

Enhanced bacterial growth in OxLDL-treated macrophages

To determine whether OxLDL accumulation in alveolar macrophages influenced the growth of *M. tuberculosis*, we treated primary guinea pig alveolar macrophages with purified OxLDL prior to *in vitro* infection. Macrophages were either treated with non-oxidized low density lipoprotein or copper-oxidized low density lipoprotein prior to *in vitro* infection. One group of macrophages did not receive any treatment prior to infection. Our results showed an approximately 10 fold ($p < 0.005$) increase in bacilli in OxLDL-treated macrophages at days 3 and 7 after the initial infection (Figure 5.8) compared to non-treated macrophages or those pre-treated with non-oxidized lipoproteins.

Discussion

Tuberculosis is a chronic inflammatory disease characterized by systemic oxidative stress and elevated markers of free radical damage in patients with active

tuberculosis (Kwiatkowska et al 1999; Lamsal et al 2007). Considering a variety of host macromolecules including proteins, lipids and nucleic acids that are susceptible to ROS mediated damage, we hypothesized that oxidatively modified LDL were among the products that accumulate during experimental *M. tuberculosis* infection in guinea pigs. In an earlier study, we showed that malondialdehyde (MDA), a byproduct of lipid peroxidation, was expressed by a variety of cell types early in *M. tuberculosis* infected guinea pigs. Further evidence of excessive ROS generation during infection was a marked depletion of the total antioxidant capacity of the serum and specifically the critical antioxidant tripeptide glutathione (see Chapter IV).

One mechanism by which the free radical generation might play a role in the pathogenesis of tuberculosis is by mediating oxidative modification of LDL via the myeloperoxidase-hydrogen peroxide-nitrite system (MPO-OxLDL) (Podrez et al 1999). Unlike LDL which is quickly degraded, and does not accumulate within cells, OxLDL is stored and accumulates within cells especially macrophages (Steinberg 2005). The lipid components of OxLDL include esterified and non-esterified cholesterol, phospholipids and triglycerides that make up approximately 80% of the total LDL content (McNamara et al 1996; Teng et al 1983). As cholesterol and other host lipids may serve as a carbon source for *M. tuberculosis* (Miner et al 2009), we asked whether these oxidized lipids account for the stored product associated with vacuolated macrophages *in vitro* and *in vivo*.

Our data builds on our previous study that showed that systemic and pulmonary oxidative stress is a prominent feature of experimental tuberculosis in guinea pigs. We demonstrated that not only does OxLDL accumulate in the serum and lesions of *M.*

tuberculosis-infected guinea pigs but the expression of the scavenger receptors CD36 and LOX1 also increased in cells that make up primary lesions with necrosis. Even though CD36 and LOX1 bind multiple ligands, the elevation of OxLDL levels during *M. tuberculosis* infection suggests they may play an important role in oxidized lipid uptake and accumulation. The increase in OxLDL expression in lung lesions was accompanied by a significant increase in serum levels. Overall, these findings lend support to our hypothesis that free radical mediated-oxidation of host LDL during *M. tuberculosis* infection results in uptake and accumulation of OxLDL by macrophages that express the scavenger receptors CD36 and LOX1.

A unique subpopulation of macrophages with abundant, vacuolated cytoplasm is a prominent feature of chronic inflammatory lesions like tuberculosis and atherosclerosis. We hypothesized that this morphological characteristic was due in part to OxLDL accumulation. These data indicate however that OxLDL expression was not just restricted to foamy macrophages since epithelioid and foamy cells had similar staining characteristics by immunohistochemistry. This discrepancy could reflect the degree and duration of the lipid accumulation over the course of the disease with foamy macrophages representing mature or more chronic accumulations. OxLDL staining was also observed in airway epithelial and vascular endothelial cells especially in the early time points. This observation may have important implications in ROS-mediated damage to other cell types during the early phase of disease.

Vaccination of guinea pigs with BCG prior to infection significantly delayed the increase in serum and lesion OxLDL levels seen in mock-vaccinated animals thus maintaining levels similar to non-infected animals in the early stages of infection.

However, BCG was less effective at preventing the local accumulation of OxLDL and CD36 and LOX1 receptor expression. These data are consistent with the ability of BCG vaccination to delay disease progression but its inability to prevent the ultimate death of animals from progressive pulmonary and extra-pulmonary disease (McMurray & Bloom 1994; Smith et al 1970).

