Elucidation of the role of S100A8/A9 and neutrophils in chronic tuberculosis

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Elucidation of the Role of S100A8/A9 and Neutrophils in Chronic Tuberculosis

By

Ninecia Roshonda Scott

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The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
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ABSTRACT OF THE DISSERTATION

Elucidation of the role of S100A8/A9 and neutrophils in chronic tuberculosis

By

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Professor Shabaana Khader, Chair

Tuberculosis (TB), caused by Mycobacterium tuberculosis (Mtb), is the number one killer due to a single infectious agent. Although, there is a vaccine against TB and antimicrobial treatments available, approximately 1.8 million people die each year. This underlines the importance of identifying and understanding mediators of TB pathogenesis to develop new diagnostics for early detection and therapies to treat individuals progressing to active disease. One of the cellular mediators that may play a role in TB pathogenesis are neutrophils. Neutrophils have been associated with active Tuberculosis (ATB); however, the role that neutrophils play within Mtb infection and disease is still unknown. To further understand the role that neutrophils play within TB pathogenesis, neutrophils were depleted in the acute and chronic stages of infection. Neutrophil depletion did not impact susceptibility during acute stages; however, susceptibility was significantly decreased during chronic stages. This suggests neutrophils promote Mtb infection and pathogenesis during the chronic stage of infection. This body of work demonstrates that decreasing neutrophil accumulation in the lung and promoting host protection can be mediated through neutrophil proteins, such as S100A8/A9, as well as TGF-β, an anti-inflammatory cytokine. Results demonstrate that S100A8/A9 promotes chronic increases
susceptibility and may be mediated through CD11b dependent lung neutrophil accumulation and \textit{Mtb} uptake. While not a neutrophilic protein, TGF-\(\beta\) inhibition significantly decreases susceptibility and neutrophil accumulation as chronic disease progressed. Overall, these results provide evidence that S100A8/A9 and TGF-\(\beta\) contribute to neutrophil mediated accumulation and \textit{Mtb} infection and pathogenesis.
Chapter 1

Introduction to tuberculosis and neutrophils

1.1 TB: A Global Burden

Mycobacterium tuberculosis (Mtb), an intracellular bacterium, is implicated as the causative agent of the disease tuberculosis (TB). Approximately one fourth of the world's population (1.3 billion people) is latently infected with Mtb (1, 2). Roughly 10% of infected individuals can progress towards active TB (ATB) disease. While the overall TB incidence is decreasing at an approximate rate of 2% each year, there is still a quarter of the world’s population who are at risk of progressing to active disease (2). The majority of infected individuals are diagnosed and treated for ATB; however, an estimated 5.6 million infected individuals are believed to be undiagnosed by the current limitations of the diagnostic tests and are therefore left untreated, posing a risk for disease spread. Despite the use of M. bovis Bacillus Calmette–Guérin (BCG) as a preventative vaccine, TB continues to cause 1.3 million deaths each year (2). According to the World Health Organization (WHO), identification of new treatment strategies that decreases latently infected individuals from progressing to ATB and development of rapid diagnostic testing is of priority, along with the generation of an effective TB vaccine (2). In order to meet these goals, it is necessary to understand immune responses that provide humans with protection against Mtb infection. Thus, research directed towards deciphering the protective host immune mechanisms and identification of biomarkers of protection are critical for effective translational applications including developing novel therapeutics and reliable diagnostics.

1.2 TB Disease Outcomes

TB is spread to others through the inhalation of Mtb laden aerosols. Once inhaled, Mtb is able to replicate and localize and/or disseminate from the lungs (3). The dynamic disease spectrum of
*Mtb* infection is continuous and best characterized as follows: clearance, latency, reactivation and active status. Host response plays an essential part in determining whether an individual is able to effectively clear the infection, control the infection or progress to active disease states. The innate and adaptive host immune responses are able to influence each of these outcomes. Alveolar macrophages and neutrophils have been suggested to be important for influencing early control and clearance (4-9). For the adaptive immune response, T cells and IFN-γ production have been important for *Mtb* control (10, 11). Granulomas, a hallmark characteristic of TB, are thought to be protective and functions to confine *Mtb* growth and prevent *Mtb* spread. Although granulomas can be an indicator of protection, granulomas can promote pathogenesis by creating an environment favorable for *Mtb* growth (12, 13). Similar to the outcomes of TB, granuloma formation, organization and its ability to control *Mtb* can promote pathogenesis or protection depending on the immune cellular responses and composition.

1.2.1 *Mtb* Clearance

Individuals exposed to *Mtb* but are capable of successfully clearing the pathogen may not have a positive tuberculin skin test (TST) or interferon-γ release assay (IGRA) results (14-17). Early clearance can be denoted as individuals who have cleared *Mtb* before the successful mounting of an adaptive immune response (18). Although there are several innate cellular populations, alveolar macrophages and neutrophils have been implicated in protection and disease progression (4, 19-21). It has been suggested that alveolar macrophages may play a role in early clearance (4, 22). Upon infection alveolar macrophages are amongst the first cells to recognize and respond to *Mtb* by producing pro-inflammatory cytokine and chemokines as well as secreting antimicrobial peptides (4, 7-9, 22, 23). Neutrophils and their cell specific proteins also contribute to effective *Mtb* killing (5, 6). Thus, an effective host innate immune response is
capable of controlling *Mtb* growth and the progression of TB disease. In addition to innate immunity, the adaptive immune response, especially IgM antibodies have been implicated in “resisting” progression to latent disease in individuals who are highly exposed to *Mtb* (24). In a recent 2019 study, a cohort of Ugandan individuals who were highly exposed to *Mtb*, yet were not positive for TB (via TST and IGRA), possessed IgG and IgM antibodies against *Mtb* proteins and IFN-γ independent T cell responses (24). *Mtb* clearance has been shown to be dependent and independent of the innate and adaptive immune response. Further research needs to continue to elucidate the mechanisms that promote and enhance *Mtb* clearance.

### 1.2.2 Latent TB

Individuals who have untreated active TB (ATB) are the source for transmitting aerosols to uninfected and susceptible individuals, thus increasing individuals that can become latently infected (25). Those who are latently infected (LTBI) are classified as having T-cells that are sensitized to *Mtb* antigens and do not have clinical signs and symptoms (3). T-cell sensitization is measured using the TST or IGRA (3). Quantiferon-TB Gold, an IGRA in vitro blood assay, tests an individual’s sensitization to *Mtb* antigens like early secretory antigen target-6 (ESAT-6), culture filtrate protein 10 (CFP-10) amongst others (26). Even though these tests are valuable at determining those who have been exposed to *Mtb*, they are unable to determine if the infection is prior, persistent or static. Low neutrophil blood counts are associated with IGRA positivity status in TB contacts -indicating neutrophils may have a role in control or pathogenesis (6). Historically, latency has been thought to be due to entrapment of *Mtb* within granulomas, decreased *Mtb* metabolism and *Mtb* replication (27). Although it is possible to identify individuals that are latently infected, it is important to screen those who would most likely progress to active disease and treat them prophylactically with anti-TB therapeutics.
1.2.3 Reactivation and Active Infection

Latently infected individuals have a 5 to 10% risk of reactivating and progressing to active disease (28). Reactivation may be associated with *Mtb* strains (geographically located) and disease severity at the time of diagnosis (29, 30). In the case of latency, the immune system mounts a robust response to contain the infection and *Mtb* prevents itself from being cleared. However, when the immune response is not sufficient in controlling the infection, reactivation occurs (31). Additionally, *Mtb* coinfection with HIV increases an individual’s risk of developing TB by 26-31 times (32). Diabetes, a comorbidity often associated with TB, enhances an individual’s risk of developing TB by three times (32). Even though loss or overabundance of protective immune responses, such as the production of TNF-α and anti-TNF-α immunotherapy, has been shown to increase bacterial burden and negatively impact granuloma structure and pathology, it has also been demonstrated to lead to reactivation (33-38). Although there are multiple animal models that aid in understanding mechanisms of reactivation, the NHP model is the gold standard. The NHP model is considered as the gold standard because NHPs are susceptible to the same *Mtb* strains that cause human pathogenesis, exhibit similar clinical signs, symptoms and histopathology, able to embody TB disease outcomes, have similar triggers needed for human *Mtb* reactivation, are genetically diverse and have similar immune responses as humans (39, 40). Although mice have similar immunological responses to humans and NHPs, the mouse model typically assesses *Mtb* persistence instead of reactivation. The Cornell model, a mouse model of persistent infection, treats *Mtb* infected mice with anti-mycobacterial drugs to undetectable levels and allows the growth of persistent *Mtb* to spontaneously occur (41).

Tuberculosis can be generally characterized by three phases – subclinical, prediagnostic and clinical (42). The subclinical phase is composed of individuals who may be TST or IGRA negative
but have positive bacterial cultures and lack symptoms (42-44). Whereas, individuals in the pre-diagnostic phase have symptoms but do not seek medical attention and treatment. The clinical phase is where individuals have symptoms and seek treatment (42). Although each of these phases has distinct differences, TB disease progression continues through each of these phases. The innate and adaptive immune responses aim to mount a sufficient response to control \textit{Mtb} growth and infection. Macrophages (innate immune cell) first aim to control \textit{Mtb} infection by phagocytosing \textit{Mtb} directly or indirectly via other \textit{Mtb} infected cells (e.g. neutrophils), and processing \textit{Mtb} antigens. If the macrophage is unable to kill \textit{Mtb}, \textit{Mtb} replicates resulting in the death of the macrophage. Dendritic cells (innate immune cell) are a bridge between the innate and adaptive immune system because they are able to phagocytose \textit{Mtb} and traffic them to a T cell rich lymph node where T cells (adaptive immune cell) recognize bacterial antigens and initiates the adaptive immune response (45). Once T cells recognize \textit{Mtb} antigens, they proliferate and migrate from the lymph node to the lungs by activating macrophages to kill \textit{Mtb} using T-cell specific cytokines (e.g. IFN-\(\gamma\) and TNF-\(\alpha\)) (35, 46-49). The arrival of CD4\(^+\) and CD8\(^+\) T cells capable of producing and secreting IFN-\(\gamma\), correlates with AB (12, 50, 51). Both, IFN-\(\gamma\) and TNF-\(\alpha\), have been demonstrated to be important in the control of TB by human and mice studies that show deficiencies in IFN-\(\gamma\) and TNF-\(\alpha\) production as well as T cells increase TB susceptibility, promotes \textit{Mtb} growth and impacts granuloma formation (35, 46, 50-52).

\textbf{1.2.4 Animal models of TB disease}

Animal models are widely used to study TB disease pathogenesis and disease outcome (53). Clinical signs, systemic bacterial burden, disease progression and protective/pathological immune factors are some of the features animal models help elucidate (53). The most common animal models of TB are mice, guinea pigs, rabbits and non-human primates (53). The ease of genetic
manipulation, cost and availability of humanized mice makes the mouse model of TB disease particularly good to assess anti-TB chemotherapy treatments and understand mediators of protection and pathogenesis (54-59). Due to their susceptibility to aerosolized Mtb and the ability to form human-like granulomas guinea pigs are good models to assess the efficacy of vaccines (53, 60). Similar to guinea pigs, NHPs also are susceptible to Mtb and are able to form humanized granulomas, but NHPs are a great model because they are able to undergo wide spectrum of diseases - latent, active, reactivation, and clearance, which has not been observed in guinea pigs (61). Thus, making NHPs a good model for understanding mechanisms of disease, assessing the efficacy of anti-TB treatments and vaccines (62-65). In this work, I have used the NHP and mouse models of Mtb-infection to further determine how neutrophils impact Mtb infection and TB disease.

1.3 Innate immune response

The immune system is made up of two arms namely the innate and adaptive arms that are able to respond to pathogens invading the host. Innate immune cells, macrophages, neutrophils and dendritic cells, has been implemented in providing protection against contracting Mtb and preventing active disease conversion (18, 66). Before the adaptive immune response is activated, the innate immune system responds to Mtb. Mtb is recognized by pattern recognition receptors (PRRs) (e.g. TLR2, TLR4, DC-SIGN, etc.) expressed on macrophages, dendritic cells (DC), neutrophils and natural killer (NK) cells leading to the release of pro-inflammatory chemokines, cytokines (e.g. IL-6, IL-1β, and TNF-α), and damage associated molecular patterns (DAMPs) (67-70). The initiation of this response results in alveolar macrophages, neutrophils, recruited macrophages and DCs migrating towards the lung site of infection to aim to kill Mtb, initiate granuloma formation to contain Mtb and promote the mounting of an adaptive immune response.
Although, the adaptive response is important to controlling *Mtb*, the innate response is necessary to prime the adaptive response (70). One of the main components of granulomas and an immune cell play within TB is the macrophage.

Macrophages play a significant role in *Mtb* pathogenesis because *Mtb* has been shown to infect this population during both early and chronic infection (71, 72). Macrophages have the ability to limit *Mtb* growth through the production of reactive oxygen species (ROS), cytokines and phagolysosome maturation as well as other cellular processes. *Mtb* growth and control is mitigated in macrophages when *Mtb* is able to evade phagosome maturation and lysosomal degradation (73). Studies have shown that receptors, specifically the mannose receptor, which recognize *Mtb* can impair phagosome maturation resulting in the survival of *Mtb* in the macrophage cellular environment (74, 75). When *Mtb* is unable to prevent phagosome maturation, it is localized within the phagolysosome compartment. The phagolysosome compartment is an acidic and nutrient deficient environment filled with anti-microbial peptides to limit *Mtb* growth (76, 77). During this maturation process, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is recruited and assembled to generate ROS (78, 79). ROS has been shown to limit growth in *Mtb* mutant strains deficient in *sodA* and *katG* (enzymes that counteract ROS effects) (80). Macrophages are able to produce cytokines, such as IL-1β, TNF-α, and IL-12, which have been demonstrated in human and mouse studies to contribute to *Mtb* control (81-84).

