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Lung Levels of Neutrophil-Related Chemokines S100A8 and CXCL5 Do Not Correlate With Mycobacterium tuberculosis Levels in 8 Mouse Strains

Emily McGlone
Worcester Polytechnic Institute

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LUNG LEVELS OF NEUTROPHIL-RELATED CHEMOKINES S100A8 AND CXCL5 DO NOT CORRELATE WITH MYCOBACTERIUM TUBERCULOSIS LEVELS IN 8 MOUSE STRAINS

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by

Emily McGlone

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APPROVED:

__________________________
Gillian Beamer, VMD, PhD, DACVP
Department of Infectious Disease and Global Health
Tufts Veterinary School
MAJOR ADVISOR

__________________________
David Adams, PhD
Dept. Biology and Biotechnology
WPI Project Advisor

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ABSTRACT

Tuberculosis is one of the top ten causes of death, and there is no simple way of predicting disease outcomes. The purpose of this project was to determine whether neutrophil-related proteins, S100A8 and CXCL5 correlate with the levels of *Mycobacterium tuberculosis*, and explore whether these proteins could be used as indicators of disease progression. Their concentrations in the lungs were calculated using sandwich ELISAs. The bacterial burdens in the lungs were calculated from Colony Forming Units (CFUs) grown on agar plates. Mice were weighed weekly. Final analyses showed no correlation between neutrophil chemokines and *M. tuberculosis* burden, and that mice had no weight loss throughout the experimental timeframe. Therefore, when there is a weight gain, lung concentrations of S100A8 and CXCL5 cannot be used as indicators of disease progression in TB.
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BACKGROUND

Tuberculosis Introduction

Tuberculosis (TB) is an infectious disease of the lungs, caused by the bacterium Mycobacterium tuberculosis and is common in third world countries. According to the World Health Organization, TB is one of the top ten causes of death worldwide (WHO, 2018). Each year, 9 million people are diagnosed with TB, and 1.5 million die from the disease (Nouailles et al., 2014). TB is also more common and deadly for those co-infected with HIV due to their already compromised immune system. Therefore, TB is the leading cause of death in HIV-positive patients (WHO, 2018). The majority of TB cases occur in Africa and Asia, with about half of the new cases occurring in Bangladesh, India, Pakistan, China, India, and Indonesia (Dye, 2006). Since TB is such a large global health issue, the World Health Organization has set a goal to reduce the number of TB cases worldwide by 95% by 2035 (Sulis et al., 2014).

According to the World Health Organization, only 10% of those exposed to M. tuberculosis will contract the disease, and the other 90% will never get sick (WHO, 2018). Men are more than two times more likely than women to develop active TB (Dye, 2006). The bacterium Mycobacterium tuberculosis has co-evolved with humans for thousands of years and humans are the main host for M. tuberculosis (Niemann & Supply, 2014). The main method for contracting M. tuberculosis is exposure to someone else with active TB. The bacteria spreads person to person through aerosolized droplets generated by coughing, sneezing, or talking (American Lung Association, 2018). The bacteria can stay in the air for a couple of hours, increasing the risk of another person inhaling the bacteria (Heemskerk et al., 2015).

Following exposure via aerosols, bacilli spread from the lungs to other parts of the body including the pleura, lymph nodes, bones, and urogenital system in susceptible individuals (Adzic-Vukicevic et al., 2017). The common symptoms of TB include a lasting cough, coughing up blood, malaise, fatigue, weight loss, chills, anorexia, fever, and night sweats (Campbell & Bah-Sow, 2006). Without treatment, TB is fatal for 53%-83% of patients diagnosed with pulmonary TB (Tiemersma et al., 2011).

Once the M. tuberculosis enters the lung alveoli, they are engulfed by macrophages. The macrophages either lyse the bacteria resulting in no infection, or the bacteria replicate inside the macrophages. In the latter case, when the macrophage dies, the multiplied M. tuberculosis is released into the surrounding area infecting more cells, resulting in pulmonary TB (CDC, n.d.). The presence of M. tuberculosis in the lungs results in a recruitment of T cells and inflammatory cells. These T cells and inflammatory cells surround the bacteria creating granulomas. In susceptible individuals, M. tuberculosis infection of the lungs results in necrosis of both the macrophages and the pulmonary tissue, which makes lung necrosis a good indication of the progression of the infection (Kramnik & Beamer, 2016). Necrosis occurs at these granulomas due to the death of macrophages that occurs from M. tuberculosis stimulating signal pathways that induce cell death. The areas of necrosis become ideal locations for the M. tuberculosis to replicate (Kramnik & Beamer, 2016). The bacteria are also taken up by the bloodstream within the first 3-6 weeks of infection, before the start of necrosis, which spreads the infection to
different parts of the body. This process is known as dissemination and leads to extrapulmonary TB.