The scavenger receptor CD36 may have several important functions in the pathogenesis of tuberculosis. Previous studies have shown that CD36 functions in the uptake of *M. tuberculosis* by macrophages (Philips et al 2005). Increased receptor expression by platelets has been shown to be pro-thrombotic in several mouse models of microvascular thrombosis (Podrez et al 2007). Because CD36 is an important receptor for the uptake of OxLDL in other diseases, the increased expression of CD36 and OxLDL in *M. tuberculosis* infected guinea pigs may reflect their importance in the pathogenesis of tuberculosis as well. The expression and pro-thrombotic activity of CD36 on platelets may contribute to the microvascular thrombosis thought to contribute to primary lesion necrosis in experimental tuberculosis. (Scapa et al, unpublished data). OxLDL contains lipid peroxidation products that may also contribute directly to the pathogenesis of lesion necrosis (Matsunaga et al 2009; Rodriguez et al 2004). These data suggest that CD36 may function to bind and facilitate the uptake of OxLDL generated during the oxidative stress conditions associated with *M. tuberculosis* infections similar to what has been described in other chronic inflammatory diseases (Febbraio et al 2001; Podrez et al 2000). Collectively, these data suggest a number of pathways in which OxLDL and CD36 receptor expression might be involved in the pathogenesis of tuberculosis and in

particular the unfavorable consequences of lesion necrosis (Canetti 1955; Lenaerts et al 2007).

Our *in-vitro* study using alveolar macrophages confirms the ability of alveolar macrophages to bind and accumulate cytoplasmic OxLDL. The reason OxLDL treated macrophages favored bacilli growth is unknown but may be related to the utilization of intracellular host lipids especially cholesterol as an energy source (Pandey & Sasseti 2008). Our study also illustrates yet another specific role oxidative stress plays in the pathogenesis of tuberculosis.

Elevated OxLDL levels may contribute to the pathogenesis of tuberculosis by altering the immune function of the host cells leading to decreased phagocytic/bactericidal activity as demonstrated in foamy macrophages (Peyron et al 2008) or by the induction of excessive pro-inflammatory cytokines that can worsen the local tissue damage. The pathway of CD36 or LOX1-mediated accumulation of OxLDL could provide attractive targets for the development of novel therapeutics that can interfere with this pathway to host tissue damage and conditions that favor *M. tuberculosis* persistence.

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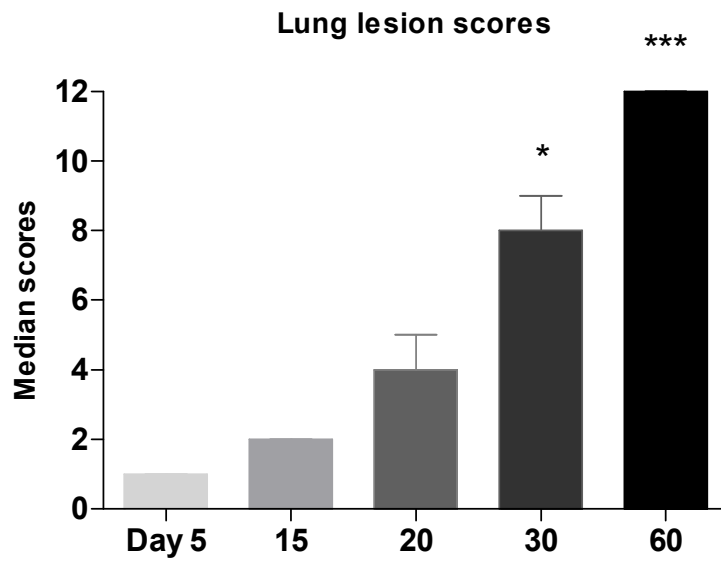


Figure 5.1 The figure illustrates individual calculated lung histopathology lesion scores of Mtb-infected guinea pigs at different time points of infection. The bars represent median values and range for each group (n=5). The stars denote statistically significant increase compared to day 5 after infection (* = $p < 0.05$ and *** = $p < 0.001$).