While macrophages are able to sense *Mtb*, respond to an infection and kill *Mtb*, dendritic cells (DCs) are able to prime the adaptive response through antigen presentation. DC-specific ICAM-grabbing non-integrin (DC-SIGN), Dectin-1, TLRs and Nod-like receptors are receptors expressed on DCs, that are able to recognize *Mtb* (85-90). After the recognition of *Mtb*, DCs are able to
process its antigens, migrate to the lymph nodes, and present them to, prime and activate T cells (91, 92). This priming of T cells by DCs initiates the specific adaptive response driven by T cells. After alveolar macrophages recognize and respond to \textit{Mtb}, neutrophils migrate to the lung and respond by releasing DAMPs (e.g. S100A8/A9), neutrophilic proteins (e.g. myeloperoxidase (MPO) and elastase) and chemokines and cytokines (93-95). The release of some of these proteins has led to \textit{Mtb} control (96). Unfortunately, by responding to \textit{Mtb} infection, neutrophils are also able to promote immunopathology within the lung (97). Although, many studies have focused on the cells of the innate immune system that contribute to \textit{Mtb} infection, disease and pathology, the role that neutrophils play in TB immunopathology is just beginning to be delineated. This thesis will mainly focus on the role that neutrophils perform TB pathogenesis.

\textbf{1.4 Introduction to neutrophils}

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are innate immune cells and are cellular components of the inflammatory immune response (98). The immune system is capable of inducing an inflammatory response that aids in responding to pathogens (70, 99). Although, neutrophils are associated with homeostasis and pathogen clearance, they are also associated with inflammatory diseases, such as acute lung injury, chronic obstructive pulmonary disease (COPD) and arthritis (100-103). They are short lived and are the most abundant leukocyte in the blood (104-106). They are characterized by the presence of granules in the cytoplasm and their nuclear structure. Neutrophils are able to develop and mature in the bone marrow and migrate and respond to infections throughout the body.

\textbf{1.4.1 Neutrophil migration}

Neutrophils are derived from myeloid precursors in the bone marrow through the process of granulopoiesis (70, 99). Neutrophils are produced and matured within the bone marrow from
myeloid lineage progenitor cells, specifically common myeloid progenitor cells (CMPs) (99). Eventually CMPs differentiate to become a granulocyte macrophage progenitor cell (GMP) (107-110). Maturation in humans takes approximately 5-6 days; whereas, 2-3 days in rodents (111-113). Upon maturation, they are released and make up approximately 50-70% of leukocytes in circulation (105, 112, 114). Once in circulation, neutrophils are able to migrate towards sites of infection and respond to microbial pathogens (115). There are two neutrophil pools within the vasculature – an intravascular blood and tissue specific pool (104, 116-118). Intravascular blood and vasculature neutrophils are different due to changes in the cytoskeleton and actin formation (119). Neutrophil migration from the bone marrow into the blood is largely affected by actin cytoskeleton rearrangement (120). Cytoskeleton rearrangement is critical to the migration of neutrophils on endothelial surfaces, which is mediated through selectins (e.g. P-selectin) and integrins (e.g. LFA-1 and Mac-1) expressed on neutrophils that bind to the endothelium adhesion molecules (e.g. ICAM-1) (120-122). This selectin mediated interactions allows for neutrophil to migrate across the vascular endothelial surface. CXCR4 is an important chemokine receptor needed in mice and primates for neutrophils to migrate to the lung (104, 123). There are studies that suggest that the lung has a resting population of neutrophils that may be used during a need for rapid mobilization (104). Under homeostatic conditions, neutrophils undergo apoptosis and are removed by mononuclear phagocytes (e.g. macrophages) or return to the bone marrow via a CXCR4-dependent mechanism for clearance (115, 124-126).

1.4.2 General neutrophil responses

After migrating to a site of infection, neutrophils have several mechanisms of recognizing and responding to pathogens. Neutrophils are able to respond to infection via: 1) phagocytosis, 2) degranulation, 3) deploying neutrophil extracellular traps (NETs), and producing chemokines and
cytokines (127, 128). Neutrophils are able to recognize and phagocytose microbes via Fc-γ, complement receptors and toll-like receptors (TLRs) (70, 115, 129, 130). Neutrophil pathogen recognition and phagocytosis induces changes in cellular surface receptors and molecules, generates ROS and causes the degranulation of antimicrobial peptides and enzymes. Generally, when pathogens fuse to neutrophil phagosomes they are either fused with granules that contain antimicrobial peptides and proteases, initiate NADPH-oxidase to produce superoxide anions, hydrogen peroxide, and ROS (70, 99, 115). In addition to their phagocytosis abilities, they also use defense mechanisms such as traps NETs (70, 115, 121, 122, 127, 131). NETs aid in immobilizing and killing microbes by releasing chromatin fibers from the nucleus that also contain granules rich in antimicrobial proteins (132-134). Often times, antimicrobial peptides and proteases are also damaging to the host by inducing inflammation and tissue damage (115). Neutrophil activation and priming is highly modulated through cytokines, chemokines and growth factors, such as pathogen associated molecular patterns (PAMPs), TNF-α, IFN-γ (70, 115, 135).

1.5 Neutrophils and tuberculosis

1.5.1 Neutrophil studies and other models

Human and murine neutrophils have differences that may play a role in the interpretation of experimental data. As stated above, maturation rates are different as well as the percentage of neutrophils in the blood, morphology, granule components, chemokine and chemokine receptor expression, as well as surface marker expression are different in humans versus murine systems (99, 136-139). Studies to determine the role of neutrophils during Mtb infection have been done in various animal models (e.g. rats, guinea pigs, zebrafish, etc.). Neutrophils have been described and defined based on characteristics, such as cell-surface markers, functions, localization and maturity in both healthy and infected individuals (Silvestre-Roig C 2019). Staphylococcus aureus
and *Candida albicans* studies have shown that neutrophil subpopulations are present during infection that are either proinflammatory (CD11b⁺CD49d⁺IL-12⁺) or anti-inflammatory (CD11b⁻CD49d⁻IL-10⁻) (140). Most studies have been done with human peripheral neutrophils, which are at their resting stage (137). This can present disadvantages for understanding the functional aspects of neutrophils during infection. One difference is that neutrophil surface phenotype and functional activity depends on the genetic background of the model studied – mouse or human. When taking human neutrophils genetically heterogeneous populations as well has large sample sizes need to be considered.

**1.5.2 Impact of neutrophils on TB infection and disease**

As noted in literature, neutrophils are activated to respond to TB infection and disease, but their definitive role within *Mtb* infection is currently unclear and controversial. As with most bacterial infections, neutrophils are the first immune cells recruited to the lung during *Mtb* infection (22, 115, 141-143). Neutrophil recruitment during the acute phase typically subside in pulmonary TB (PTB) contacts after 6 weeks (6). This indicates that there is an acute inflammatory response that occurs in response to *Mtb* infection (6). The roles of neutrophils are controversial because many studies provide evidence that indicate neutrophils can contribute to both protection and pathology within *Mtb* infection and disease. The caveat to these studies is that the RB6-8C5 antibody depletes or reduces the number of cells expressing the Gr-1 (Ly6G/Ly6C) cellular marker, which includes neutrophils, plasmactoid dendritic cells, monocytes and CD8⁺ T cells; thereby, making it unclear whether neutrophils are protective during *Mtb* infection. To determine if neutrophils impacted *Mtb* infection, studies have used RB6-8C5 depleting antibody, which depletes neutrophils, to transiently deplete neutrophils during early stages of *Mtb* infection. RB6-8C5 neutrophil depletion during early *Mtb* infection increased bacterial burden. This finding suggested
that neutrophils are beneficial during *Mtb* infection (20, 144, 145). To rectify the ambiguity of whether neutrophils are protective during *Mtb* infection, two studies used 1A8 (Ly6G), a neutrophil specific antibody to deplete neutrophils during early *Mtb* infection. Both studies showed no difference in bacterial burden (142, 143). However, the study by Blomgran and colleagues indicated neutrophils play an indirect role in immune function by demonstrating that when neutrophils are depleted dendritic cell migration to the local lymph node decreases and delays in the adaptive response occur by decreasing *Mtb* antigen specific CD4+ T cell proliferation and activation. CD4+ T cell lung proliferation and activation due to the lack of neutrophils was also shown in the Kang et al. study (143). The two previous studies specifically depleted neutrophils and demonstrate the impact of neutrophils; however, both used lab adapted *Mtb* strains. In addition to lab strains, hypervirulent (capable of increased levels of resistance to drugs, increased pathogenesis and transmissibility) *Mtb* strains are emerging which may provide to be more informative in investigating the protective or pathological contributions of neutrophils during *Mtb* infection and disease (146-149).

1.5.3 Neutrophil Mechanisms in TB

One of the primary effector functions of neutrophils in response to a bacterial infection is to phagocytose bacteria. Neutrophils express pattern recognition receptors (PRRs), such as TLRs, C-type lectins, Fcγ and complement receptors to aid in survey, recognize and respond to *Mtb* and other pathogen invaders (70, 115, 130, 150). When localized in the lung, TLR2, 4 and 9 recognize and phagocytose *Mtb* (71, 151-153). Fc receptors, FcγRII, FcγRIIib and FcγR1, in addition to complement receptors are also present on neutrophils and are highly expressed in neutrophils of TB infected individuals (130, 154). Several studies have shown that neutrophils phagocytose *Mtb* (5, 155, 156). Studies have shown that after neutrophils recognize and phagocytose *Mtb*, they
undergo neutrophil apoptosis as a mechanism of controlling *Mtb* growth (115, 157). When a neutrophil undergoes apoptosis, they have decreased cell function, and reduced antimicrobial and pro-inflammatory capacities (115, 158, 159). *Mtb* has been known to mediate neutrophil apoptosis by activating caspase-1,3 and decreasing FcγR1 surface expression (115, 160-162). Proinflammatory molecules that prolong neutrophil survival typically prime neutrophils for enhanced function (115). Apoptotic neutrophils have been shown to aid indirectly in killing *Mtb* via activated macrophage phagocytosis (96). Tan et. al. demonstrated that the addition of apoptotic neutrophils to *Mtb* infected alveolar macrophages resulted in increased antimicrobial activity (96). Neutrophils are able to secrete cytokines and chemokines (e.g. IL-6, CXCL-1, CXCL-10) that are able to recruit more neutrophils or enhance the innate immune response to microbes (163). Upon observing whether neutrophil infiltration associated with susceptibility, Eruslanov et al. demonstrated that early in infection neutrophils infiltration begins 1-week post infection (wpi) and peaked at 2-3 wpi in I/St mice infected with an high dose of H37Rv (155). Neutrophils are recruited throughout the acute and chronic stages of *Mtb* infection through the expression of CXCL-1 and CXCL-5 by IL-17-RA induction by non-hematopoietic cells (164). Neutrophils isolated from *Mtb* susceptible mice have higher migratory capabilities, survive longer and contain more intracellular mycobacteria (155). This suggests that neutrophils may provide a niche for *Mtb* to grow and hide from host immune response. *Mtb* susceptible (I/St mouse strain) and resistant (A/Sn mouse strain) neutrophil motility in response to *Mtb* cellular components or infected cells was found to be increased in *Mtb* susceptible mouse strains (155). *Mtb* infected resistant neutrophils were shown to be resistant to apoptosis (CD95+/Fas+) and have an impaired life span. In addition to motility, susceptible neutrophils (I/St) were able to phagocytose increased amounts of *Mtb* compared to resistant neutrophils (A/Sn) indicating that neutrophil host immunity can impact susceptibility
With increased neutrophil infiltration in the lung and lifespan, it has been suggested that neutrophils promote infection through the “Trojan Horse” model. The “Trojan Horse” model speculates that since neutrophils are able to take up Mtb, hide from the immune system and allow for its growth. Mtb induces death responses in neutrophils and when infected, neutrophils become apoptotic (annexin v+) and unable to kill Mtb (161, 165). Mtb has a unique set of genes (e.g. ESAT-6 and CFP-10) encoded in the RD1 (region of deletion 1) that is important for Mtb virulence and pathogenesis (165-170). Inhibition of neutrophil apoptosis and limiting of Mtb growth has been demonstrated to be RD1-dependent (165, 167). This finding demonstrates that neutrophils are able to limit Mtb growth within specific contexts or deficiencies in Mtb. Data discussed here also suggests that impaired neutrophil infiltration, motility, life span and resistance to apoptosis is beneficial for combating against susceptibility (155). Thus, it is important that neutrophil recruitment and elimination is balanced during infection and disease.

Studies have demonstrated that NETs are also present in active pulmonary tuberculosis patients compared to healthy controls (171, 172). In human neutrophils that are incubated with Mtb, the morphology of the neutrophil is changed – inducing aggregation and the release of fibrous material and granules (171). NETs are comprised of cellular DNA, histones and neutrophil granule proteins (70). Ramos-Kichik et al. demonstrated that NETs are capable to trapping Mtb but not able to kill Mtb (171). NETs immobilizing Mtb in an extracellular environment where neutrophils are also able to produce nitric oxide as well as its byproducts, hypochlorous acid and chloramines may aid in mediating Mtb control (70).

Although there have been many studies to determine whether neutrophils are able to kill Mtb, in vitro studies have not consistently concluded whether or not neutrophils are able to control Mtb growth. In the Mycobacterium marinum (Mm) zebrafish model, neutrophils have been shown to
aid in decreasing bacterial burden and control infection (21). When Mtb infected mice transiently depleted neutrophils and monocytes via administration of RB6-8C5 antibody, bacterial burdens were significantly decreased (20). This finding was also see in Mtb infected rats (173). These finding suggests that neutrophil depletion during early infection promotes Mtb growth. Individuals who have chronic granulomatous disease (CGD), a disorder where neutrophils are impaired, are susceptible to TB (174, 175). Even though there are studies that demonstrate neutrophils contribute to Mtb control, there are other studies that demonstrate the opposite. Berry et al. demonstrated that ATB individuals an enhanced IFN-inducible gene profile mediate by neutrophils (43). In many Mtb mouse studies increased neutrophil accumulation has been positively correlated with Mtb burden (155, 176). By identifying host and microbe factors that aid in neutrophils killing of Mtb researchers would be able to design treatments to target and enhance the neutrophil’s efficiency in killing Mtb.