Tuberculosis is diagnosed using either a rapid molecular test, a sputum smear, or a culture-based method (WHO, 2018b). The best method for diagnosing TB is through a rapid molecular test, which provides the most accurate results within two hours. The sputum smear test, used for over 100 years, requires the collection of a sputum sample that is then analyzed with a microscope to examine for \textit{M. tuberculosis}. The culture-based method is used more as a secondary reference because it can take up to 12 weeks to determine the results (WHO, 2018b). The use of rapid molecular tests is becoming more common worldwide, but the sputum smear test and culture-based methods are still used to monitor the disease progression.

\textbf{TB Treatment and Prevention}

Tuberculosis is curable and preventable in some individuals with proper treatment. TB is treated by giving a combination of antimicrobials over six to nine months. The common drugs prescribed include isoniazid, rifampin, ethambutol, and pyrazinamide (CDC, 2016). However, if this treatment plan is not completed, the disease can reoccur, and the surviving bacilli can be drug resistant (Kurz et al., 2016). \textit{M. tuberculosis} resistant to both of the first-line treatments, isoniazid and rifampin, are considered to be multidrug resistant TB. Treating multidrug resistant TB is difficult and requires chemotherapy on top of a second line drug treatment (WHO, 2018). Treating multidrug resistant TB only has a 48% success rate (Kurz et al., 2016).

Bacille Calmette-Guerin (BCG) is the only currently approved vaccine for tuberculosis, and it is usually given to children in high risk areas where TB is prevalent. This vaccine is an attenuated form of \textit{Mycobacterium bovis} bacteria, and was first used in children almost one hundred years ago (Orme, 2014). This vaccine is good at preventing disseminated TB in children, but has variable protection against pulmonary TB in adults, who make up most of the TB cases (Van Der Meeren et al., 2018). The efficacy of the BCG vaccine on pulmonary TB in adults is between 0-80\% (Pai et al., 2016). Since the BCG is not very effective in adults, there is currently much research focused on finding a new TB vaccine or finding ways to improve the BCG vaccine to generate durable immunity. Due to the variability in TB infections, researching new vaccines is very costly, time consuming, and has more obstacles than most vaccine research. One of the biggest obstacles is the lack of an animal model that shows the full variability of TB seen in human patients (Pai et al., 2016). Therefore, much research also focuses on creating genetically diverse mouse models that accurately reflect the wide variability of \textit{M. tuberculosis} infections seen in humans.

Despite these obstacles, there are many TB vaccines in clinical trials. One vaccine currently in phase IIB clinical trials is M72/AS01E vaccine, which is designed to prevent active TB in uninfected individuals or those with latent TB (Pai et al., 2016). The major component of this vaccine is two proteins that are present in the \textit{M. tuberculosis} bacteria (Van Der Meer en et al., 2018). During the Phase IIB clinical trial, the efficacy of the vaccine was tested on individuals in Africa, concluding the vaccine had a 54\% efficacy with no major side effects (Van Der Meeren et al., 2018). Therefore, more research will be continued with this vaccine in the future.
TB Susceptibility

The degrees of TB disease lie on a spectrum. One end of the spectrum is known as latent TB, where the individual is believed to be infected with *M. tuberculosis* but is asymptomatic. These individuals are non-contagious, but are at risk of developing active TB. It is estimated that 90% of those infected with *M. tuberculosis* are in the latent phase, or the bacteria gets destroyed by the immune system (Heemskerk et al., 2015). In latent TB, macrophages engulf and surround the bacteria to produce areas called granulomas. Granulomas can exist for a long period, potentially the rest of the person's life, and sometimes never turn into active TB (CDC, n.d.). However, in susceptible individuals *M. tuberculosis* can eventually overpower the immune system causing the granulomas to break, creating active TB (CDC, n.d.).

Incipient TB is an intermediate phase of TB. Incipient TB refers to the early stages of the infection during its change from latent to active. During incipient TB the patient is still asymptomatic, but is considered to be in a disease state (Achkar & Jenny-Avital, 2011). Incipient TB is defined by the presence of a 2 cm² opacity seen in a chest X-ray on an asymptomatic immunocompetent individual, where the only potential explanation for the opacity is TB (Achkar & Jenny-Avital, 2011).

In active TB, the *M. tuberculosis* replicates to very high levels, and the infection is contagious. Active TB most commonly occurs in the lungs and is also known as pulmonary TB. During pulmonary TB, the individual experiences coughing, coughing up blood, weight loss, and fever. These individuals might have a positive sputum smear test (Campbell & Bah-Sow, 2006). When an individual has active TB, the infection can also spread to other parts of the body, known as extra-pulmonary TB.