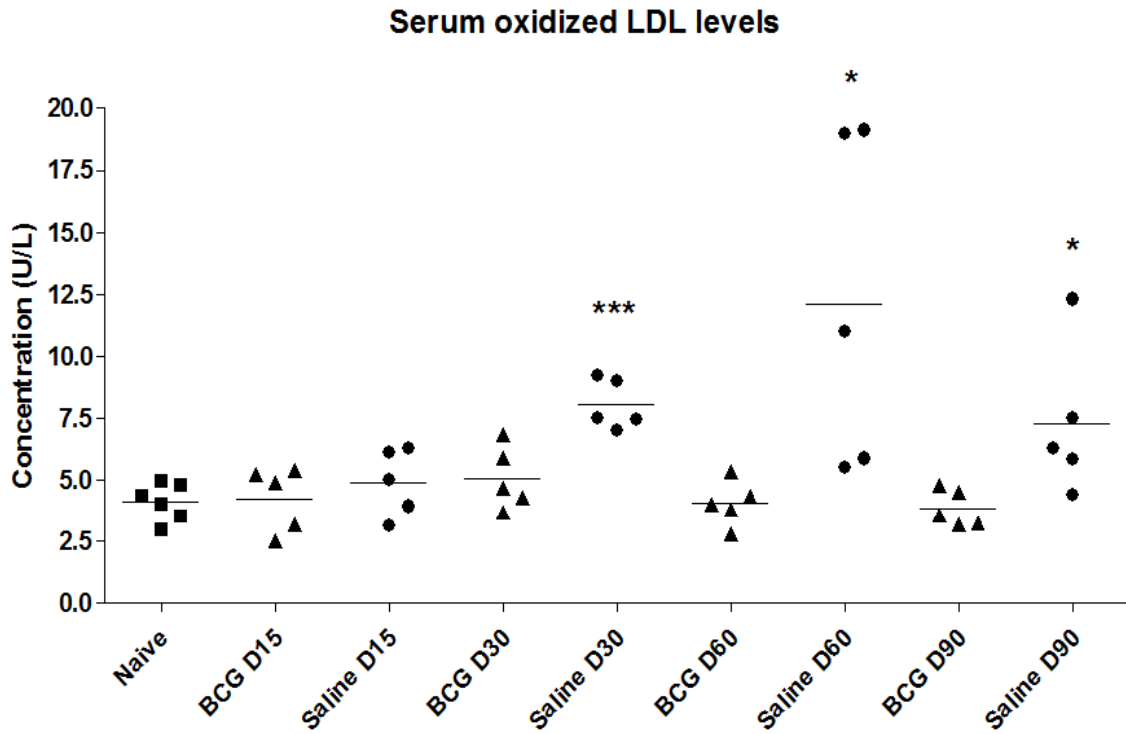


Figure 5.2 The figure illustrates the concentration of OxLDL measured by competitive ELISA in the serum of the infected guinea pigs that are either saline or BCG-vaccinated at different time points after infection. The serum OxLDL concentration in the naive non-infected animals is also illustrated within. The stars denote statistically significant increase compared to the naive animals (* = $p < 0.05$ and *** = $p < 0.001$).