1.5.4 Neutrophils and granulomas

Granulomas consist of myeloid and lymphoid cells that aggregate together to limit dissemination (177). Although this response functions to limit mycobacterium growth and dissemination, excessive lung damage also occurs. Neutrophil recruitment is not only associated with pathogenesis, but also TB granulomas (93, 172, 178, 179). The structure of the granuloma allows for immune cells to be in physical contact or influenced by chemokines and cytokines in order to mediate this defense. Having IFN-γ, IL-2, IL-12 and TNF-α localized within a granuloma promotes Mtb control within a confined area. TNF-α and IFN-γ are important in granuloma formation, maintenance and the induction of chemokine production (e.g. CXCL9, CXCL10 and CXCL11) (46, 180, 181). Granulomas formation does not occur visibly until immune responses are activated which in mice is typically three weeks from initial infection (182). Granulomas that
are protective and within resistant mice are classified as well organized; whereas, *Mtb* infected macrophages are surrounded by activated T cells, mainly Th1 and Th17 cells, which have become activated through dendritic cell priming (13, 183). Granulomas with ectopic lymphoid structures have been associated with protection and latently infected individuals (184). These structures can be described as a T cell encompassed Mtb infected macrophage center (184). In addition to ectopic lymphoid structures being associated with disease, decreased amounts of neutrophils and S100A8/A9 has also been described as protective characteristics of granulomas (93). In contrast, susceptible mice have loosely organized granulomas with random and sparsely distributed macrophages throughout the granuloma (183, 185, 186). These granulomas that lack structural organization and ectopic lymphoid structures are also associated with susceptibility, disease progression and transmission (184, 187). Granulomas during active disease typically have increased amounts of bacteria in them compared to latent granulomas (188). Lesions that have increased fibrous and calcified environment are associated with bacterial control; whereas, caseous granulomas are associated with active disease (188, 189). Necrotic granulomas are associated with higher bacterial burdens (13). Th1 responses have been demonstrated to drive granuloma necrosis (13). Foamy macrophages are typically a component of these types of granulomas and have defective phagocytosis, antigen presentation processing, have increased production of TGF-β, an immune suppressive cytokine and have been associated with disease progression (190, 191).

1.5.5 Chemokines and cytokines

Neutrophils are able to produce a wide array of chemokines and cytokines – ones that both are able to act on neutrophils in an autocrine manner as well as able to act on other immune cells (e.g. dendritic cells and macrophages) (70, 192, 193). G-CSF, IL-1β and TNF-α are cytokines made by neutrophils that have been shown to be important for neutrophil migration, degranulation and the
response of the neutrophil to infection (70, 129, 194-196). After *Mtb* is recognized by neutrophil cell surface receptors (e.g. TLR4), proinflammatory cytokine production (e.g. IL-1β) is induced (130, 197). IL-1β has been shown to increase phagocytosis and the anti-microbial activity of neutrophils and macrophages (198). *Mtb* infected mice lacking IL-1 receptor 1 (IL-1R1) were unable to control lung bacterial burden and had a significantly increased mortality rate (199). This limited *Mtb* control correlated with an increase in *Mtb* infected cells and inflammation, which included neutrophils (199). This suggests that IL-1 cytokine production and signaling could play an important role in *Mtb* infection. Additionally, Berry et al. showed that ATB individuals have an enhanced type I interferon gene profile that is driven by neutrophils (43). This finding provides evidence that neutrophil-dependent gene regulation and production may mediate TB disease.

In addition to pro-inflammatory cytokines, anti-inflammatory cytokines, specifically TGF-β, have also been shown to play a role in TB disease. Stimulated neutrophils have been shown to produce TGF-β (200). TGF-β has been associated with TB based on lung samples of individuals with ATB (201, 202). In the Aung et al study, data was shown that TGF-β was expressed in over half of patients that died within late stage ATB (201). Several studies have shown TGF-β protein levels are increased in TB patients, associated with TB reactivation and were decreased during anti-TB treatment (203).

### 1.5.6 Neutrophil proteins

Despite the evidence that suggests that neutrophil granules and proteins can kill *Mtb* and may be beneficial during *Mtb* infection, the presence of neutrophils and their proteins have also been shown to contribute to pathology during *Mtb* infection and disease. Studies have shown that exacerbated neutrophil accumulation is not protective and this finding may extend to neutrophilic proteins such as S100A8/A9 (176, 204-206). Neutrophils have a wide variety of bactericidal
proteins and peptides that are released into the infection environment (70). Human and mouse studies have explored the contribution of granule associated neutrophilic proteins to killing Mtb (70). Cathepsin G (CG), neutrophil elastase (NE), proteinase 3 (PR3), defensins, cathelicidin (LL-37), lactoferrin and lysozyme are amongst the proteins that have been interrogated (70, 207). Neutrophilic proteins, such as neutrophil human protein 1 (NHP-1), have been shown to directly and indirectly contribute to a decrease in bacterial burden. When NHP-1, a neutrophilic protein generated in humans, was given to mice intravenously infected with a high dose of H37Rv, lung bacterial burden decreased (208). Activated macrophages that phagocytose neutrophilic granules have been indirectly shown to be effective in killing Mtb (96). Interestingly, chronic granulomatous disease (CGD) neutrophils incubated with Mtb and demonstrated the ability to limit Mtb growth compared to normal neutrophils (174, 175). In general, the presence of neutrophilic proteins has been associated with protection and Mtb control. Lower neutrophil counts and protein levels of lipocalin-2 was associated with the Black African population and a higher susceptibility rate (6). When CG deficient mice (CGKO) were infected during the acute phase of infection, they had significantly higher amounts of bacteria in the lungs, but not the spleen or lymph nodes (LN) (207). This suggests that CG is important in controlling bacteria in the lungs during the acute stage. However when CG and NE are both deficient, lung bacterial burden is even higher than the CGKO, indicating that NE has an additive effect in controlling mycobacterial growth (207). The CG/NE double knockouts also have increased granuloma size and TNF-α protein levels compared to wildtype and CGKO infected mice (207). CG was demonstrated to have mycobactericidal effects, in both aerobic and anaerobic conditions, because bacterial burden was significantly decreased in cultures with CG (207). The Steinwede et al. have demonstrated that CG and NE proteins were beneficial in limiting Mtb growth when given in liposomes (207). Also, lower plasma protein
levels of human neutrophil protein 1, 2 and 3 is shown to correlate with an increase in MDR-TB (209). These findings suggest that neutrophils that are deficient in specific neutrophilic proteins are still able to control Mtb (174).

S100A8 (MRP8) and S100A9 (MRP14) are constitutively expressed in neutrophils in high amounts and can be often found as a heterodimer (S100A8/A9), which is known as calprotectin (93, 210, 211). Individually and as a heterodimer, these proteins have been shown to be TLR4 and receptor for advanced glycation end products (RAGE) ligands (212, 213). S100A8 and S100A9 have been shown to be important for neutrophil chemotaxis and have antimicrobial capabilities (214). S100A9 is able to increase neutrophil recruitment and CXCL-10, a neutrophil chemokine, in the lung (214). S100A9 and S100A8/A9 promote neutrophil recruitment to the lung of naïve mice (214). Deletion of S100A8 is embryonically lethal (215). S100A9 deficient mice are considered normal, but have lower amounts of S100A8 and are less responsive to chemoattractants (216, 217). When adenoviral S100A9 was given to TLR4 deficient mice, lung inflammation and pathology increased to wild-type levels (213). Gopal et. al. showed that S100A8/A9 proteins are associated with neutrophil accumulation and inflammation in human, NHP and mouse models (93). This finding is interesting and needs to be explored because other neutrophilic proteins are known to be protective, but S100A8/A9 enhances TB susceptibility (218-220). S100A8/A9 has been shown to be associated with increased susceptibility the role that S100A8/A9 in TB disease and infection has not been elucidated. This is a major focus of this thesis.

1.5.7 TB diagnostics and biomarkers

Currently, diagnosis of ATB is determined by a sputum culture, X-ray or Computed Tomography (CT) scan, TST and clinical signs (53). The gold standard for determining whether an individual is infected with Mtb is a sputum test, but takes approximately 3-4 weeks for
confirmation occurs (53). Although an estimated 54 million people have been diagnosed and treated for TB, there are still unidentified individuals that are infected that have not been identified (2). Studying neutrophilic proteins, specifically S100A8/A9, presents a unique opportunity to assess whether it would be a diagnostic biomarker for TB. It has been suggested that underdiagnosed individuals is due to lack of healthcare or lack of diagnosis (2). To aid this problem, the identification of optimal biomarker(s) would need to have a high specificity and sensitivity for determining individuals that are healthy, latently and actively infected and be available at a low cost. The identification of biomarker resulting to an earlier diagnosis of an individual with TB is extremely important for decreasing those impacted.
Chapter 2

S100A8/A9 regulates neutrophil recruitment during chronic tuberculosis

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**Conflict of Interest Statement**

T.J.S. is co-inventor of a patent of the 16-gene ACS signature. All other authors declare no competing financial interests

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2.1 Abstract

Neutrophil accumulation is associated with lung pathology during active tuberculosis (ATB). However, the molecular mechanism(s) by which neutrophils accumulate in the lung and contribute to TB immunopathology is not fully delineated. Using the well-established mouse model of TB, our new data provides evidence that the alarmin S100a8/a9 mediates neutrophil accumulation during progression to chronic TB. Depletion of neutrophils or S100a8/a9 deficiency resulted in improved *Mycobacterium tuberculosis* (*Mtb*) control during chronic but not acute TB. Mechanistically, we demonstrate that following *Mtb* infection, S100a8/a9 expression is required for upregulation of the integrin molecule CD11b specifically on neutrophils, mediating their accumulation during chronic TB disease. These findings are further substantiated by increased expression of *S100A8* and *S100A9* mRNA in whole blood in human TB progressors when compared to non-progressors. Importantly, we demonstrate that S100A8/A9 serum levels along with chemokines are useful as biomarkers to distinguish between ATB and asymptomatic *Mtb*-infected latent individuals. Thus, our results support targeting S100A8/A9 pathways as host-directed therapy for TB.

2.2 Introduction

*Mycobacterium tuberculosis* (*Mtb*), the causative agent of the disease tuberculosis (TB), is estimated to infect one fourth of the world's population and results in ~1.6 million deaths each year (2). In addition, the emergence of multi-drug and extensively drug resistant *Mtb* strains and variable efficacy of the currently used vaccine, *Mycobacterium bovis* BCG, are barriers to global control of TB. Thus, more research is needed to fully understand the mechanisms of TB immunopathogenesis that can be targeted to improve host control of *Mtb* infection.
Neutrophil accumulation has been associated with TB disease in humans and mouse models of TB (176, 221). Neutrophils are the primary Mtb-infected cell population in the sputum, bronchoalveolar lavage (BAL), and cavities of Mtb infected patients (222). In mouse models, neutrophils are amongst the first cells infected with Mtb, and are capable of recognizing and responding to Mtb infection and displaying effector functions (142, 151). Calprotectin, also known as S100A8/A9, is mainly expressed by myeloid cells, specifically neutrophils and monocytes, and is a feature of chronic inflammatory diseases such as autoimmune diseases and TB (93, 210, 211). S100A8 and S100A9 covalently form the hetero-complex protein S100A8/A9, and bind to receptors such as Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE), which mediates a pro-inflammatory response through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling (223, 224). We recently demonstrated that S100A8/A9-expressing neutrophils accumulated within TB granulomas in human and animal models of TB, and that S100A8/A9 serum levels coincided with increased neutrophil numbers and enhanced TB disease in active TB (ATB) patients (93). However, we did not fully understand the mechanism(s) by which S100A8/A9 mediates neutrophil accumulation that may promote TB disease severity. In the current study, we demonstrate that S100a8/a9 expression mediated accumulation of lung neutrophils in chronically Mtb-infected mice. Additionally, the observed accumulation is mirrored by increased S100A8 and S100A9 mRNA in whole blood transcriptional profiles of latently infected individuals (LTBI) who progress to ATB disease (progressors), when compared with individuals who do not progress to TB disease (non-progressors). These studies suggest that in animal models and human progressors, S100a8/a9 expression levels increase during TB progression or chronic TB disease. Consistent with a detrimental role for S100A8/A9, mice
depleted of neutrophils or deficient in S100a9 exhibit decreased Mtb burden in the lungs during chronic infection and TB reactivation. The improved Mtb control in S100a9KO mice coincided with reduced lung neutrophil accumulation within TB granulomas, and decreased expression of the integrin CD11b on neutrophils. Our mechanistic in vitro studies show that S100a9KO neutrophils have defective Mtb uptake, as well as reduced ability to upregulate CD11b integrin expression. Importantly, we demonstrate that S100A8/A9 serum levels along with chemokines are useful as biomarkers to distinguish between ATB patients and LTBI individuals. Our results suggest that targeting S100A8/A9 as host directed therapy in TB would provide novel pathways that can be used as adjunct therapy to improve Mtb control.

2.3 Results

Depletion of neutrophils during chronic but not acute TB improves Mtb control

A neutrophil associated transcriptional signature was reported in ATB patients and neutrophil accumulation was associated with TB disease in mouse models (93, 176, 221). Consistent with this, neutrophils accumulated in the blood during ATB but not in asymptomatic latency in macaques infected with Mtb (Figure 2.1A). However, it is unclear if neutrophils play distinct roles during the acute and chronic stages of Mtb infection. Thus, we studied the kinetics of neutrophil accumulation in the well-established mouse model of low dose aerosol Mtb infection in C57BL/6J (B6) mice. Neutrophils accumulated in the lung following infection with Mtb HN878 early, starting at 30 days post infection (dpi). Accumulation further increased during chronic stages of infection, starting around 60 dpi and continuing until 300 dpi (Figure 2.1B). As we observed increased neutrophil accumulation during both acute and chronic stages, we next addressed the functional role for neutrophils at the two distinct phases of Mtb infection. We found that treatment with 1A8 antibody, which specifically depletes neutrophils (225), during acute Mtb infection did
not impact overall lung and spleen \textit{Mtb} burden (Figure 2.1C). Treatment with 1A8 depleted lung neutrophils significantly, but also coincided with increased monocyte and macrophage populations and increased production of the chemokine, granulocyte-colony stimulating factor (G-CSF) (Figure 2.1D and Supplemental Figure 2.1A). However, despite changes in myeloid cell accumulation, the total inflammatory area measured in the lung was not significantly altered (Figure 1E). In contrast, 1A8 treatment during chronic \textit{Mtb} infection led to decreased \textit{Mtb} burden, in both the lung and spleen (Figure 2.1F). Neutrophil depletion during chronic TB did not impact other myeloid populations (Supplemental Figure 2.1B), but there was decreased production of pro-inflammatory cytokines, such as interleukin (IL)-6 and TNF-\(\alpha\) (Figure 2.1G). The area of lung cellular inflammation was still not impacted in mice when neutrophils were depleted during chronic infection (Figure 2.1H). These data together suggest that despite early accumulation of neutrophils, neutrophils do not significantly impact \textit{Mtb} control during acute stages of infection. In contrast, neutrophil accumulation during chronic \textit{Mtb} infection may have a functional role in promoting higher \textit{Mtb} burden and contributing to increased susceptibility.