Dissemination of *M. tuberculosis* can also occur very rapidly, which results in fulminant TB. During fulminant TB there is a rapid onset of the disease which can lead to septic shock and multiorgan failure (Bridges & Bedimo, 2006). Fulminant TB is a rare form of the disease, but it occurs more frequently in those who are HIV-positive since they are already immunocompromised (Bridges & Bedimo, 2006).

This complex symptomology results in a range of super-susceptible, susceptible, and resistant individuals. It is widely accepted that this range of susceptibility is due to genetics. The idea that tuberculosis susceptibility is partly genetic is supported through the studies of monozygotic and dizygotic twins infected with *M. tuberculosis*. Monozygotic twins are equally susceptible to TB, whereas the susceptibility of TB in dizygotic twins is variable (Smith et al., 2016). However, the mechanism by which genes play a role in the susceptibility remains unknown. Genetic variability among strains of the *M. tuberculosis* bacteria are also responsible for the range in susceptibility and virulence (Pai et al., 2016).

TB Mouse Models

Two major types of mouse models are used in medical research, inbred strains and outbred strains. Inbred strains have been used for decades in infectious disease research, including infection experiments with *M. tuberculosis*. Inbred strains are often used because they are cost effective, immunocompetent, readily available for purchase, genetically stable, and easily genetically manipulated. Inbred mice are genetically stable because each individual mouse
is genetically identical the others, allowing for experimental reproducibility (Yeadon, 2013). However, since the inbred strains are composed of identical individuals, there is also a lack of diversity in the mouse populations, which does not match the diversity of the human population. Therefore, recent research has focused on creating a diversity outbred (DO) population of mice. The DO mouse population is created from eight inbred strains known as the “founder strains,” which were used in this project. These DO populations are genetically heterogeneous, creating a large genetic diversity within the population (Churchill et al., 2012). Since there is a large genetic diversity among DO mice, they are a better model for studying the relationship between infectious disease and genetics.

Since the naturally occurring host for *M. tuberculosis* is humans, it is well known that one or two inbred strains mice cannot model the variable susceptibility observed across the human population. Unfortunately, scientists in the TB field have generalized the responses of 1-2 inbred mouse strains to all mice, creating the following dogma: Mice are more resistant to TB than humans; Mice can handle higher bacterial burden than humans; and mice form different granulomas compared to humans (Dharmadhikari & Nardell, 2008; Cooper, 2015). Finally, it is already known that the susceptibility of TB largely depends on both the host and bacterial genetics. Many different mouse strains are affected differently by TB due to their genetics, which leads to inconsistent results among studies (Fonseca et al., 2017). Also, since mice are not natural hosts of *M. tuberculosis*, laboratories often use modified strains of *M. tuberculosis* that can infect the mice (Fonseca et al., 2017).

Despite these setbacks, mouse models are still widely used for TB research since they are the most understood model, and researchers continue to create a more humanized mouse model that reflects human *M. tuberculosis* infection (Fonseca et al., 2017). One advantage of mouse models is the ability to engineer the mice to over-express (knock-in) or under-express (knock-out) a desired gene. This ability to genetically modify the mice has played a big role in determining which genes play a role in TB susceptibility (Dharmadhikari & Nardell, 2008). Therefore, the creation of a mouse strain that mimics the susceptibility of *M. tuberculosis* in humans would help in research.

**TB Mouse Model Founder Strains**

As mentioned above, the DO mouse population was created from eight inbred founder strains for the purpose of increasing genetic diversity in the experimental mouse model. The eight founder strains were: CAST/EiJ, PWK/PhJ, NOD/ShiLtJ, A/J, C57BL/6, NZO/HtJ, 129S1/SvImJ, and WSB/EiJ. Within each strain, individual mice are genetically identical and homozygous at all loci. Across each strain, these eight founders are highly genetically different which is why they were selected to then subsequently breed together to generate the DO population. The CAST/EiJ, PWK/PhJ, and WSB/EiJ strains were originally derived from wild mice captured from different locations across the globe (Collaborative Cross Consortium, 2012). The CAST/EiJ mice were derived by inbreeding mice found in a warehouse in Thailand (https://www.jax.org). The WSB/EiJ strain was formed by inbreeding mice captured in Maryland, and the PWK/PhJ strain was inbred in the Czech Republic (https://www.jax.org). The NOD/ShiLtJ strain is often used when studying the effects of being immunocompromised, because the NOD/ShiLtJ strain has defective cytokine productions from macrophages, as well as insufficient natural killer (NK) cell production, and defective antigen presentation under certain stimuli (https://www.jax.org). All eight of these founder strains were used in this MQP project.
S100A8 as an Inflammatory Marker for Active TB