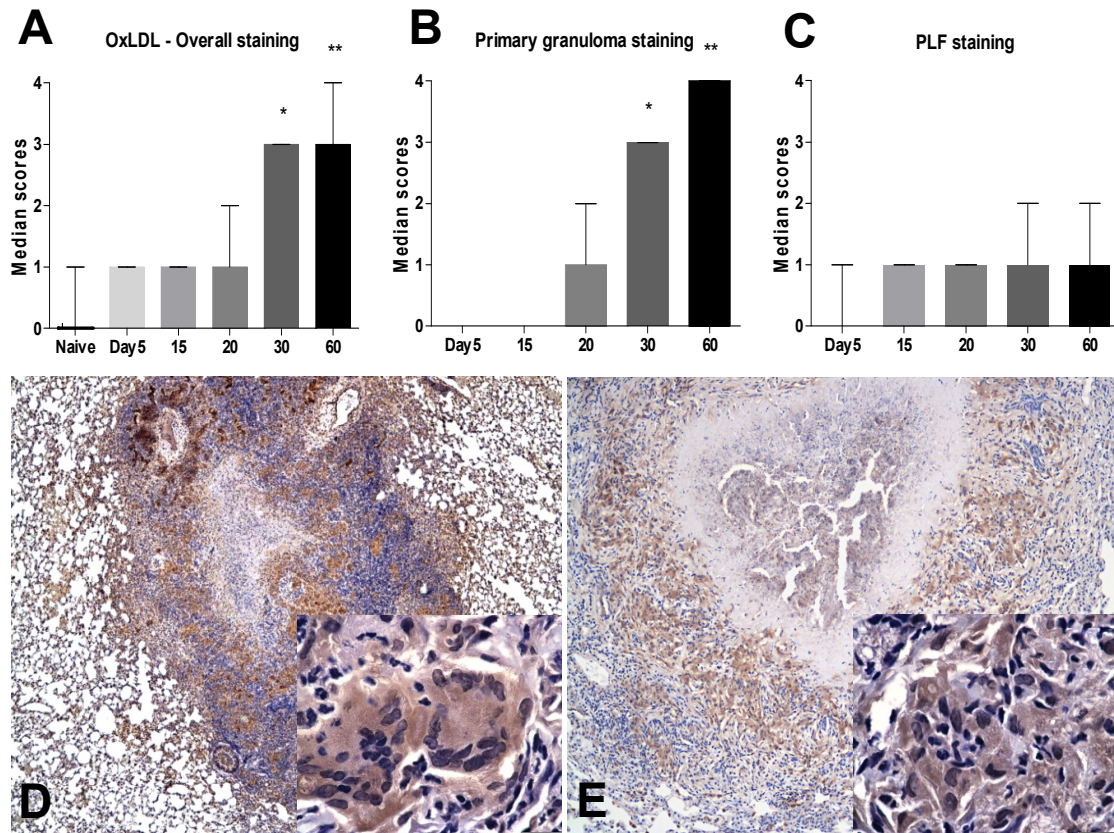


Figure 5.3 The figures A, B and C represent OxLDL immunostaining scores within overall, primary granuloma and primary lesion free (PLF) lung areas respectively at different time points of Mtb infection in guinea pigs. The bars represent median values and range for each group (n=5). The stars denote statistically significant increase compared to the naive animals (* = $p < 0.05$ and ** = $p < 0.01$). The photomicrographs D and E represent immunostaining of OxLDL predominantly within primary granulomas with necrotic core on day 30 and day 60 of infection respectively (40x). The inserts (200x) show intracellular staining within macrophages.

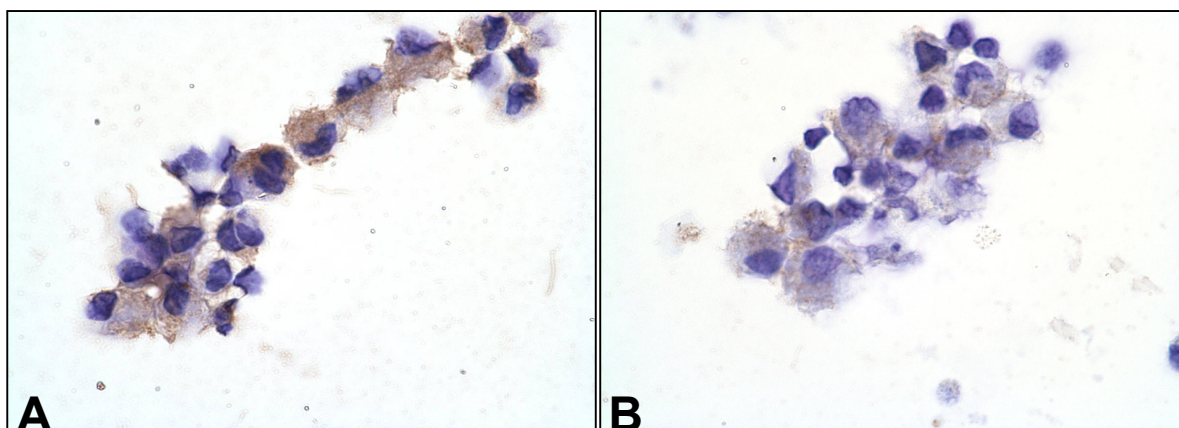


Figure 5.4 A and B represent OxLDL immunostaining in BAL cells collected from Mtb infected guinea pigs at day 30 and day 60 after infection respectively (1000x magnification). Predominantly macrophages and occasionally granulocytes show intracellular staining.