\textbf{S100a8/a9 is induced during progression to TB disease in animal models and in humans}

S100A8/A9 makes up 40\% of the cytoplasmic protein of a neutrophil (226). In addition, we have shown that increased serum S100A8/A9 levels correlate with increased neutrophils in peripheral blood of ATB patients (93). Accordingly, we found that accumulation of S100a8/a9 levels in \textit{Mtb}-infected mouse lungs also closely followed the accumulation of neutrophil influx in the infected lung. Early increased production of S100a8/a9 was detected between 15-30 dpi and sustained production during chronic infection was observed after 30 dpi (Figure 2.2A). As TB disease progressed, S100a8/a9 levels increased, coinciding with the increased accumulation of neutrophils observed in chronically \textit{Mtb}-infected lungs (Figure 2.1B and 2.2A). Thus, we next addressed if
S100A8/A9 could be tested as a biomarker for TB disease progression in humans with LTBI. Therefore, we analyzed the whole blood transcriptional mRNA levels of S100A8 and S100A9 in participants enrolled in the Adolescent Cohort Study (ACS) of TB progressors and matched Mtb-infected controls (227). Transcript levels of both S100A8 and S100A9 mRNA significantly increased in the TB progressors about six months prior to TB diagnosis compared to expression levels in matched healthy controls (Figure 2.2B). Thus, our results show that both in mice and in humans, an increase in S100a8/a9 expression coincides with TB disease progression.

S100a9 deficiency improves Mtb control during chronic infection by limiting neutrophil accumulation

Our new data here shows that S100a8/a9 levels increase during chronic Mtb infection in mice and human TB progressors, and depletion of neutrophils during chronic infection in mice improves Mtb control (Figure 2.1 and 2.2). Therefore, we next assessed the long-term outcome of Mtb infection in B6 and S100a9KO mice (217) (which lack functional S100a8/a9) during chronic stages of infection with Mtb HN878. While lung and spleen Mtb HN878 CFU was comparable at 50 dpi, we found that during chronic infection from 100 dpi until 300 dpi, Mtb CFU in the lungs of S100a9KO mice were significantly lower, when compared to B6 HN878-infected mice (Figure 2.3, A and B). This improved Mtb control was also observed during infection with another clinical strain, HN563, but not when the lab-adapted Mtb strain H37Rv was used (Supplemental Figure 2.2, A and B).

S100A8/A9 plays a key role in neutrophil recruitment and accumulation (228-230) and thus the absence of S100a8/a9 may promote improved Mtb control by altering cellular accumulation to the lung and regulating inflammation. Coinciding with the timing of improved Mtb control in the lungs of S100a9KO mice at 100 dpi, we found that neutrophil accumulation was reduced, while other
myeloid subsets such as alveolar macrophages (AMs), recruited macrophages (RMs), monocytes and dendritic cells (DCs) were not significantly different in lungs of $S100a9$KO mice, when compared with B6 infected mice (Figure 2.3, C and D). However, at the later stage of chronic disease, AMs, DCs, monocytes, and neutrophils were all reduced in $S100a9$KO mice when compared with B6 infected lungs (Figure 2.3, C and D). The localization of MPO-expressing neutrophils within TB granulomas during chronic infection was also decreased in $S100a9$KO mice, suggesting that S100a8/a9 expression promoted the accumulation of neutrophils in the lung, specifically localization within TB granulomas (Figure 2.3E). Presence and maintenance of B cell follicles within lung granulomas has been associated with effective $Mtb$ control (184). Consistent with improved protection in the lungs of $S100a9$KO infected mice at chronic stages, lung B cell follicle area was significantly enhanced within TB granulomas (Figure 2.3F). However, despite altered accumulation of neutrophils within granulomas in $S100a9$KO mice, overall inflammatory area or the specific type of inflammation (myeloid or lymphocytic) in the lungs of $S100a9$KO $Mtb$-infected mice was not significantly different when compared with $Mtb$-infected B6 mice (Supplemental Figure 2.3, A and B).

$S100A8/A9$ binds to RAGE (231), and delivery of RAGE inhibitor (FPS-ZM1) to $Mtb$-infected mice during the chronic stages of infection significantly decreased $Mtb$ burden similar to levels observed in $S100a9$KO mice (Figure 2.4A). Although inflammation was not significantly decreased in RAGE-treated mice, B cell follicle size was significantly increased within TB granulomas (Figure 2.4, B and C). These results together suggest that absence of $S100A8/A9$ expression or inhibition of its receptor function both improve $Mtb$ control during chronic TB. To further experimentally test the role of $S100A8/A9$ in TB reactivation and disease progression, we used the Cornell mouse model of TB reactivation where mice were infected with $Mtb$, treated with
antibiotics, following which reactivation of *Mtb* was assessed. Our data show that while B6 mice that received antibiotic chemotherapy reactivated significantly elevated *Mtb* CFU in the lung, *S100a9KO* mice exhibited significantly lower rates of reactivation and harbored lower lung *Mtb* CFU when compared with B6 mice (Figure 2.4D). In conjunction with lower *Mtb* CFU, while inflammation was not altered, neutrophil accumulation was significantly decreased in lung granulomas of *S100a9KO* mice (Figure 2.4, E and F). These results suggest that while *S100A8/A9* expression coincides with neutrophil accumulation and improved *Mtb* control, *S100A8/A9* expression did not directly regulate inflammation during TB.

**S100a8/a9 expression regulates CD11b expression on neutrophils during chronic tuberculosis**

As neutrophil accumulation was significantly decreased in *S100a9KO* *Mtb*-infected mice, we next determined whether surface expression of the integrin CD11b, a key molecule in leukocyte adhesion and migration (232), was differentially expressed in myeloid cells in *S100a9KO* *Mtb*-infected mice when compared with B6 *Mtb*-infected mice. We observed that CD11b surface expression was significantly decreased within lung neutrophils in *S100a9KO* *Mtb*-infected mice during chronic stages of infection, whereas lung monocytes and RMs had no significant changes in CD11b expression (Figure 2.5A). Gr-1 (Ly6G/Ly6C) surface expression was also assessed, but no significant changes were seen in any lung myeloid cell population during both acute and chronic stages of infection in *S100a9KO* mice when compared with B6 *Mtb*-infected mice (data not shown). As myeloid cells such as neutrophils and monocytes are among the primary producers of *S100A8/A9* (211, 233), we addressed if adoptive transfer of CD11b+ cells producing S100A8/A9 can reverse the improved *Mtb* control seen in *S100a9KO* *Mtb*-infected mice. Accordingly, CD11b+ cells transferred into *S100a9KO* mice resulted in increased lung *Mtb* burden, suggesting that a
CD11b myeloid population was contributing to the increased susceptibility during \textit{Mtb} infection (\textbf{Figure 2.5B}). These data were corroborated by decreased lung protein levels of neutrophil attracting chemokines CXCL-1 and MIP-2 in \textit{S100a9KO} mice during chronic infection (\textbf{Figure 2.5C}). Taken together, absence of S100A8/A9 during chronic \textit{Mtb} infection reduces neutrophil localization, decreased expression of CD11b expression on neutrophils, and adoptive transfer of CD11b cells reversed the protection observed in \textit{S100a9KO Mtb}-infected lung.

\textbf{S100A8/A9 regulates CD11b expression on neutrophils during Mtb infection}

Our data points to a critical role for S100a8/a9 in regulating CD11b expression specifically on neutrophils to modulate accumulation into the lung. To mechanismically address the role of S100a8/a9 in regulation of CD11b expression on neutrophils and \textit{Mtb} control, bone marrow derived neutrophils were infected with reporter-expressing \textit{Mtb}, and bacterial uptake and neutrophil marker expression was assessed. While B6 neutrophils were more readily infected with \textit{Mtb}, \textit{S100a9KO} neutrophils exhibited less ability to take up \textit{Mtb} (\textbf{Figure 2.6A}). Furthermore, CD11b expression was significantly upregulated on B6 \textit{Mtb} \textsuperscript{hi} neutrophils but not as highly induced in \textit{S100a9KO Mtb} \textsuperscript{hi} neutrophils (\textbf{Figure 2.6B}). Indeed, \textit{S100a9KO} neutrophils also expressed lower levels of CD11b even in uninfected controls, as published before (217), suggesting that while S100a8/a9 regulates the expression of CD11b at homeostatic levels, this effect is much more profound upon \textit{Mtb} infection. Similar results were obtained when Gr-1 and CD18 levels were measured on \textit{S100a9KO Mtb}-infected neutrophils, when compared with B6 \textit{Mtb}-infected neutrophils (\textbf{Figure 2.6B}). To address if live \textit{Mtb} infection was required for CD11b upregulation, we then tested if stimulation with heat killed (HK) \textit{Mtb}, or components of \textit{Mtb} will similarly regulate the S100a8/a9-dependent effects on CD11b regulation. We found that HK \textit{Mtb} and stimulation with culture filtrate protein (CFP) and cell wall components (CW) of \textit{Mtb} similarly
induced an S100a9-dependent CD11b upregulation on neutrophils, suggesting that live *Mtb* infection was not necessary (Figure 2.6C). Since S100a8/a9 is known to induce NF-κB signaling, we also wanted to understand the role of NF-κB signaling in mediating CD11b upregulation. We isolated neutrophils from IKK\(^{−/−}\) LysM\(^{Cre}\) mice, which lack canonical IKK and NF-κB signaling in LysM-expressing cells. We found that lack of NF-κB signaling reduced *Mtb* infection in neutrophils (Figure 2.6D), and NF-κB signaling was required for upregulation of CD11b and Gr-1 on *Mtb*-infected neutrophils (Figure 2.6E). These results together demonstrate that absence of S100a8/a9 expression in neutrophils significantly impacts *Mtb* uptake and CD11b induction following *Mtb* infection, in part dependent on NF-κB signaling. These results together provide novel insights into the molecular mechanisms that mediate CD11b upregulation in a S100a8/a9 dependent manner.

**S100A8/A9 can function as a biomarker of TB disease progression**

Our data demonstrate that S100A8/A9 mRNA levels increased during progression to TB in humans. Thus, we wanted to next address if serum S100A8/9 levels can be used as biomarkers to distinguish ATB patients from healthy LTBI and uninfected healthy controls (HC). S100A8/A9 serum protein levels were significantly higher in ATB patients compared to HCs (Table 2.1, Figure 2.7A). Importantly, we also found significantly increased S100A8/A9 serum levels in LTBI Tuberculin Skin Test (TST\(^{+}\)) and Quantiferon\(^{+}\) (QFT\(^{+}\)) house hold contacts of ATB patients, and LTBI TST\(^{+}\) QFT\(^{+}\) individuals with occupational exposure to TB, when compared with uninfected HCs (Figure 2.7A). S100A8/A9 levels were also higher in LTBI TST\(^{+}\)QFT\(^{+}\) individuals with occupational exposure to TB when compared with LTBI TST\(^{+}\) QFT\(^{-}\) individuals with similar occupational exposure to TB (Figure 2.7A). These results suggest that S100A8/A9 levels could serve as an easily measurable biomarker of TB disease progression in LTB
individuals. Additionally, we assessed if increased S100A8/A9 serum protein was only increased during ATB or was also increased in other acute and chronic inflammatory pulmonary diseases. While S100A8/9 serum protein levels were not higher in patients with Chronic Obstructive Pulmonary Disease (COPD), S100A8/A9 serum levels were high in influenza infected patients (Figure 2.7A). These results suggest that S100A8/A9 serum measurements may be used as biomarkers for TB, while taking into consideration clinical symptoms which may confound the measurements.

As CXCL-1 and CXCL-10 serum levels were additionally increased in ATB patients (Table 2.1), we next sought to understand whether combining S100A8/A9 with CXCL-1 and CXCL-10 serum protein levels will improve biomarker performance for distinguishing ATB from LTB than S100A8/A9 alone (93). Median serum biomarker concentrations, with interquartile ranges, across the three groups of participants is included in Table 2. The medians were the lowest in the uninfected HCs for all biomarkers, followed by levels in LTB individuals, and the highest expression was noted in the ATB group. Overall and pairwise comparisons were listed in Table 3, where the differences in the distribution of the biomarkers across all levels were significant when comparisons were made between ATB and LTB, as well as ATB and HC. For a threshold of 114 pg/ml, CXCL-1 could differentiate between ATB and HC groups with a sensitivity of 88.4% (95% CI, 0.78-0.96) and a specificity of 61.5% (95% CI, 0.42-0.80); while for CXCL-10, a threshold of 302 pg/ml would differentiate between ATB and HC with a sensitivity of 86.5% (95%CI, 0.76-0.94) and a specificity of 57.7% (95% CI, 0.38-0.76). For S100A8/9, a threshold of 1805 pg/ml would differentiate between ATB and HC with a sensitivity of 92.3% (95% Cl, 0.84-0.98) and a specificity of 76.9% (95% CI, 0.57-0.92). Notably, S100A8/A9 levels were the only significant determination when LTB and HCs were compared pairwise and not when CXCL-1 and CXL-10
were determined for pairwise comparisons between the LTB vs HC (Table 2.2). Furthermore, receiver operating characteristic (ROC) analysis was applied to appraise the diagnostic values of the 3 biomarkers individually and their combination (Table 2.3 and 2.4). Combining S100A8/A9 along with CXCL-1 and CXCL-10 into a biomarker signature improved differentiation between ATB and HCs (0.9467, 95% CI, 0.88-1.0) when compared with utilizing CXCL10 and S100A8/A9 as combined biomarker signatures (0.9268, 95% CI, 0.85-0.99, Table 2.3 and 2.4, Figure 2.7B). These results together suggest that use of S100A8/A9 in combination with other chemokines should be further pursued for use as a biomarker for TB disease and TB disease progression.