S100A8, also known as calgranulin-A, is a calcium-binding protein containing two binding motifs. It is encoded by the S100A8 gene in humans and in mice. The genes that encode S100A8 are in the S100 family of proteins (Schäfer & Heizmann, 1996). S100A8 can form a heterodimer with protein S100A9 to form a protein called calprotectin. S100A8 and A9 are prominent players in innate immunity (Nacken et al., 2003; Roth et al., 2004). S100A8 is upregulated in the serum of individuals with inflammatory diseases or sepsis, which means it can be used as a biomarker for inflammatory disease and during disease diagnosis (Wang et al., 2018). Previous research indicates that the concentration of S100A8 in the serum of active TB patients directly correlates with lung damage and morbidity as experienced by TB patients (Gopal et al., 2013). Therefore, S100A8 can be used to determine the progression of active TB. Currently, there are no simple and fast tests to determine the difference between latent and active TB, so perhaps assaying S100A8 levels could provide such a test (Gopal et al., 2013).

The S100A8 protein is stored in neutrophils and monocytes. During trauma or infection, the neutrophils and monocytes upregulate and secrete S100A8 to create an inflammatory response (Wang et al., 2018). The release of S100A8 also results in the recruitment of leukocytes, and signals the neutrophils and macrophages to release cytokines (Wang et al., 2018). While inflammation is part of our internal defense against pathogens, excess and prolonged inflammation can lead to cell death and tissue damage (Wang et al., 2018). The amount of S100A8 is normally regulated through a negative feedback loop, but when this negative loop is not working, the excessive inflammation can cause damage. High levels of S100A8 are often found in patients with septic shock and inflammatory damage (Wang et al., 2018).

Since excess S100A8 associates with disease progression, perhaps it could be used as a target protein for disease therapy. Therapeutically decreasing the levels of S100A8 could decrease inflammation, which could help decrease the stress on the organs, and decrease the damage caused by inflammation (Wang et al., 2018). Therefore, S100A8 could potentially be used as a target for treating active TB. Decreasing S100A8 in active TB patients could decrease lung damage caused by the inflammation, without affecting adaptive immunity against the M. tuberculosis (Gopal et al., 2013).

CXCL5 and TB

CXCL5 is lipopolysaccharide-induced ligand from the CXC cytokine family, responsible for neutrophil recruitment (Mei et al., 2010). CXCL5 is commonly found in areas of inflammation, especially in the lungs (Mei et al., 2010). In mice, the CXCL5 is produced regularly by the platelets in the blood. During times of inflammation, the alveolar cell types I and type II upregulate the production CXCL5 in the lungs (Nouailles et al., 2014). Although it is known that CXCL5 is involved in lung inflammation, the specific details have not been fully studied (Mei et al., 2010). In the case of Escherichia coli pneumonia lung infections, the CXCL5 recruits more neutrophils to the area compared to other members of the CXC cytokine family. In humans, there is often a large abundance of CXCL5 in the lung fluids during a time of infection (Mei et al., 2010). The increased number of neutrophils results in inflammation in the lungs. Therefore, there is a correlation between CXCL5 concentration and lung damage, which can lead
to death of the host (Mei et al., 2010). Currently, CXCL5 has been predominantly studied in murine models. Murine CXCL5 closely resembled human CXCL5, and human CXCL5 is also produced by the alveolar cells, but research has not demonstrated that human CXCL5 also results in neutrophil recruitment during lung infections (Nouailles et al., 2014).

CXCL5 is upregulated in the lungs of patients with active TB. In a previous study, work from the Beamer laboratory showed that some DO mice infected with *M. tuberculosis* had high concentrations of CXCL5 in the lungs that occurred only in DO individuals that developed early morbidity and died after only a few weeks after infection (Muhammad et al., 2015). The same work and additional unpublished observations (not shown) have identified lung CXCL5 as a strong and positive correlate of pulmonary TB disease in mice.

**ELISAs**

Enzyme-linked immunosorbent assay (ELISA) is a method to quantify molecules including protein, peptides, or hormones in a complex sample. In this project, ELISAs were used to quantify S100A8 and CXCL5 in lung samples from *M. tuberculosis*-infected mice. This assay is commonly performed on a 96-well polystyrene plate. The sandwich ELISA is the most sensitive type of ELISA (ThermoFischer Scientific, n.d.). During a sandwich ELISA, a capture antibody against the target protein is coated onto the ELISA well. Then, the sample containing the target protein is added. The target protein binds to the antibody coat, and the rest of the unbound proteins are washed away. Next, a secondary detection antibody is added. The detection antibody binds to the other end of the target protein (Sino Biological, n.d.), sandwiching the target protein between two antibodies. The secondary antibody is conjugated to an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), that forms a color when mixed with a colorless reagent solution. The amount of color produced in the well is proportional to the amount of target protein present. The ELISA plate is read with a spectrophotometer to produce absorbance values associated with the concentration of the desired reagent. When combined with standard curves of known protein concentrations, the mathematical relationship between absorbance of CXCL5 or S100A8 in lung samples can be derived and calculated using linear or polynomial regression models.
PROJECT PURPOSE