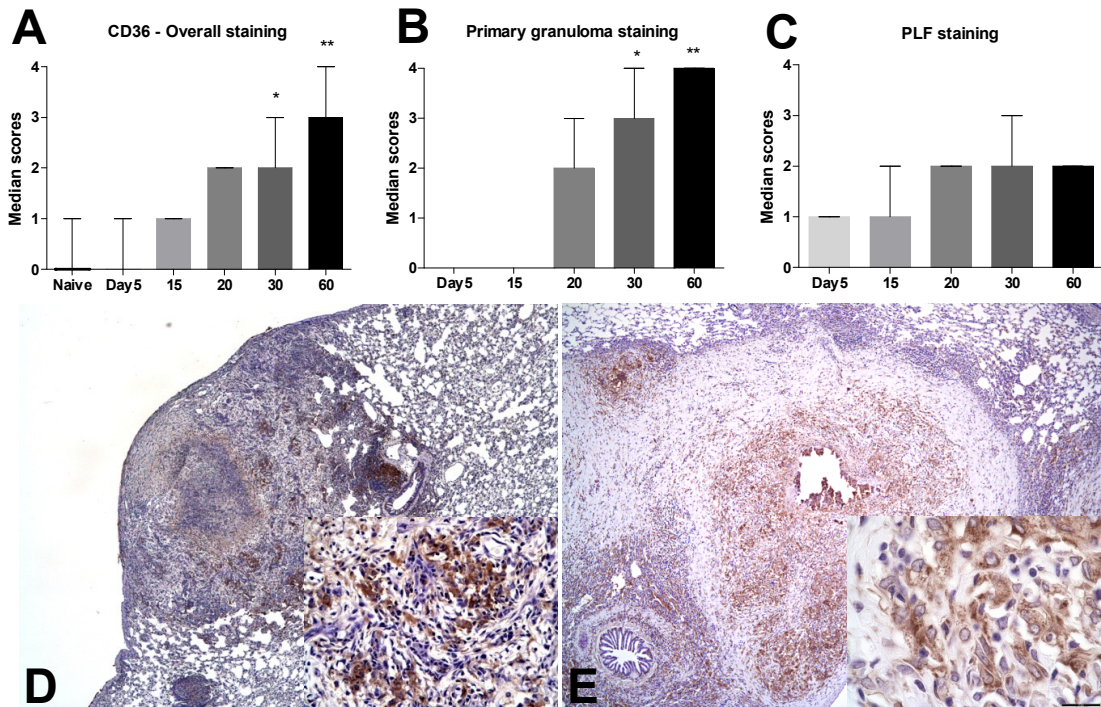


Figure 5.5 The figures A, B and C represent CD36 immunostaining scores within overall, primary granuloma and primary lesion free (PLF) lung areas respectively at different time points of Mtb infection in guinea pigs. The bars represent median values and range for each group (n=5). The stars denote statistically significant increase compared to the naive animals (* = $p < 0.05$ and ** = $p < 0.01$). The photomicrographs D and E represent immunostaining of CD36 predominantly within primary granulomas with necrotic core on day 30 and day 60 of infection respectively (40x). The inserts (200x) show intracellular staining within macrophages.

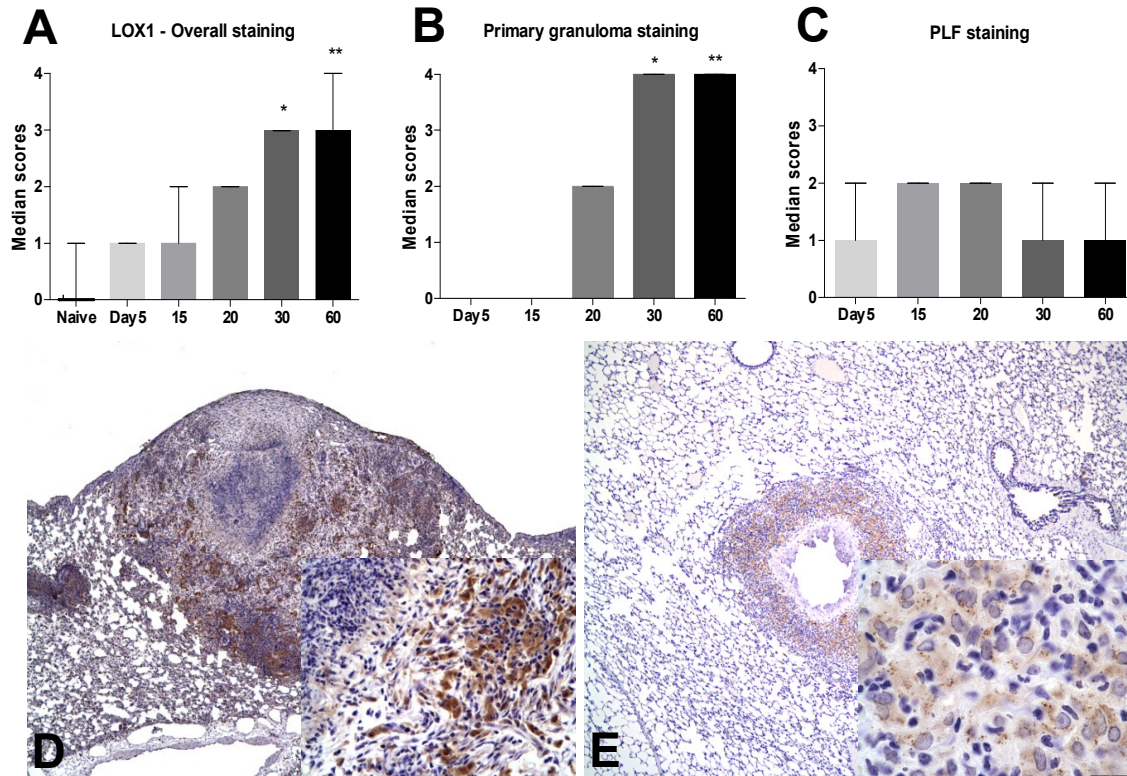


Figure 5.6 The figures A, B and C represent LOX1 immunostaining scores within overall, primary granuloma and primary lesion free (PLF) lung areas respectively at different time points of Mtb infection in guinea pigs. The bars represent median values and range for each group (n=5). The stars denote statistically significant increase compared to the naive animals (* = $p < 0.05$ and ** = $p < 0.01$). The photomicrographs D and E represent immunostaining of LOX1 predominantly within primary granulomas with necrotic core on day 30 and day 60 of infection respectively (40x). The inserts (200x) show intracellular staining within macrophages.