2.4 Discussion

Neutrophils have recently been implicated as drivers of immunopathogenesis of TB in human and animal models (93, 221, 234). However, the molecular mechanisms by which neutrophils regulate TB immunopathogenesis are not clearly understood. In this study, we demonstrate that neutrophils accumulate during progression to TB disease in Mtb-infected humans and mice, and play a detrimental role during chronic TB disease in mice. Additionally, S100a8/a9 levels mirror the neutrophil accumulation in the lung in mice, and S100a9 deficiency in mice results in improved Mtb control during chronic disease. We show that the mechanism by which S100A8/A9 contributes to increased TB disease pathogenesis is by regulating the expression of the integrin CD11b, which is required for neutrophil accumulation in the lung (217, 229). Furthermore, S100A9 deficiency also reduces TB reactivation in mice, and use of RAGE inhibition results in improved Mtb control. Finally, our human studies suggest that S100A8/A9 levels along with expression of chemokines such as CXCL-1 and CXCL-10 may serve as biomarkers to identify LTBI and ATB from HCs. Together, our results demonstrate that S100A8/A9 play pivotal
functions in regulating neutrophil accumulation during TB, primarily through their effects on CD11b expression, thus providing novel insights into the immunopathogenesis of TB.

Although neutrophil accumulation has been associated with increased disease in mouse and NHP models, as well as human TB, it is still unclear whether neutrophils have protective or pathological functions in *Mtb* infection (5, 96, 155, 164, 221). Despite neutrophils being one of the first cell types infected with *Mtb* (142), our results show that neutrophil depletion during acute *Mtb* infection has no significant impact on *Mtb* control or TB pathology. These results provide formal evidence that neutrophils do not play a protective role during acute *Mtb* infection. In contrast, our data showing that depletion of neutrophils during chronic *Mtb* infection improves *Mtb* control clearly establishes a detrimental role for neutrophils during the chronic stages of *Mtb* infection. Interestingly, while both neutrophil depletion and S100A8/A9 deficiency improved *Mtb* control, overall inflammatory lung disease was not impacted. In the case of neutrophil deficiency, it is possible that transient depletion of neutrophils was not sufficient to impact overall inflammation and prolonged neutrophil depletion may be required to impact lung pathology. During S100A8/A9 deficiency, while overall inflammatory disease was trending towards decreased inflammation, these data were not significant. However, the reduced accumulation of neutrophils into TB granulomas coincided with increased formation of B cell containing granulomas, which our studies have previously shown to be associated with improved *Mtb* control (184). Together, our results demonstrate that neutrophils and S100A8/A9 may play key roles in modulating TB immunopathogenesis by driving neutrophil accumulation.

A mechanism by which S100A8/A9 regulates TB pathogenesis is likely by directly regulating CD11b expression on neutrophils. While *S100a9* deficiency did not impact CD11b expression on neutrophils during the early stages of *Mtb* infection, deficient mice express lower CD11b levels.
on neutrophils during chronic TB. In contrast, in S100a9KO bone marrow neutrophils, CD11b expression is significantly lower in uninfected controls, and upon Mtb infection, upregulate CD11b to a lesser extent than B6 bone marrow neutrophils. Considering these baseline differences in CD11b expression in bone marrow neutrophils, it is intriguing why differences in CD11b expression do not occur in S100a9KO mice until day 100 following Mtb infection. The nature of the microenvironment (e.g. presence of other pro-inflammatory chemokines, prolonged Mtb infection and stimulation, etc.) may contribute to differences observed in in vitro studies and ex vivo isolated neutrophils. Additionally, it is of considerable interest that while S100a9KO mice are protected from infection with a clinical Mtb isolate, HN878, S100a9KO mice do not show any protection when infected with a lab adapted Mtb strain, H37Rv. Thus, our data suggest that S100a8/a9 may play homeostatic roles in regulating CD11b expression in bone marrow neutrophils, which following infection and induction of inflammatory cytokine signals, these effects may be amplified. Neutrophil chemotaxis has been associated with cytoskeletal reorganization and actin polymerization and regulation. S100a9 deficient neutrophils had actin defects when responding to IL-8 stimulation, suggesting that S100a9 has a role in cytoskeletal dynamics and reorganization (217). Functional actin coordinates cell surface integrin regulation (e.g. CD11b/CD18), which could impact neutrophil migration and adhesion. Together, this suggests that S100a8/a9 expression in the lung may induce chemokines that mediate neutrophil accumulation, potentially directly act as a chemoattractant for neutrophils (229), but also by regulating the expression of CD11b to regulate additional neutrophil migration into the lung.

S100A8/A9 is known to interact with TLR4 and RAGE, while CD11b expression can be driven by interactions with TLR4 on neutrophils (212, 235). Thus, S100A8/A9 may engage TLR4 and RAGE to upregulate CD11b expression on neutrophils. Adoptive transfer of CD11b cells increased
susceptibility in S100a9KO mice, suggesting that CD11b immune populations, namely neutrophils or monocytes expressing S100a8/a9, are the likely cellular population mediating increased susceptibility during chronic TB. The utilization of RAGE inhibitor, FPS-ZM1, is protective in animal models of emphysema and Alzheimer's disease (224, 236). During chronic TB, we show that transient use of RAGE inhibitors is protective and is sufficient to decrease lung Mtb burden, but not lung inflammation. Whether use of RAGE inhibitor limits neutrophil accumulation and thus reduces the Mtb niche is not fully explored and should be the focus of future studies. Excitingly, our data showing that S100a8/a9 deficiency delays TB reactivation suggests that RAGE inhibitors may be potentially used as host directed therapeutics in combination with current antibiotic regimens to improve Mtb control and should be further studied.

The rapid diagnostic test for detection of TB recommended by the World Health Organization is an automated PCR assay that detects mycobacteria in sputum expectorated by patients (WHO). However, access remains restricted in low resource settings, and sputum-based microscopy to identify Mtb still remains the most commonly used diagnostic for TB, but this method is primarily capable of identifying ATB patients. Therefore, new non-sputum based screening tools for identifying individuals with ATB are required so that they can be prioritized for clinical investigation and treatment. Blood biomarkers that can differentiate progression of LTB to subclinical disease and ATB, failure of TB treatment and TB relapse would provide additional tools to improve TB diagnosis and combat the global TB epidemic. Several studies have explored the use of host transcriptional biosignatures as diagnostic biomarkers for progression of TB and to predict risk of human TB disease (221, 237). Although these studies propose the use of transcriptional gene signatures as screening or triage tests for TB, the costs and technology associated with RT-PCR or RNA sequencing may still limit use of these biomarkers as diagnostic
in low resource setting. Our published studies have shown that ATB patients in India, Mexico and South Africa all reliably exhibit increased expression of serum S100A8/9 proteins when compared with HC (93), and now in QFT+LTBI individuals. Serum is more amenable to developing a simple screening test that may be easier to translate into a point of care format (e.g. the Quantum Blue assay, which works also for sputum and urine). Additionally, our new studies also show that S100A8/A9 along with chemokine such as CXCL-1 and CXCL-10 serum protein levels reliably able to differentiate between ATB and HC, and although less effective but still significantly can discriminate between ATB and QFT+ LTBI. Furthermore, that S100A8/A9 is not significantly increased during other chronic pulmonary diseases such as COPD cohort suggests that S100A8/A9 may be useful as a biomarker for a triage test to rule out individuals who do not have TB, such that biomarker+ individuals then get followed up for further TB diagnosis. Thus, further validation of the use of S100A8/A9, CXCL-1, and CXCL-10 in different geographical cohorts and in treatment failure and relapse studies will be useful and timely.

In summary, our studies have mechanistically described a pivotal role for S100A8/A9 proteins in mediating TB pathogenesis through regulation of CD11b and neutrophil recruitment. Additionally, our experimental studies targeting the S100A8/A9 signaling pathway project a novel pathway for host directed therapeutics for TB. A more mechanistic understanding of the role of S100A8/A9 proteins further validates the development of S100A8/A9 and related biomarkers as novel diagnostics for TB.

2.5 Methods

Mice and NHP Mtb infection

C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME). S100a9KO was obtained from Dr. Thomas Vogl (217)and mice were bred within the Washington University in St.
Louis animal facility. Both sexes between the ages of 6-8 weeks were used. All mice were maintained and used in accordance with the approved Institutional Animal Care and Use Committee (IACUC) guidelines at Washington University in St. Louis. Mtb strains were cultured and mice were aerosol infected with ~100 colony forming units (CFU), as described previously (184). At specific time points post infection, lungs were harvested, homogenized, and serial dilutions of tissue homogenates plated on 7H11 agar plates to determine Mtb CFU. For reactivation experiments, mice were treated with rifabutin (Sigma) (100 mg/L) and isoniazid (Sigma) (200 mg/L) for 6 weeks and mouse tissue was then harvested at 140 dpi. For rhesus macaques infected with Mtb, we used clinical data stored in the Animal Records System (ARS) at the Tulane National Primate Research Center. Macaques were chosen if they were experimentally infected with Mtb (either CDC1551, Erdman or H37Rv strains) during 2007-2015 and exhibited either ATB or were latently infected. Post-infection, data were obtained weekly from animals until euthanasia or necropsy. Neutrophil percentages in the blood were obtained from complete blood counts performed at the same time as serum chemistry.

**Adoptive transfer of CD11b⁺ cells**

Lung cell suspensions were prepared from the lungs of B6 mice 100 dpi following HN878 infection. CD11b⁺ cells were enriched from the lung suspension using magnetic selection with CD11b microbeads (Miltenyi Biotec, Auburn, CA) per manufacturer’s instructions. 50 µl (1x10⁶ cells) of this suspension (72.4% purity) was administered intratracheally to HN878-infected mice at 100 dpi. These mice were harvested on 120 dpi as indicated.

**RAGE-inhibitor treatment**
RAGE signaling was inhibited starting 205 dpi as previously described (224) through daily intraperitoneal (i.p.) injection of 1 mg/kg RAGE-specific blocker FPS-ZM1 (Tocris) or DMSO (control). Mice were euthanized 15 days after treatment.

**Neutrophil Depletion**

Neutrophils were depleted as described (143) using 300 µg anti-mouse Ly6G (BioXcell) or isotype IgG (Sigma). Briefly, for in acute infections (less than 21 dpi), mice were given 300 µg of anti-mouse Ly6G every other day between 10-20 dpi intra-peritoneally (i.p.). In chronic infections, mice were given 300 µg of anti-mouse Ly6G every other day between 95-105 dpi via intraperitoneal (i.p.) injections.

**Lung Cell Preparation and Flow Cytometry**

Lung cell suspensions were prepared, stained, collected and analyzed for flow cytometry as described before (184). Fluorochrome-labeled antibodies specific for CD11b (M1/70, BD), CD11c (HL3, BD), Gr-1 (RB6-8C5, eBioscience) were used in this study. Cells were collected using a FACSJazz with FACS Software software. Cell populations were gated based on their forward by side scatter characteristics and the frequency of specific cell types was analyzed using FlowJo version 7.6.5 (Tree Star Inc, CA). Lung alveolar macrophages were gated as CD11c⁺ CD11b⁻, lung myeloid dendritic cells were gated on CD11c⁺ CD11b⁺, neutrophils as CD11b⁺ Gr-1^{hi}, recruited macrophages were annotated as CD11b⁺ Gr-1^{lo}, and monocytes were gated on CD11b⁺ Gr1^{int} cells as in Dunlap et al 2018 (22).

**In vitro neutrophil infections**

Neutrophils were isolated from the bone marrow using the mouse neutrophil isolation kit (Miltenyi Biotec, Auburn, CA). Neutrophils were infected at an MOI of 1 for three hours. Neutrophil uptake was derived from neutrophil infection with *Mtb* HN878-mCherry. CD11b MFI was also assessed.
in neutrophils treated with heat-killed *Mtb* HN878 (1x10^6 CFU) or *Mtb* HN878 culture filtrate protein and cell wall extracts (10 µg/ml). Fluorochrome-labeled antibodies specific for CD11b (M1/70, BD), CD18 (M18/2, Biolegend), Gr-1 (RB6-8C5, eBiosciences), were used in this study.

**Lung histology**

Lungs from *Mtb*-infected mice were perfused with 10% neutral buffered formalin and were paraffin embedded. Lung sections were stained with hematoxylin and eosin (H&E) and processed for light microscopy. Images were obtained using Zeiss Axioplan 2 microscope and were recorded with a Zeiss AxioCam digital camera. Sections were probed with rabbit anti-MPO (PB9057, BosterBio; dilution 1:100) and biotinylated rat anti-mouse Ly6G (clone IA8, Biolegend; dilution 1:100) to detect neutrophils or with anti-B220 (clone RA3-6B2, BD Pharmingen; dilution 1/100) to detect B cells. Primary antibodies were detected with Cy3 donkey anti-rabbit Ig G(711-166-152, Jackson ImmunoResearch Laboratories; dilution 1:200) and Alexa Fluor 488 streptavidin (A21208, Thermo Fisher Scientific; dilution 1:200). B cell follicles were assessed through the automated tool of the Zeiss Axioplan 2 microscope (Zeiss, Thornwood, NY, USA), and total area and average size was calculated in squared microns. Myeloid and lymphocyte areas were acquired with stained slide images using a Hamamatsu Nanozoomer 2.0 HT system with NDP scan image acquisition software and was quantified using Visiomorph image processing software (Visiopharm, Broomfield, CO).