The purpose of this project was to determine whether neutrophil-related proteins, S100A8 and CXCL5 correlate with the levels of *M. tuberculosis* in the lungs, and explore whether these proteins could be used as indicators of disease progression in the eight founder strains of the DO population. The concentrations of S100A8 and CXCL5 in the lungs were calculated using sandwich ELISAs. The bacterial burdens in lungs were calculated from Colony Forming Units (CFUs) grown on agar plates and the mice were weighed weekly. Based on work in the DO population (Muhammad et al., 2015) and previous work using the eight founder strains (Smith et al., 2016) we expected that the concentrations of S100A8 and CXCL5 would correlate with weight loss, early morbidity, and with *M. tuberculosis* lung burden. Since little *M. tuberculosis* studies have been done on mouse strains other than C57BL/6, 129, and A/J strains, any results obtained with new strains will be interesting. If this project is successful, it will help develop a method for determining the disease state of TB patients.
METHODS

Mouse Infection and Sample Collections

CAST/EiJ, PWK/PhJ, NOD/ShiLtJ, A/J, C57BL/6, NZO/HiLtJ, 129S1/SvImJ, and WSB/EiJ mouse strains were purchased from The Jackson Laboratory in Bar Harbor, Maine. The mice were kept in a Biosafety Level 3 laboratory at The Ohio State University. The mice were infected by aerosolized *M. tuberculosis* Erdman so that each mouse was exposed to 25-50 CFUs of *M. tuberculosis* (Beamer et al., 2008). The mice were weighed every week. Groups of 3-5 mice were euthanized by CO₂, and blood and lungs from each mouse were collected at 20 days, 30 days, and 40 days post infection. The blood was clotted and centrifuged for 10 minutes at 3,000 RPMs, and then serum collected, filter sterilized, and frozen. Three lung lobes were homogenized in sterile phosphate buffered saline, serially diluted, and plated in technical replicates on 7H11 agar supplemented with OADC, incubated at 37°C for 3 weeks, and *M. tuberculosis* colony forming units were counted (Beamer et al., 2008). The remaining lung homogenates were then filter sterilized and stored -80°C. The remaining lung lobes were inflated with 10% neutral buffered formalin, fixed for 3 weeks, and embedded in paraffin, sectioned, and stained using hematoxylin and eosin. Infection, tissue harvest, and CFU counts were performed at The Ohio State University. The frozen lung and serum samples were sent to the Tufts Cumming School of Veterinary Medicine for analysis. These experiments were approved by Tufts University Institutional Animal Care and Use Committee numbers (IACUC numbers G2012-53; G2012-151; G2015-33).

ELISA

Filtered lung homogenates were tested for cytokines. S100A8 and CXCL5 ELISA kits were purchased from *R&D Systems: A Bioteche Brand*, and optimized for our laboratory conditions before testing samples. The 96-well plates were first coated with 100 μL of 4 μg/mL of the capture antibody. For the CXCL5 assay, two vials of capture (Lot numbers GNZ0413111 and GNZ0516081) were used. The coated ELISA plates were incubated at 4°C overnight. The next day, the unbound capture antibodies were removed from the plate. Then, all of the ELISA plates were blocked with 200 μL of 1% BSA and left at room temperature to incubate for one hour. The BSA was then removed from the plate and the samples were added. For each plate, the first three columns were the CXCL5 standard with a 4 ng/mL concentration in the first row, and then a one to one serial dilution proceeding down the column, until the last row which a blank filled with 1% BSA. The remaining wells were filled with 100 μL of 1:10, 1:100, and 1:1,000 serially diluted lung or serum samples. The samples were incubated at 4°C for 72 hours. After the incubation period, the samples were removed from the plates, and all of the plates were washed 6 times with 1X PBST. After washing, the 100 μL of a 0.05 μg/mL detection antibody (Lot numbers BES0716081 and BES0715071) was added to each of the wells. The detection antibody was incubated at room temperature for two hours. After the incubation period, the plates were washed again with 1X PBST 6 times. Then, 100 μL of 5 μL/mL streptavidin-HRP was added to each of the wells and incubated at room temperature for 20 minutes under tin foil to prevent exposure to light. The HRP was then removed from the plates, and the plates were washed again using the 1X PBST 6 times. Next, 100 μL of TMB was added to each of the wells, and the plates incubated at room temperature for 20 minutes under tinfoil. After 20 minutes, 100
μL of 2.5% HCl was added to each well to stop the reaction. The optical density of each plate was then measured using a microplate reader set to 450 nm. The exact same procedure was followed for the detection of S100A8. The S100A8 capture antibody had lot number CHFW0117111 and the detection antibody was lot number WKF0417111.