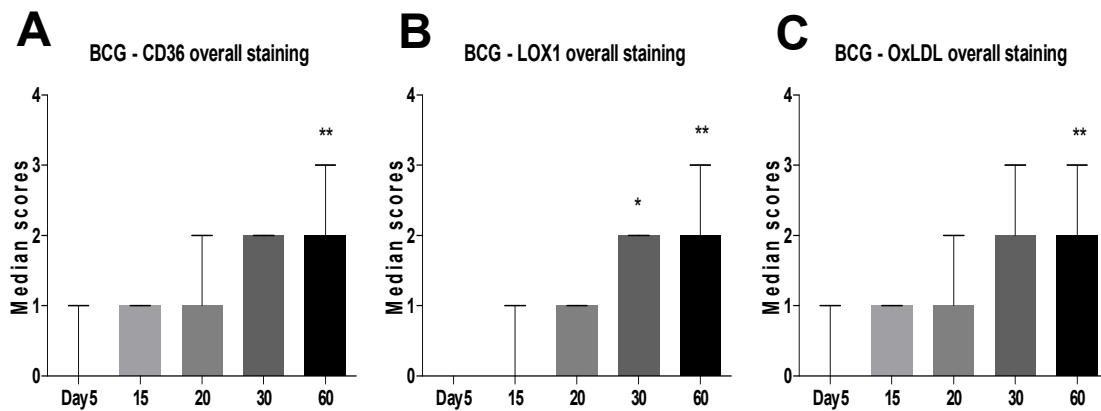


Figure 5.7 The figures A, B and C represent CD36, LOX1 and OxLDL immunostaining scores within overall lung areas respectively at different time points in BCG-vaccinated-Mtb infected animals. The bars represent median values and range for each group (n=5). The stars denote statistically significant increase compared to day 5 after infection (* = $p < 0.05$ and ** = $p < 0.01$).

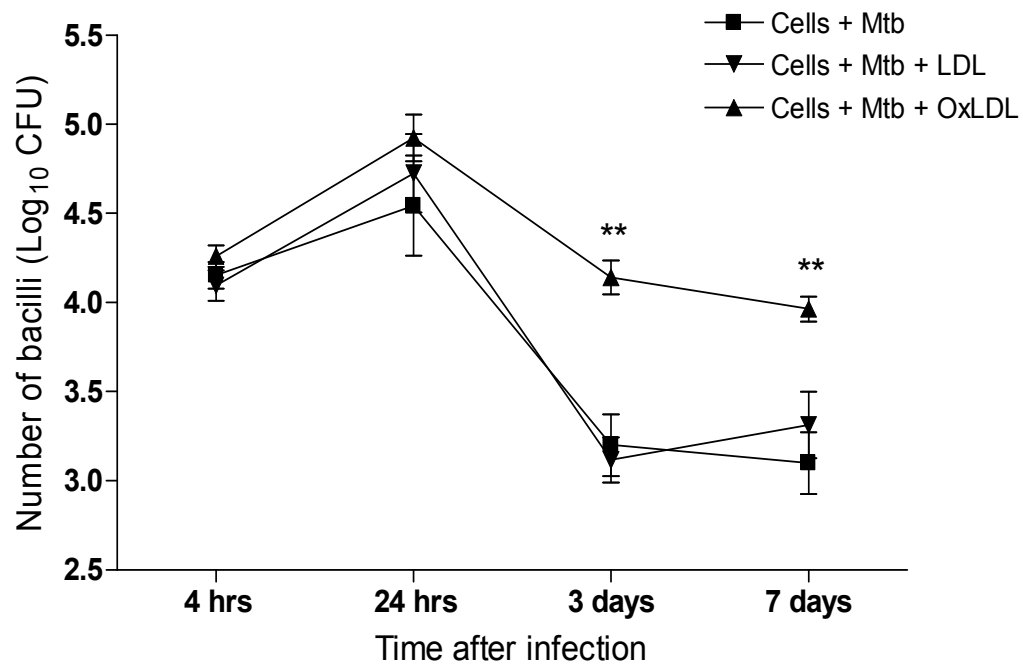


Figure 5.8 The graph shows the number of bacilli (mean \pm SD) grown from the primary guinea pig alveolar macrophages at various time points after infection with *M. tuberculosis*. Three groups represented include control cells that received no treatment, cells that are treated with LDL or OxLDL (n=4). The stars denote statistically significant increase compared to the control group at respective time points (** = $p < 0.01$).