**RNA sequencing data from Adolescent Cohort Study progressors and controls**

We compared gene-level mRNA expression levels of *S100A8* and *S100A9* in *Mtb*-infected adolescents enrolled into the Adolescent Cohort Study (ACS) who remained healthy during 2 years of study follow-up (controls) (n=106) or who progressed to microbiologically confirmed, active TB (progressors) (n=44), as described (227, 237). Adolescents who were *Mtb*+ at enrollment, or
developed ATB disease more than 6 months after *Mtb* infection was first detected were included in our analyses. Each progressor had two healthy matched controls based on age, gender, ethnicity, school of attendance, and presence or absence of prior episodes of tuberculosis disease. Participants were excluded if they were ATB+ within 6 months of enrollment or QFT and/or TST conversion, or if they were HIV infected. Study protocols were approved by The Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town. Written informed consent was procured from parents/legal guardians as well as from every adolescent. Participants with diagnosed or suspected tuberculosis disease were referred to a study-independent public health physician for treatment according to national tuberculosis control programs of the country involved. RNA was extracted from PAX gene tubes and RNA-sequencing performed as described (227, 237). Prospective RNA-Seq data of progressors were realigned to the time point at which active TB was diagnosed. Differences in gene-level mRNA expression between each progressor sample and the average of demographically matched control samples were computed using the published ACS metadata (227, 237). Time To Diagnosis values were assigned to each sample according to the original definitions. The log$_2$ fold change values between progressor and control biomarkers were modeled as a nonlinear function of Time To Diagnosis for the entire population using the smooth-spline function in R with three degrees of freedom. Ninety-nine percent confidence intervals for the temporal trends were computed by performing 2000 iterations of spline fitting after bootstrap resampling from the full dataset.

**ATB cohort**

We recruited patients (n = 52) with confirmed ATB. The diagnosis of ATB was made by the conventional microscopic detection of acid-alcohol resistant bacteria as well as culture of *Mtb* in Lowenstein-Jensen medium in serial, non-concentrated sputum samples, radiological studies,
physical examination and clinical history. All patients with ATB were residents of the urban and metropolitan areas of Mexico City and some patients were residents from different states of the south and central area of Mexico including Veracruz, Oaxaca, Puebla and Chiapas. Patients with ATB, excluding patients with HIV and cancer, were recruited at the INER Tuberculosis Clinic in a period between 2015 and 2017.

At the same time, household contacts of ATB patients who are TST‘QFT’ LTBI (n = 36) were recruited in the first two months of diagnosis. The criteria for inclusion of contacts of ATB patients were: 1) Subjects of legal age (18 to 54) willing to sign the letter of consent; 2) Subjects in close contact with the index case of ATB and 3) Subjects willing to provide the blood samples needed to participate in the study. In addition, a group of individuals (n = 28) belonging to health personnel (resident physicians of the pulmonology specialty) and administrative medical staff of the INER, considered as an occupational risk population of which both TST‘QFT’+ (n=19) and TST‘QFT’- (n=9) were included. Also, a group of uninfected healthy individuals (TST‘QFT’-) were also recruited (n=26). Only patients with ATB, LTBI, and healthy controls who agreed to participate in the study and who signed an informed consent letter were included. The study was reviewed and approved by the Institutional Research Committee with protocol number B04-15. After the consent signature, 20 ml of blood anticoagulated with EDTA and 10 ml of blood without anticoagulant was used to isolate serum for the purpose of performing the experimental analysis.

**G-CSF, IL-6, TNF-α, MIP-2, S100A8/A9, CXCL-1 and CXCL-10 protein quantification**

Levels of G-CSF, IL-6 and TNF-α, CXCL-1 and MIP-2 in mouse lung homogenates were measured using a mouse Luminex assay (Linco/Millipore). Circulating levels of S100A8/A9 were measured in serum samples by ELISA using the DuoSet ELISA Development kit for human S100A8/S100A9 heterodimer (Cat: DY8226, R&D Systems Inc., Minneapolis, MN, USA),
according to the manufacturer’s instructions. Serum samples were diluted 1:4000 in regent diluent (1% BSA in PBS). Absorbance was read at 450 nm and 540 nm (for wavelength correction) using the Synergy HT microplate ELISA reader (BioTek Inc., Winooski, VT, USA). Serum levels of CXCL-1 were measured by CXCL1 Quantikine ELISA Kit (Cat: DGR00B, R&D Systems Inc.) according to the manufacturer’s instructions. For this assay, samples were diluted 1:2. Absorbance was read at 450nm and 540nm using the Synergy HT microplate ELISA reader (BioTek Inc.). Serum levels of CXCL-10 were assessed by Luminex using the Bio-Plex Pro CXCL10 Set (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer’s instructions. The samples were read in a BioPlex-200 instrument. The results were analyzed using the BioPlex software V 4.1 (Bio-Rad Laboratories).

Statistics

Differences between the means of two groups were analyzed using the two tailed Student’s t-test in GraphPad Prism 5 (La Jolla, CA). Multiple groups were analyzed using 1-way ANOVA and Tukey’s post-test analysis. Differences are noted as significant when a p-value is less than or equal to 0.05 (*), less than or equal to 0.01 (**), less than or equal to 0.001 (***) and less than 0.0001 (****).

The distribution of the biomarkers in human samples was examined and expressed as a median and interquartile range (IQR). The differences in the distributions across the groups was evaluated using the Kruskal-Wallis nonparametric test where the Dwass, Steel, Critchlow-Fligner Method was used to check for Pairwise Two-Sided Multiple Comparison Analysis (238-240). The diagnostic efficiency of the individual biomarkers and their combinations was assessed by receiver operating curve (ROC) analysis where the AUC and associated 95% confidence intervals (CIs) were determined. A nonparametric approach was used to compare the correlated receiver operating characteristic (ROC) curves without adjustments for pairwise comparisons (241). Logistic regression analysis was performed to determine
the predictive probability of these biomarkers on the each of the outcome combinations. The level of significance was set to 0.05 and all analysis were 2-sided. Statistical analyses and figures were performed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

**Study Approval**

For human studies, written informed consent from participants was received under a protocol approved by the Ethics Committee of the Instituto Nacional de Enfermedades Respiratorias (Mexico City) and the Human Research Ethics Committee of the Faculty of Health Sciences (Cape Town). Protocols involving the use of animals were approved by IACUC at Washington University in St. Louis and Tulane National Primate Research Center. All of the experiments were performed in accordance with the protocols.

**Authors Contributions**

Conceptualization: SAK, Mouse experiments: NRS, RDG, JRM, PT; NHP experiments: ANB, SM; Human analysis: NRS, NA, ACL, LJA, MMT, KBL; writing of the original draft of the manuscript: NRS, NA, RDG, and SAK; review and editing of the manuscript: NRS, NA, JRM, TV, DK, TJS, JZ and SAK; funding acquisition: DK, TJS, JZ, and SAK; study supervision: SAK.

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2.7 Figure Legends
Figure 2.1. Neutrophil depletion during chronic TB improves *Mtb* control. (A) The number of blood neutrophils from Rhesus macaques with ATB (n=8) or LTBI (n=10) were measured at dpi following
Mtb infection or at necropsy (N). B6 mice were aerosol-infected with ~100 CFU Mtb HN878. (B) Neutrophil accumulation was determined by flow cytometry at 0, 3, 5, 14, 20, 30, 60, 100, 150, 300 dpi. B6 mice were infected with Mtb HN878 and administered IgG (n = 5-10) or 1A8 (n = 5-10, 300 µg/dose) i.p. every other day. (C) Bacterial burden in the lung and spleen was determined by plating on 21 dpi. (D) Lung homogenates were analyzed for G-CSF protein expression by ELISA on 21 dpi. (E) Pulmonary inflammation was quantified on formalin-fixed paraffin embedded (FFPE) lung sections from 21 dpi samples stained with H&E. B6 mice were infected with HN878 and administered IgG (n = 4-5) or 1A8 (n = 4-5, 300 µg/dose) i.p. every other day. (F) Bacterial burden in the lung and spleen was determined by plating on 106 dpi. (G) Lung homogenates were analyzed to measure levels of IL-6 and TNF-α proteins at 106 dpi using Luminex assays. (H) Pulmonary inflammation was quantified on FFPE lung sections from 106 dpi tissues stained with H&E. (A-G) Student’s t-test between isotype and 1A8 treated mice. The data points represent the mean (±SEM) of values. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***) and P < 0.0001 (****).
**Figure 2.2.** *S100A8/A9* mRNA levels are indicators of TB disease progression in mice and humans with TB. B6 mice (n=3-5) were aerosol-infected with ~100 CFU *Mtb* HN878. (A) Lung S100A8/A9 levels were determined by ELISA at 0, 5, 8, 12, 15, 30, 50, 100, 150, 300 dpi. (B) Kinetics of *S100A8* and *S100A9* mRNA expression over time, expressed as log2 fold change between bin-
matched progressors (n=44) and controls (n=106) and modeled as non-linear splines (dotted lines). Light green shading represents 99% CI and dark green shading 95% CI for the temporal trends, computed by performing 2000 spline fitting iterations after bootstrap resampling from the full dataset. The deviation time (day), calculated as the time point at which the 99% CI deviates from a log2 fold change of 0, is indicated by the vertical red line. P ≤ 0.05 (*) and P < 0.0001 (****).
Figure 2.3. S100a8/a9 deficiency protects mice during chronic TB. B6 (n=5-10) and S100a9KO (n=5-9) mice were aerosol-infected with ~100 CFU *Mtb* HN878. (A, B) Lung and spleen bacterial
burden was determined by plating at different 50,100,150,300 dpi. Lung myeloid population cell
counts were enumerated in B6 and *S100a9KO* HN878-infected mice using flow cytometry at
50,100,150,300 dpi and (C) neutrophils and other (D) other myeloid cells are shown. FFPE lung
sections were used to carry out immunofluorescence staining for Ly6G (green), MPO (red), and
DAPI (blue) or B220 (green), CD3 (red) and DAPI (blue). (F) Total area occupied by B cell
follicles was determined using the morphometric tool of the Zeiss Axioplan microscope. AMs
alveolar macrophages, DCs dendritic cells, RM recruit macrophages. (A-B, D-G) Student’s t-
test between B6 and *S100a9KO*, (C) 1-way ANOVA with Tukey’s post test. The data points
represent the mean (±SEM) of values from 5-10 mice. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***)
and P ˂ 0.0001 (****).
Figure 2.4 Targeting the S100A8/A9 pathway limits susceptibility to chronic TB and TB reactivation. *Mtb*-infected B6 and *S100a9KO* (n=10) mice were treated with RAGE inhibitor (1
mg/kg RAGE-specific blocker FPS-ZM1 (n=5) or DMSO (n=5)) at 205 dpi for 15 days and (A) lung bacterial burden at 220 dpi was determined by plating, (B) pulmonary inflammation was quantified on FFPE lung sections stained with H&E and (C) area occupied by B cell follicles was quantified histologically. B6 (n=9) or S100a9KO (n=13) infected mice were treated with (100 mg/l) and isoniazid (200 mg/l) for 6 weeks and mice organs were then harvested and homogenized at 140 dpi to determine reactivation of Mtb infection. (D) Lung bacterial burden was determined by plating, (E) pulmonary inflammation was quantified on FFPE lung sections stained with H&E, and (F) quantification of Ly6G (green), MPO (red) neutrophils was carried out in FFPE lung sections. (A-F) Student’s t-test. The data points represent the mean (±SEM) of values from 5-13 mice. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (**), and P < 0.0001 (****).
Figure 2.5 CD11b+ cells mediate improved protection in S100a9KO Mtb-infected mice. B6 (n=5-24) and S100a9KO (n=5-24) mice were aerosol-infected with ~100 CFU Mtb HN878. (A) CD11b mean fluorescence intensity (MFI) on neutrophils, monocytes and RMs was determined.
using flow cytometry at 50, 100, 150 and 300 dpi. S100a9KO (n=6) infected mice received CD11b+ purified cells from B6 mice (50 µl containing 1x10^6 cells) intratracheally at 100 dpi. (B) Lung bacterial burden was determined by plating at 120 dpi. (C) Lung homogenates were analyzed by Luminex for KC/CXCL-1 and MIP-2 at 300dpi. (A and C) Student’s t-test between B6 and S100A9KO per timepoint, (C) 1-way ANOVA with Tukey’s post test. The data points represent the mean (±SEM) of values from 5-24 mice. P ≤ 0.05 (*), P ≤ 0.001 (**), and P < 0.0001 (****).
Figure 2.6 S100A8/9 regulates CD11b expression on neutrophils. Bone marrow neutrophils were isolated and infected with mCherry labeled HN878 (MOI of 1) for 3 hours. (A) *Mtb* uptake
by neutrophils was determined in B6 (n=5) and S100a9KO (n=5) neutrophils using flow cytometry. (B) MFI of CD11b, Gr-1 and CD18 expression on uninfected (UI) and highly infected (Mtb\textsuperscript{hi}) neutrophils was determined by flow cytometry. Neutrophils from B6 mice were left untreated (UI) or treated with heat killed Mtb (HK), Mtb culture filtrate protein (CFP) and Mtb cell wall preparations (CW) (C) MFI of CD11b expression on uninfected or treated neutrophils for 3 hours and CD11b MFI was determined by flow cytometry. Bone marrow neutrophils from IKK\textsuperscript{fl/fl} LysM\textsuperscript{Cre} mice and IKK\textsuperscript{fl/fl} mice were infected with Mtb (MOI 1) and (E) Mtb uptake, (F) CD11b and Gr-1 MFI was assessed using flow cytometry. (A-F) Student’s t-test. The data points represent the mean (±SEM) of values from 5 biological replicates. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***) and P < 0.0001 (****).
Fig 2.7 S100A8/A9 proteins can be used as a surrogate biomarker to distinguish between 
ATB and healthy controls. Heathy uninfected controls (TST-QFT) (n=26), LTBI household
contacts (TST+QFT+LTBI) (n=36), LTBI occupational exposed individuals who are TST+QFT+ (n=19), LTBI occupational risk individuals who are TST+QFT- (n=9), ATB (n=52), patients with chronic obstructive pulmonary disease (COPD) (n=16), and influenza (Flu) (n=18) patient serum were collected and (A) S100A8/A9 levels were determined by ELISA. (B) ROC curves of single (top panel) and top 3 biomarker combinations (bottom panel) for ATB vs HC (left panel) and ATB vs LTBI (right panel) shown. P ≤ 0.05 (*) and P < 0.0001 (***)
Supplemental Figure 2.1 Neutrophil depletion in chronic *Mtb* infection does not alter accumulation of other myeloid cell populations in the lung. B6 mice were infected with HN878.
and administered IgG (n = 10) or 1A8 (n = 9-10, 300 µg/dose) i.p. at 10, 12, 14, 16, 18 and 20 dpi.

(A) Lung myeloid cell populations were enumerated in isotype and 1A8 treated mice using flow cytometry at 21 dpi. B6 mice were infected with HN878 and administered IgG (n = 4-5) or 1A8 (n = 4-5, 300 µg/dose) i.p. at 95, 97, 99, 101, 103 and 105 dpi. (B) Lung myeloid cell populations were enumerated in isotype and 1A8 treated mice using flow cytometry at 106 dpi.