**Light Microscopy**

One lung sample from each strain was examined under an Olympus light microscopy. The samples collected 30 days post infection were used, except for WSB/EiJ. The sample collected 20 days post-infection was used for WSB/EiJ. The areas of inflammation were examined at 4X, 10X, and 20X magnification. Images of all of these slides were taken using Infinity Capture software. The relative area of inflammation in each strain was compared at 4X magnification.

**Statistics**

Before analysis, all of the data was checked for single outliers using Grubbs’ test on GraphPad Outlier Calculator. Eight outliers were identified and censored. Analysis of multiple variables (for example mouse strains, proteins, and time) ANOVA was used following Tukey’s multiple comparison test to determine statistical significance. To identify correlations between S100A8 and CXCL5 and *M. tuberculosis* burden; and S100A8 with CXCL5, Spearman’s test was used. All data with a p-value of 0.05 or lower was deemed to be statistically significant. All of the final graphs presented were made using Prism 8.
RESULTS

The purpose of this MQP was to determine whether the concentrations of neutrophil-related chemokines S100A8 and CXCL5 correlate with \textit{M. tuberculosis} lung burden, or with each other, or with disease indicators in the eight founder strains. \textit{M. tuberculosis} burden was measured by colony forming units (CFU). The concentrations of S100A8 and CXCL5 were quantified by sandwich ELISAs, and correlated with \textit{M. tuberculosis} CFUs in lung samples. Eight different mouse strains, CAST/EiJ, PWK/PhJ, NOD/ShiLtJ, A/J, C57BL/6, NZO/HiLtJ, 129S1/SvImJ, and WSB/EiJ, now referred to as CAST, PWK, NOD, A/J, B6, NZO, 129, WSB respectively, were analyzed to mimic the wide-ranging genetic variance seen in the human population.

Before this project began, all mouse strains were infected with an average of 31 CFUs of aerosolized \textit{M. tuberculosis} Erdman at The Ohio State University. During those experiments, mice were weighed at least weekly, and 3-5 mice per strain were euthanized and lungs and serum collected at 20 days, 30 days, and 40 days post-infection. Lungs were processed to determine \textit{M. tuberculosis} CFUs. Figure-1 below shows the CFU results from this previous research. The A/J strain shows significantly higher \textit{M. tuberculosis} burden at day 30 post-infection relative to day 20 post-infection (p-value= <0.0001) and day 40 post-infection (p-value= <0.0001). The NZO strain showed significantly lower levels of \textit{M. tuberculosis} at day 40 post-infection relative to days 20 and 30 post-infection (p-values= 0.0049 and 0.0001).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{\textit{M. tuberculosis} Bacterial Burden in Mouse Lungs at 20, 30, and 40 Days Post-Infection. \textit{M. tuberculosis} CFUs from serially diluted lung homogenates of individual mice were assayed by growth on agar, and colonies counted after 3 weeks incubation at 37ºC. Results shown are averages of 3-5 mice per strain per time-point, and standard error. The first bar for each strain is 20 days post infection. The second bar is 30 days post infection. The third bar is 40 days post infection. The statistical significance between time points was done through a Tukey’s multiple comparison test and is denoted by the asterisks.}
\end{figure}
All of the mice were weighed in grams at days 20, 30, and 40 days post-infection. Figure-2 shows the average weights of all the strains over time. All eight of the strains gained weight over time, with the CAST strain showing statistically significant weight gain when comparing the weight at day 40 post-infection with days 20 and 30-post infection (p-values = 0.0007 and 0.0075 respectively). No weight data was available for WSB. The weight increase corresponds with a decrease in *M. tuberculosis* burden in CAST, NOD, B6, NZO, and 129.

**Figure-2: Weight (grams) of mice at 20, 30, and 40 Days Post-Infection.** This figure shows the average weight in grams over time. The error bars represent the standard error of the mean. The first bar for each strain is 20 days post-infection, the second bar is 30 days post-infection, and the third bar is 40 days post-infection. The statistical significance between time points was done through a Tukey’s multiple comparison test and is denoted by the asterisks.
The concentrations of neutrophil-related chemokines S100A8 (Figure-3) and CXCL5 (Figure-4) present in the lungs of *M. tuberculosis* infected mice were assayed by sandwich ELISAs. The average concentrations are shown in pg/mL at 20 days, 30 days, and 40 days post infection. For S100A8, 7 of the 8 strains tested showed an increase at day 30 post-infection relative to day-20, and the increase is statistically significant in the CAST and A/J strains (p-values= 0.0065 and 0.0140 respectively). The pattern of increasing S100A8 at day 30 post-infection reflects the pattern of *M. tuberculosis* in only two strains, PWK and A/J. In the other 6 strains the patterns of S100A8 and *M. tuberculosis* were not similar.