Chapter Six

Conclusion

In my efforts to elucidate the pathogenesis of tuberculosis in guinea pig model, I have addressed a few critical pieces of the puzzle. Granuloma necrosis is a unique feature of tuberculosis which is noted in both human and guinea pigs. Understandably lesion necrosis is a complex biological process that is likely to involve intricate interplay between host and bacterial factors. In my studies, I have shown the importance of the ability of the bacteria to cause necrosis as a virulence determinant, the role of oxidative stress in progression of the disease and development of lesion necrosis in addition to evaluating comparative virulence of various clinical mycobacterial isolates and characterizing certain macrophage cell populations within the granuloma.

I began my initial studies to address the discrepancies or lack of sufficient information in the following areas

- Most of the candidate vaccine or drug evaluation studies in tuberculosis research community heavily rely on laboratory strains of *M. tuberculosis* namely H37Rv and Erdman and it is unclear if the results obtained using these laboratory strains can be applicable in "real world" situations where the candidate drug or vaccine has to face highly virulent mycobacterial strains.
- There is a lack of information regarding the pathogenicity, infectiousness and *in-vivo* growth characteristics of various clinical isolates.
- Prevalence of MDR-tuberculosis warrants characterization of the MDR isolates. An earlier study suggested the loss of virulence as a trade-off for acquiring drug resistance and another study using mice studies showed competent growth of

MDR bacilli in lungs of infected animals contradicting the earlier hypothesis (Mitchison et al 1960; Ordway et al 1995; Orme 1999). These contradictions need to be addressed.

- Significant number of studies use the number of culturable bacilli (CFU) alone as measure of virulence and drug/vaccine efficacy (McMurray 2001) and CFU may not be an accurate correlate of virulence.

The conclusions drawn from my first study in which I compared the virulence of *M. tuberculosis* strains H37Rv, Erdman K01, HN878 and CSU93 are as following

- CFU is an unreliable correlate of virulence as the number of bacilli in lungs, spleen and lymph node did not correlate with the virulence of the strains as reflected by the survival studies.
- Granuloma necrosis in lungs and extra-pulmonary sites correlated with the virulence of *M. tuberculosis* strains as indicated by the survival studies.
- The incidence of heart lesions, which are indicative of extensive extra-pulmonary dissemination, correlated with virulence.
- A laboratory strain, Erdman K01, was the most virulent strain in our study based on survival studies. Erdman K01 strain rather than H37Rv is likely to be a better candidate for challenge in vaccine and drug evaluation studies in mice and guinea pig models.
- This study further proves that tuberculosis is truly a multi-systemic disease in the guinea pig model with involvement of spleen, liver, heart, pancreas, adrenal, mediastinal and peripancreatic lymph nodes besides lungs.

The second study in which I compared the virulence of several more strains of *M. tuberculosis* including highly virulent clinical strains that belong to W-Beijing family and a few MDR strains, led to the following conclusions.

- MDR strains of *M. tuberculosis* used in the study TN7642 and TN5904, were the least virulent of all strains tested suggesting that there indeed might be a trade-off between virulence and drug-resistance.
- Organ pathology and necrosis again reliably paralleled virulence as reflected by survival studies.
- There are no intrinsic differences in virulence between W-Beijing and non-Beijing strains of *M. tuberculosis* in my studies.
- The highly virulent strains in this study caused remarkable necrosis and cavitation of lymph nodes which were previously not seen in the guinea pig model of tuberculosis.
- SA161 and SA629 are likely to be excellent candidate *M. tuberculosis* strains that can be used to test new vaccines and drugs in development.

These aforementioned studies provided critical insight into the importance of organ pathology, specifically necrosis in tuberculosis pathogenesis. The greatest number of bacilli are often present within the areas of necrosis (Canetti 1955). When the necrotic core reaches an airway it can lead to the transmission of bacilli via coughing and when it reaches a blood vessel it can lead to the dissemination of bacilli within the host to other organs via blood circulation. Considering the aforementioned significance of lesion necrosis, I decided to further study this particular aspect of tuberculosis pathogenesis. As discussed earlier, the development of lesion necrosis during tuberculosis is likely multi-

factorial and complex. I hypothesized that excessive free radical generation during the oxidative stress conditions that are likely prevalent during the development of tuberculosis lesions, contribute to the progression of lesions to necrosis.