AMs alveolar macrophages, DCs dendritic cells, RMs recruited macrophages. The data points represent the mean (±SEM) of values. Student’s t-test between isotype and 1A8 treated mice. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***) and P < 0.0001 (****).
Supplemental Figure 2.2 S100A8/A9 deficiency does not impact susceptibility with lab-adapted *Mtb* H37Rv infection. B6 (n=4-5) and S100a9KO (n=5) mice were aerosol-infected with
~100 CFU of drug resistant W-Beijing strain HN563. (A) Lung and spleen bacterial burden was determined by plating at 50 and 100 dpi. B6 (n=4-5) and S100a9KO (n=5) mice were aerosol-infected with ~100 CFU Euro-American lab-adapted strain H37Rv. (B) Lung and spleen bacterial burden was determined by plating at 50 and 100 dpi. The data points represent the mean (±SEM) of values from 4-5 mice. Include statistical information. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***) and P < 0.0001 (****).
Scott et al Supp. Fig. 3
Supplemental Figure 2.3 Lack of *S100A9* during chronic TB does not impact overall lung inflammation.

B6 (n=5-7) and *S100a9KO* (n=5-8) mice were aerosol-infected with ~100 CFU HN878. (A) Pulmonary inflammation was assessed and quantitated on H&E stained FFPE lung sections from B6 and *S100a9KO* mice. (B) Lymphoid or myeloid inflammation quantification of FFPE lung section in B6 and *S100a9K* mice. (A-B) Student’s t-test between B6 and S100A9KO. The data points represent the mean (±SEM) of values from 5-8 mice. P ≤ 0.05 (*), P ≤ 0.01 (**), P ≤ 0.001 (***) , and P < 0.0001 (****).

**Table 2.1 Descriptive Statistics (Median and Interquartile Range) of biomarkers in Active TB, Latent TB and Healthy controls**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Active TB</th>
<th>Latent TB</th>
<th>Healthy C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>Median</td>
<td>IQR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td>145.92</td>
<td>122.23, 184.57</td>
<td>129.66</td>
</tr>
<tr>
<td>CXCL10</td>
<td>1140.8</td>
<td>469.07, 2231.9</td>
<td>254.94</td>
</tr>
<tr>
<td>S100A8/A9</td>
<td>5990.6</td>
<td>2906.09, 10050.35</td>
<td>1595.7</td>
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<sup>a</sup>Interquartile Range (IQR)

**Table 2.2 Differences in the distribution across the three individual biomarkers**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Active TB vs Latent TB</th>
<th>Active TB vs Healthy C</th>
<th>Latent TB vs Healthy C</th>
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<tr>
<td>CXCL1</td>
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<td>0.0198</td>
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<td>0.1539</td>
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<td>CXCL10</td>
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<td>&lt;.0001</td>
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</table>

<sup>a</sup>Kruskal Wallis Test

<sup>b</sup>Dwass, Steel, Critchlow-Fligner Method

**Table 2.3 Area Under the Curve (AUC) of the Model Biomarker Combinations**

<table>
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<th>Models</th>
<th>AUC</th>
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<th>UL 95% CI</th>
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<td>S100A8/A9</td>
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<td>Models</td>
<td>pvalue for pairwise and overall c statistic comparisons</td>
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<tr>
<td>------------------------------------</td>
<td>--------------------------------------------------------</td>
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<tr>
<td><strong>Active TB vs Health Controls</strong></td>
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<tr>
<td>S100A8/A9+CXCL1+CXCL10 vs CXCL10+S100A8/A9*</td>
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<td>Overall p value</td>
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<td><strong>Latent TB vs Health Controls</strong></td>
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</table>

*Bolded are the top 3 model combinations in terms of the AUC*
<table>
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<th>Comparison</th>
<th>p value</th>
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<tr>
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<tr>
<td>S100A8/A9+CXCL1+CXCL10 vs S100A8/A9</td>
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<tr>
<td>S100A8/A9+CXCL10 vs S100A8/A9</td>
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<tr>
<td>Overall p value</td>
<td>0.3768</td>
</tr>
</tbody>
</table>

*a*significant comparisons are in bold
Chapter 3:

TGF-β inhibitor (CWHM 12) treatment controls acute tuberculosis

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3.1 Introduction

Mycobacterium tuberculosis (Mtb), the causative bacterial pathogen of the disease tuberculosis (TB) causes approximately 1.6 million deaths each year and infects approximately one fourth of the world's population (2). In addition, multi-drug and extensively drug resistant Mtb strains have become prevalent, making TB extremely difficult to treat. Currently, BCG vaccine which is derived from Mycobacterium bovis BCG is available as a preventable vaccine against Mtb infection; however its efficacy is variable. It is imperative to understand Mtb infection, TB disease and elucidate host protective mechanisms in order to improve host defenses against TB. Understanding such immune mechanisms will also enrich the discovery and implementation of host directed therapies against TB.
Transforming growth factor (TGF)-β comes in three isoforms and has been demonstrated to contribute to several biological processes, including immune regulation and inflammatory responses (242). TGF-β is necessary for lung organogenesis and hemostasis and is associated with various respiratory diseases, such as pulmonary fibrosis and emphysema (243, 244). TGF-β exerts its activity by being activated and binding to TGFβRI or II cell surface receptors initiating the Smad signaling pathway, resulting in the transcription of transcription factors, coactivators and corepressors (245, 246).

Increased serum levels of TGF-β and expression in TB lesions in lung samples of individuals with active TB disease (ATB) have been shown (201, 202). In the Aung et al study, TGF-β expression was shown to be located close to alveolar walls and not in the granuloma (201). Aung et al. observed TGF-β was expressed in over half of patients that died within late stage ATB (201). Additionally, Bonecini-Almeida showed TGFβRI and TGFβRII expression was increased in TB patients compared to patients with other lung diseases and healthy volunteers (202). When stimulated with *Mtbc* antigens, monocytes from TB patients produce increased levels of TGF-β (203). Several studies also show that TGF-β serum levels are increased in TB patients, are associated with TB reactivation and decrease during treatment (203). TGF-β is a known promoter of T regulatory cells and a suppressor of inflammation (247-249). Since increased TGF-β levels are associated with TB disease, limiting TGF-β signaling should have a beneficial effect on the host. One such therapeutic could be CWHM 12, a TGF-β inhibitor. CWHM 12 is a small molecule inhibitor of αv integrins. Integrins, especially αv, are important mediators of pulmonary fibrosis. Signaling through the TGF-β receptors requires matured TGF-β isoforms. Lack of integrins, such as αv, has been shown to mimic deficiencies of mice that lack TGF-β1 and TGF-β3 (250). Henderson et al. demonstrated that αv is a mediator of organ fibrosis, specifically pulmonary
fibrosis (251). Mice treated with CWHM 12 during bleomycin induced pulmonary fibrosis were significantly decreased. Since lung fibrosis can be a characteristic of TB (252), we sought to determine if CWHM 12 treatment inhibits pulmonary fibrosis and enhances \textit{Mtb} control during infection.

In this study, we assessed the effect of CWHM 12 on the host responses during \textit{Mtb} infection. We used the C3Heb/FeJ (FeJ) \textit{Mtb} infection model because it is able to recapitulate necrotic and hypoxic granulomas, which are a characteristic of human TB (253, 254). We observed that CWHM 12 treatment in FeJ infected mice decreased bacterial burden during early infection. The most effective treatment resulted in a 1 log bacterial burden decrease. This decrease in bacterial burden also resulted in decreased accumulation of alveolar macrophages and neutrophils and increased recruited macrophages. Enhanced protection resulted from decreased iNOS production, increased collagen in the whole lung and granulomas as well as significant decreases in MIP-2 and IL-10 production. This study provides evidence that inhibition of TGF-\(\beta\) and using CWHM 12 may be a host directed therapy to TB disease.

3.2 Results

\textbf{TGF\(\beta\) inhibitor targeting \(\alpha_v\) integrin improves \textit{Mtb} control in mice}

FeJ mice have been shown to be increasingly susceptible to \textit{Mtb} starting at 15 dpi, where lung bacterial burden is significantly increased compared to parental mouse strains, C3H and B6, and necrotic lesions are observed (Kramnik 1998). These necrotic lesions, mainly composed of macrophages, neutrophils and lymphocytes, very similar to what is seen in humans (Irwin 2015, Smith 2016) Previously, CWHM 12 was shown to decrease bleomycin induced pulmonary fibrosis; however, the effect of CWHM 12 in \textit{Mtb} pulmonary infection is unknown. Using treatment timepoints 0-14 dpi (start of infection), 14-28 dpi (progression from acute to chronic)
and 30-44 dpi 9chroinc TB disease) would allow us to assess at which stage of Mtb infection would CHWM 12 be effective in controlling bacterial burden and fibrosis. We delivered CWHM 12 or our vehicle control to HN878 Mtb infected FeJ mice at three different stages of Mtb infection. Treatment with CWHM 12 delivered the start of infection resulted in a significant decrease in lung bacterial burden compared to the control group (Fig. 3.1A). Lung and spleen bacterial burden control was further increased when treatment was delivered after the establishment of infection and the innate immune response (14-30 dpi) (Fig 3.1B). Upon CWHM 12 treatment in the later stages (30-44 dpi) after the establishment of infection, there was no differences in lung Mtb bacterial control (Fig. 3.1C). These data suggest that CWHM 12 treatment is most effective during the acute stages of infection.

**CWHM 12 treatment mediates improved Mtb control by decreasing myeloid cell recruitment.**

CWHM 12 treatment is effective against Mtb growth when delivered up to 30 dpi; however, we also wanted to assess whether this improved Mtb control was associated with cellular recruitment and changes in lung inflammation. Even though mice treated at the start of infection did had similar levels of inflammation, when treatment was given after the establishment of infection inflammation increased Fig. 3.2A). However, when CWHM 12 treatment was given at during chronic infection, inflammation was significantly increased (Fig. 3.2A). Although, significant inflammation did not occur due to CWHM 12 treatment during acute stages of Mtb infection, changes in cellular recruitment was observed. To determine which cellular population(s) were modulated during CWHM 12 treatment of Mtb infected FeJ mice, we measured the accumulation of lung alveolar macrophage, dendritic cell, neutrophil, monocyte and recruited macrophages. When CWHM 12 treatment increased Mtb control correlated with a significant decrease in alveolar
macrophage and neutrophil populations; whereas, recruited macrophages were increased in the lung (Fig. 3.2B). Total lung cellular accumulation between the groups showed that a significant increase in cellular influx in the CWHM 12 treated group when compared to controls, which further supports the increased inflammation observed in the lungs of these mice (Fig. 3.2A).

**CWHM 12 suppresses iNOS and cytokine production, but enhances collagen in lung**

Since we saw significant lung macrophage accumulation, we determine whether CWHM 12 treatment influence macrophage activation through iNOS production. To determine this, we stained for iNOS and observed that the CWHM 12 treated group had significantly less iNOS+ cells (Fig. 3.3A). Since we saw significant changes in macrophages and iNOS production it may suggest macrophages within the treated lungs are polarizing toward a M1 state.

Previously, Henderson et al. demonstrated that CWHM 12 reduces bleomycin induced pulmonary fibrosis; however, it is unknown how CWHM 12 impacts Mtb induced pulmonary fibrosis. Fibrosis was significantly increased in the lungs and granulomas of CWHM 12 treated mice (Fig. 3.3B-C). These data demonstrate that CWHM 12 treatment enhances pulmonary fibrosis in the Mtb mouse model.

To further understand and elucidate why recruitment of alveolar macrophages, neutrophil and recruited macrophages increased in response to treatment, we assessed cytokine production in the lung. We observed IL-10 and macrophage Inflammatory Protein 2 (MIP-2) were significantly decreased in the treatment group (Figure 3.3D). MIP-2 is a chemoattractant for neutrophils, so its decreased production in the lungs may result in a decrease in neutrophil pulmonary localization (255-257). Altogether, these data suggest CWHM 12 induces decreased pulmonary bacterial burden by modulating alveolar, neutrophil and recruited macrophages recruitment and suppressing iNOS, MIP-2 and IL-10 production.
3.3 Discussion

TGF-β is able to modulate innate and adaptive immune responses, as well as fibrosis. Active TGF-β is able to signal through TGF-β receptors 1 and 2 and signals Smad proteins to translocate to the nucleus and act as transcription factors for target genes. It has been shown through several studies that Active Tuberculosis is associated with increased production of TGF-β. *Mtb* infected mice had enhanced lung protection when IL-10 deficient (258, 259). A therapeutic that inhibits or suppresses TGF-β may aid in enhancing host immune protection and *Mtb* control. Throughout this study, we sought to establish whether CWHM 12 treatment during *Mtb* infection mediates a protective host immune response and controls *Mtb*. We have demonstrated that CWHM 12 treatment given early in infection aids in *Mtb* control, treatment is most effective after infection has been established. Inhibition of TGF-β at different stages of *Mtb* infection resulted in varying degrees of *Mtb* control, if CWHM 12 were to be used as a host directed therapeutic the time in which to administer it would need to be taken into consideration. CWHM 12 treatment, improved *Mtb* control and this was associated with decreased alveolar macrophages and neutrophils and increased recruited macrophages. Decreased numbers of alveolar macrophages and neutrophils may enhance the protective response because both populations have been shown to be reservoirs of *Mtb* (4, 22, 222). MIP-2 is produced by alveolar macrophages and is a recruitment chemokine for neutrophils (260, 261). Decreased amounts of MIP-2 suggest that reduced cellular presence of alveolar macrophages may lead to the decreased production of MIP-2 and recruitment of neutrophils.