**Figure-3: Lung Concentrations of Protein S100A8 (pg/mL) in Mouse Lungs at 20, 30, and 40 Days Post-Infection.** The average lung concentrations of S100A8 measured by ELISAs in pg/mL over time. The error bars represent the standard error of the mean. The first bar for each strain is 20 days post-infection, the second bar is 30 days post-infection, and the third bar is 40 days post-infection. The statistical significance between time points was done through a Tukey’s multiple comparison test, and is denoted by the asterisks.
For protein CXCL5 (Figure-4), 4 of 8 strains increased in CXCL5 over time (NOD, A/J, B6, and WSB). This pattern corresponds with a decrease in *M. tuberculosis* in NOD and B6. The remaining 6 strains do not show a similarity in pattern between CXCL5 and *M. tuberculosis*. Two strains showed a decreased in CXCL5 over time (NZO and 129) with it being statistically significant at days 30 and 40 post-infection relative to day 20 post-infection (p-values= 0.0402 and 0.0020 respectively). CAST and PWK show a trough at day 30 post-infection which is statistically significant in PWK when compared to day 20 and day 40 post-infection (p-values= 0.0277 and 0.0010 respectively).

*Figure-4: Lung Concentrations of Chemokine CXCL5 (pg/mL) Determined by ELISAs at 20, 30, and 40 Days Post-Infection.* The average concentrations (pg/mL) of CXCL5 measured by ELISAs in lung samples over time. The error bars represent standard error of the mean. The first bar for each strain is 20 days post-infection, the second bar is 30 days post infection, and the third bar is 40 days post-infection. The statistical significance between time points was done through a Tukey’s multiple comparison test, and is denoted by the asterisks.
Plots of the levels of *M. tuberculosis* lung burden (CFUs, X-axis) versus lung concentrations of neutrophil-related chemokines CXCL5 and S100A8 (pg/mL, Y-axis) at days 20, 30, and 40 post-infection are shown in Figure-5. Each color represents a different mouse strain, and matches the color coding in the previous figures. All 8 mouse strains show no statistically significant correlations when analyzing all 8 strains together.

![Figure 5: Correlation of Lung M. tuberculosis Burden vs CXCL5, M. tuberculosis Levels vs S100A8; and CXCL5 vs S100A8, for All 8 Mouse Strains at each time point. Panels A-C show CXCL5 versus *M. tuberculosis* burden at days 20, 30, and 40, respectively. Panels D-F show S100A8 vs burden at days 20, 30, and 40, respectively. Panels G-I show CXCL5 vs S100A8 at days 20, 30, and 40, respectively.](image)

Lung lobes of all the mouse strains were sectioned at Tufts University Cummings School of Veterinary Medicine and stained with hematoxylin and eosin. These samples were then examined under 4X magnification (Figure-6). The lung lobes of each strain were examined at 30-days post-infection, except the WSB sample was taken at 20 days post-infection. The areas that are stained a dark purple are the areas of inflammation from *M. tuberculosis*. PWK, A/J, and B6 show the largest area of inflammation at 4X magnification. The high area of inflammation corresponds with a large *M. tuberculosis* burden in A/J, but not the other strains. CAST and 129 show the smallest area of inflammation in the lungs at 30-days post-infection.
Figure-6: Light Microscopy of Lung Samples of Each Strain at 4X Magnification. This figure shows a lung sample seen at 4X magnification for each of the strains. For all of the panels, the dark purple staining is the area of inflammation. Panel A: Cast, Panel B: PWK, Panel C: NOD, Panel D: A/J, Panel E: B6, Panel F: NZO, Panel G: 129, and Panel H: WSB. All of the lung samples were taken at 30-days post-infection, except for WSB which was taken at 20-days post-infection.
DISCUSSION

Based on previous work in our laboratory and others (Beamer et al., 2008; Smith et al., 2016), we hypothesized that some inbred founder mouse strains would develop weight loss, and that *M. tuberculosis* bacterial burden would correlate with S100A8 and CXCL5 in the lungs. The latter was expected because S100A8 is secreted as an inflammatory response, and CXCL5 recruits neutrophils to the lungs during *M. tuberculosis* infection and is associated with lung damage. However, in the panel of eight inbred strains analyzed in this project (CAST, PWK, NOD, A/J, B6, NZO, 129, and WSB), no correlations were identified (Figure-5). For both S100A8 and CXCL5, the patterns of 6 out of the 8 strains did not match with a pattern seen in *M. tuberculosis* burden, and the strains that did correspond with patterns seen in *M. tuberculosis* were not the same for both S100A8 and CXCL5 (Figure-1, Figure-3, Figure-4).