To address this hypothesis, I started by establishing the prevalence of oxidative stress conditions during tuberculosis in guinea pig model. I used markers of oxidative stress such as malondialdehyde (a lipid peroxidation end product) to establish it. Next I evaluated the systemic antioxidant status during the infection in guinea pigs using a calorimetric assay. As the antioxidant levels were low during tuberculosis, I studied the effectiveness of a critical antioxidant mechanism mediated by the transcription factor Nrf2. As Nrf2-mediated specific antioxidant response mechanism was defective during the disease, I attempted to reverse this specific pathway therapeutically and thereby address my hypothesis (i.e. Therapeutic alteration of oxidant/antioxidant balance in favor of antioxidants and will reduce the extent of lesion necrosis and progression of disease. This would establish the role of oxidative stress in the development of lesion necrosis). The conclusions drawn from this study are

- Oxidative stress conditions associated with enhanced free radical generation and decreased antioxidant levels were prevalent during tuberculosis in the guinea pig model as demonstrated by markers of oxidative stress in lungs.
- Systemic antioxidant capacity during *M. tuberculosis* infection in guinea pigs was markedly decreased which was restored in BCG-vaccinated animals.
- Cytoplasmic expression of the antioxidant transcription factor Nrf2 increased as the disease progressed during the *M. tuberculosis* infection in guinea pig lungs.

- Despite this increase, the nuclear translocation of Nrf2 was deficient in tuberculous granulomas of guinea pigs and it correlated with defective Nrf2-downstream antioxidant enzyme expression.
- BCG vaccination failed to restore the deficiency of Nrf2-mediated defense locally within the lungs.
- NAC administration to induce Nrf2 translocation resulted in increased antioxidant levels and decreased lung pathology and necrosis in the drug-treated guinea pigs suggesting that the Nrf2-deficiency is likely reversible and oxidative stress does play a role in the development of lesion necrosis.

While studying the role of oxidative stress in tuberculosis pathogenesis, I learned that free radical oxidation of host lipids results in formation of lipid-laden foam cells in atherosclerotic lesions. As similar foam cells that quite often harbor significant numbers of *M. tuberculosis* bacilli are commonly seen in tuberculosis lesions of mice, guinea pig and man, I hypothesized that low-density lipoprotein (LDL), which contains host lipids (predominantly cholesterol), is likely oxidized during oxidative stress conditions to form oxidized LDL (OxLDL) and accumulates in macrophages and these lipid-laden macrophages support enhanced bacterial survival in the tuberculosis lesions.

To address this hypothesis, I measured systemic OxLDL levels using ELISA and semi-quantified OxLDL expression levels locally in lungs using immunohistochemistry. Next I evaluated the expression levels of the receptors for OxLDL, CD36 and LOX1 using immunohistochemistry and studied the effect of BCG vaccination on the accumulation of OxLDL locally and systemically. Finally I examined the functional

significance of accumulation of OxLDL in guinea pig primary alveolar macrophages during *M. tuberculosis* infection. The conclusions drawn from this study are

- OxLDL level in serum from saline-vaccinated guinea pigs infected with *M. tuberculosis* was elevated compared to BCG-vaccinated and non-infected animals.
- OxLDL accumulated in macrophages including the foam cells during *M. tuberculosis* infection in guinea pigs.
- Expression of scavenger receptors involved in the uptake of oxidized LDL (CD36 and LOX-1) markedly increased during *M. tuberculosis* infection in guinea pigs.
- BCG vaccination failed to completely inhibit OxLDL accumulation locally within the lungs even though it decreased systemic OxLDL levels in serum.
- Accumulation of OxLDL in alveolar macrophages supported significantly increased mycobacterial growth suggesting that OxLDL likely provides a nutrient-rich, specifically cholesterol-rich, microenvironment for the mycobacteria to thrive amidst the host immune response.

Overall my studies demonstrate comparative virulence of various clinical strains of *M. tuberculosis* in the guinea pig model, the importance of lesion necrosis in tuberculosis pathogenesis, role of oxidative stress in development of necrosis and its role in accumulation of intra-cytoplasmic host lipids in the macrophages. Future studies based on my work can be performed in the following areas

- Elucidation of factors (host or bacterial) involved in the differences in virulence of various clinical isolates.

- Evaluating the suitability of the high virulent *M. tuberculosis* strains such as Erdman K01, SA161 and SA629 as challenge strain in drug and vaccine evaluation studies.
- Further molecular characterization of Nrf2 deficiency in guinea pig model and testing more therapeutic agents such as sulforaphane and CDDO-Imidazole that are known to induce Nrf2 activation to reverse such deficiency.
- Incorporation of Nrf2-inducing drugs with the anti-tuberculosis drugs to efficiently clear the disease.
- Elucidating other functional alterations, such as changes in pro- and anti-inflammatory cytokine production levels in macrophages, if any, due to OxLDL accumulation during tuberculosis.
- Evaluation of the suitability of administering therapeutics that can reverse OxLDL formation and lipid accumulation in cells by inhibiting the uptake receptors as an adjunct therapy to increase the effectiveness of anti-tuberculosis therapy.

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