Previous research performed at the University of Massachusetts Medical School (UMMS) examined lung CFUs in the same 8 inbred mouse strains (CAST, PWK, NOD, A/J, B6, NZO, 129, and WSB) infected with 50-200 CFUs of *M. tuberculosis* H37Rv. In this research (Smith et al., 2016), the CFUs were examined at 3 weeks and 6 weeks post-infection, except for WSB which was examined at 3- and 4-weeks post-infection. All 8 strains showed an increase in *M. tuberculosis* CFUs over time with a significant difference only in the WSB strain (Smith et al., 2016). This result contradicts the findings presented in this MQP which shows an increase in only 3 strains (PWK, A/J, and WSB) (Figure-1). The research provided by Smith et al. also showed a weight loss in the 129 strain after 3 weeks, and in A/J and PWK strains after 6 weeks (Smith et al., 2016). On the other hand, this project shows a weight gain in all the strains for the entirety of the study.

This lack of agreement between our studies here and those of Smith et al may reflect differences in the initial dose of infection, the strain of *M. tuberculosis* used, or procedures and equipment used to infect the mice across institutions. The two institutions performing these two studies indeed used different strains of *M. tuberculosis* and different doses of *M. tuberculosis*. The Ohio State University used an average of 31 CFUs of *M. tuberculosis* Erdman dose to initially infect mice, while UMMS reported that mice were infected with a higher dose: 50-200 CFUs of *M. tuberculosis* H37Rv (Smith et al., 2016). Both institutions used a Glas-col whole body exposure aerosol machine (Smith et al., 2016; Beamer et al., 2008).

The Beamer laboratory at Tufts University Cummings School of Veterinary Medicine previously published work using the DO population of mice infected with 100 CFUs of *M. tuberculosis* Erdman (Muhammad et al., 2015), and 25 CFUs of *M. tuberculosis* Erdman (unpublished observations) using a nose-only aerosol exposure administered by a CH Technologies aerosol-generating machine. Both published and unpublished results determined that 20-50% of the DO mice developed morbidity (confirmed to be TB lung disease) within 20-40 days post-infection, and substantial weight loss in that time frame that correlated with high *M. tuberculosis* burden, and with CXCL5 and S100A8. Therefore, we expected that this project
would show similar results because the eight founder strains used in these experiments are the parents of the DO population, and the mice were infected with the same dose and strain of *M. tuberculosis*. This could reflect differences in *M. tuberculosis* susceptibility between inbred strains (composed of genetically identical individuals and homozygous at all loci) and outbred population (genetically distinct individuals that are heterozygous at most loci). The main difference between the experimental procedure at Tufts University and The Ohio State University was in the machine that delivered *M. tuberculosis* strain Erdman: CH Technologies nose-only exposure at Tufts versus Glas-col whole body exposure at The Ohio State. The main difference in the outcomes were that some DO mice developed a diseased state of TB, characterized by early morbidity and weight loss. In contrast, none of the eight inbred founder strains used in this project lost weight and all gained weight (Figure-2). Our results here did successfully detect and quantify CXCL5 and S100A8 in the lungs of the 8 founder strains, but the levels did not correlate with *M. tuberculosis* burden. Taken together, these results suggest that S100A8 and CXCL5 are present in the lungs during *M. tuberculosis* infection, and that levels accumulate prior to weight loss due to pulmonary TB. We now speculate that weight loss needs to occur in order for a correlation to be seen between the chemokine concentration and *M. tuberculosis* CFUs.

Since no correlations were found during this project, this has raised several questions for future research, including: 1) Comparing the two types of aerosol-generating equipment for delivering *M. tuberculosis* (Glas-col whole body expose and CH Technologies nose-only exposure) in the 8 strains investigated in this project and in the DO mice. 2) Discovering additional host biomarker(s) that could correlate with and/or predict disease; 3) Investigating the upstream control of S100A8, CXCL5, and *M. tuberculosis* CFUs; and 4) Pursuing studies to determine whether weight loss is necessary for seeing a correlation between *M. tuberculosis* CFUs vs S100A8 or CXCL5; 5) Performing the infections in transgenic mice lacking specific components of the immune system to determine which host responses are important for containing the infections.
BIBLIOGRAPHY