In addition to a decrease in MIP-2 production during treatment, IL-10 protein levels were also decreased. IL-10, normally associated with TGF-β, also induces an anti-inflammatory response and has also been associated with ATB (202, 258). *Mtb* infected mice had enhanced lung
protection when the mice are IL-10 deficient or IL-10 depleted (258, 262). Denis and Ghadirian have shown that decreased amounts of IL-10 contribute to increased Mycobacterial control in macrophages (262). Decreased amounts of alveolar macrophages and neutrophils not only present a reduction in niches for Mtb growth, but in the case of alveolar macrophages it also serves as an environment of Mtb control due to IL-10 suppression (262). It is promising that in conjunction with TGF-β inhibition, IL-10 production is also decreased during CWHM 12 treatment. In addition to an anti-inflammatory role, TGF-β has been computationally suggested to contribute to granuloma formation (263). Macrophages are major components of granulomas, so CWHM 12 may also be mediating the formation of a protective granuloma that is able to contain and control Mtb. Characteristics of a “protective” granuloma have increased B cell follicles, increased iNOS, decreased hypoxia and decreased perivascular cuffing (184, 264). Classical M1 stage macrophages typically are also associated with iNOS and proinflammatory cytokine production; whereas, M2 polarization is associated with anti-inflammatory cytokine and chemokine production. Although, not specifically quantified this iNOS data paired with population changes may indicate that CWHM 12 may drive macrophages to polarize toward a M1 state. In order to reaffirm this hypothesis, Mtb presence along with protective granuloma characteristics within control treated and CWHM 12 treated granulomas would need to be assessed. Additional in vitro experiments are needed to assess whether CWHM 12 has mycobactericidal abilities as a sole compound and when in the presences of macrophages. Increased TGF-β in active TB patients has been established and suggests that the diminished production of TGF-β would be associated with treatment. Mtb infected non-human primates that were treated with TB chemotherapy (rifampin and isoniazid) for two months showed that TGF-β levels decreased as antibiotic treatment progressed (265). This decrease was shown to be
associated with decreased signaling through to SMAD-2/3 (265). Interestingly within this NHP study, collagen levels increased similar to what we see in our model. Since we observe the greatest decrease in lung bacterial burden after Mtb infection has been established, it would be beneficial to assess the efficacy of CWHM 12 treatment in conjunction with standard TB treatment.

Here we have demonstrated that CWHM 12 TGF-β inhibition resulted in controlling Mtb at different stages of infection. Along with this enhanced control, iNOS decreased and collagen production increased within granulomas. Increased collagen in the lung and granuloma may indicate a more fortified granuloma that is able to prevent Mtb dissemination. Overall, this paper demonstrates that CWHM 12 treatment enhances Mtb control, mediates pulmonary cellular populations, as well as the micro and structural environment of the lung and granuloma.

3.4 Methods

Mice

C3HeB/FeJ (FeJ) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred within Washington University in St. Louis animal facility. Mice used were 6-8 weeks and also sex matched. All mice were maintained and used in accordance with the approved Institutional Animal Care and Use Committee (IACUC) guidelines at Washington University in St. Louis.

Bacterial Strains

Mtb strain HN878 (BEI Resources) was cultured in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and frozen in 2 ml aliquots at -80°C. Mice were aerosol infected with approximately 100 Mtb colony forming units (CFU). At 30 dpi, organs were harvested, homogenized and serial dilutions of tissue homogenates plated on 7H11 agar plates and CFU determined.
Preparation of lung cell suspensions

Lung cell suspension was prepared from *Mtb* infected FeJ lungs as described by Gopal et al 2014. Next, lungs were perfused with cold saline (with heparin) prior to dissecting them with sterile razor blades in chilled DMEM. Dissected lung tissue was then incubated in DMEM containing collagenase (0.7 mg/ml; Sigma-Aldrich) and DNase (30 µg/ml; Sigma-Aldrich) at 37°C, 5% CO₂ for 30 min. Digested lung tissue was passed through a 70 µm pore size nylon tissue strainer (Falcon; BD Biosciences). Single cell suspension was treated with Gey’s solution to lyse any residual red blood cells and washed twice before counting using a Coulter Counter.

Osmotic pump installation

For all studies, CWHM 12 and CWHM 96 (vehicle) were both solubilized in 50% DMSO (in sterile water) and dosed to 100 mg per kg body weight per day. Drug or vehicle (50% DMSO) was delivered by implantable ALZET osmotic minipumps (Durect, Cupertino, CA). Pumps were inserted subcutaneously 14 day post infection. Lungs were harvested after 30-45 days post infection.

Lung histology

Lungs from *Mtb*-infected mice were perfused with 10% neutral buffered formalin and were paraffin embedded. Lung sections were stained with hematoxylin and eosin (H&E) and processed for light microscopy. Sections were probed with F4/80, iNOS and DAPI. Images were obtained using Zeiss Axioplan 2 microscope and were recorded with a Zeiss AxioCam digital camera. To visualize collagen deposition, Masson’s trichrome staining (Sigma-Aldrich) was performed according to the manufacturer’s instructions. Images were obtained using Zeiss Axioplan 2 microscope and were recorded with a Zeiss AxioCam digital camera. Trichrome images were obtained using the Hamamatsu Nanozoomer 2.0 HT system with NDP scan image acquisition.
software and was quantified using Visiomorph image processing software (Visiopharm, Broomfield, CO).

**Flow cytometry**

Lung cell suspensions were prepared, stained, collected and analyzed for flow cytometry as described in Khader et al 2011. Fluorochrome-labeled antibodies specific for CD11b (M1/70), CD11c (HL3) and Gr-1 (RB6-8C5) were used in this study. Cells were collected using a FACSJazz with FACS Diva software. Cell populations were gated based on their forward by side scatter characteristics and the frequency of specific cell types was calculated using FlowJo (Tree Star Inc, CA). Lung alveolar macrophages were gated as CD11c\(^+\)CD11b\(^-\), lung myeloid dendritic cells were gated on CD11c\(^+\)CD11b\(^+\), neutrophils as CD11b\(^+\)Gr-1\(^{hi}\), recruited macrophages were annotated as CD11b\(^+\)Gr-1\(^{lo}\), and monocytes were gated on CD11b\(^+\)Gr\(^{1\text{int}}\) cells as in Desvignes et al 2012 (266).

**Quantitation of protein production**

IL-10 and MIP-2 protein levels were measured using a mouse Luminex assay (Linco/Millipore).

**Statistical analysis**

Differences between the means of two groups were analysed using the two tailed Student’s t test in GraphPad Prism 5 (La Jolla, CA). Differences with a P ≤ 0.05 were considered significant.
Figure Legends

A. Lung

B. Lung

C. Lung

Spleen

Scott et al. Figure 1
Figure 3.1 Treatment with CWHM 12 leads to decreased bacterial burden in Mtb HN878 infected FeJ mice. FeJ mice were aerosol infected with ~100 CFU of Mtb HN878. Bacterial burden in the lung and spleen were determined by plating after 30 or 45 days post infection (dpi). Lung and spleen bacterial burden from (a) 0-14 dpi, (b) 14-28 dpi, (c) 30-44 dpi mice treated with CWHM12. (b) H&E staining and (c) quantification of cellular infiltration of mice harvested 30 dpi. Original magnification for (a). Error bars represent means ± SD. *P ≤ 0.05, ** P ≤ 0.005, *** P ≤ 0.001 by (a-c) students t test.
Figure 3.2 CWHM 12 increases recruited macrophages within the lung during Mtb HN878 infection. FeJ mice were aerosol infected with ~100 CFU of Mtb HN878 and the lungs were harvested at 30 or 45 dpi. (a) Pulmonary inflammation was assessed and quantitated on H&E stained FFPE lung sections from Control and CWHM12 mice. (b) Lung myeloid cell populations The total number of (a) cells was determined using a Coulter Counter The percentage of (b) AMs alveolar macrophages, DCs dendritic cells, RMs recruited macrophages. Error bars represent means ± SD. *P ≤ 0.05, ** P ≤ 0.005, *** P ≤ 0.001 by (a,b) students t test.
Scott et al. Figure 3
Figure 3.3 CHWM 12 suppresses iNOS, IL-10 and MIP-2 and increases collagen production during Mtb infection. FeJ mice were aerosol infected with ~100 CFU of *Mtb* HN878 and treated with CWHM12 14-28 dpi. FFPE lung sections were used to carry out (a) immunofluorescence staining for F4/80 (green), iNOS (red), and DAPI (blue), (b) trichrome staining and quantifications of whole lung and granulomas. (d) Protein levels of MIP-2 and IL-10 were determined by luminex assay. Error bars represent means ± SD. group. *P ≤ 0.05, ** P ≤ 0.005, *** P ≤ 0.001 by (a-d) students t test.
Chapter 4

Conclusions and Future Directions

Previous studies have demonstrated that neutrophils respond and are effectors of immunopathogenesis of TB in human and animal models (43, 93). Neutrophilic mechanisms that contribute to TB immunopathogenesis are not fully elucidated. In this thesis, I demonstrate that neutrophil accumulation occurs during TB infection and progression to disease Mtb-infected humans and mice. Particularly, I show that neutrophils have a pathogenic role during chronic TB disease in mice. Previous data from Gopal et al. demonstrated that S100A8/A9 serum levels correlated with neutrophil cell count, but the data presented here further provides mechanistic evidence that S100a8/a9 lung concentrations parallel lung neutrophil accumulation in mice. The data presented in this thesis provides evidence that S100A8/A9 mediated TB susceptibility and reactivation and is associated with increased neutrophil accumulation to the lung and may be dependent on CD11b surface expression on neutrophils. Together, the human data collected provides evidence that S100A8/A9 serum levels in combination with CXCL-1 and CXCL-10 are able to distinguish ATB patients from HCs. Overall, data presented in this thesis on S100A9 demonstrates that S100A8/A9 promotes lung neutrophil accumulation during TB that may be mediated through CD11b expression on neutrophils, thus providing a new perspective in the role neutrophils play in the immunopathogenesis of TB.

In addition to identifying S100A8/A9 playing a role in chronic TB, this thesis also demonstrates that TGF-β plays a role in Mtb infection. Increased levels of TGF-β, a cytokine, in the serum has been associated with ATB. The second study within this thesis sought to understand how TGF-β was mediating its effect during Mtb infection and determining whether an TGF-β inhibitor is able to control infection. CWHM 12 treatment and transient TGF-β inhibition is able to aids in Mtb
control, being most effective after infection has been established. During TGF-β inhibition and \textit{Mtb} control, decreased neutrophil accumulation in the lung was observed. This finding mirrors findings from the S100a9 deficient \textit{Mtb} model, indicating neutrophils indeed have a role in \textit{Mtb} infection.

Although there are many mouse, NHP, and human studies that have associated neutrophil accumulation to TB progression and disease, it is still unclear whether neutrophils have protective or pathological functions in \textit{Mtb} infection (5, 43, 96, 155, 164). It has been well established that neutrophils are considered as one of the first cell types infected with \textit{Mtb} (142). Neutrophil depletion during the acute stage of infection showed no significant change in lung or spleen bacterial burden suggesting that neutrophils have no significant impact on \textit{Mtb} control or TB pathology during the acute stage. However, neutrophil depletion during the chronic stage of infection significantly decrease lung and spleen bacterial burden suggesting that neutrophils play a pathogenic role during this stage. Interestingly, while both neutrophil depletion and S100A8/A9 deficiency improved \textit{Mtb} control, overall inflammatory lung disease was not impacted. In the case of neutrophil deficiency, it is possible that transient depletion of neutrophils was not sufficient to impact overall inflammation and prolonged neutrophil depletion may be required to impact lung pathology. The functionality of neutrophils may also change depending on the stage of infection.

To determine if this is correct, RNAseq from lung neutrophils isolated from \textit{Mtb} infected mice would assess whether neutrophil gene expression is different depending on the stage of infection. Data from these isolated neutrophils may illuminate additional neutrophilic factors and mechanisms that promote susceptibility or protection during \textit{Mtb} infection. Since I observed that decreased neutrophil accumulation occurred during CWHM 12 treatment, it would be beneficial to assess whether the protective neutrophil gene expression profile is conserved during anti-TB
treatment. Understanding whether a neutrophil changes its gene expression profile during infection and during anti-TB treatment will expound the TB and neutrophil community.

During S100A8/A9 deficiency in the mouse model, there was an overall trend towards decreased inflammation (data not significant) even though neutrophil accumulation was significantly decreased. The reduced neutrophil accumulation within the S100a9 deficient mice also coincided with decreased amounts of neutrophils present and an increase in B cells in the TB granulomas. Previous studies published by our lab have demonstrated that increased B cells in TB granulomas were associated with improved \textit{Mtb} control (184). Additional studies into the connection between S100A8/A9 and granuloma formation should be assessed since the data presented here suggests that it promotes the formation of granulomas that do not control \textit{Mtb}. Together, our results demonstrate that neutrophils and S100A8/A9 may play key roles in modulating TB immunopathogenesis by driving neutrophil accumulation.

In both S100a9 deficient mice and TGF-\(\beta\) treated mice, neutrophil accumulation was associated with a significant decrease in lung bacterial burden. This finding in addition to the neutrophil depletion data suggests that neutrophils may provide a niche for harboring \textit{Mtb}. To further understand how S100A8/A9 contributes to \textit{Mtb} growth, in vitro bactericidal assays should be done in the presence and absence of neutrophils to assess whether S100A8/A9 solely promotes \textit{Mtb} growth or if it is neutrophil dependent. The same assays should be applied to TGF-\(\beta\) to determine its sole effect on \textit{Mtb} growth or if it is cell dependent. Elucidating how S100A8/A9 is able to promote \textit{Mtb} growth would be a novel finding because in most bacterial infections, S100A8/A9 is actually effective against killing extracellular pathogens (218, 267, 268). Currently, there is not much literature detailing how TGF-\(\beta\) limits \textit{Mtb} growth, so identifying this would also be beneficial to the TB field.
In this *Mtb* mouse model, a suggested mechanism by which S100A8/A9 regulates TB pathogenesis is through the upregulation CD11b expression on neutrophils. Interestingly, *S100a9* deficiency impacted CD11b expression only on neutrophils and not monocytes or recruited macrophages in the chronic stage of infection. In vitro and in vivo studies slightly mirror each other because both S100a9 deficient neutrophils had lower expression of CD11b when infected. It is important to note that CD11b expression differences *S100a9* KO mice did not occur until 100 dpi. The nature of the microenvironment (e.g. presence of other pro-inflammatory chemokines, prolonged *Mtb* infection and stimulation, etc.) may contribute to differences observed in *in vitro* studies and *ex vivo* isolated neutrophils. An adoptive transfer of CD11b cells increased susceptibility in *S100a9* KO mice, suggesting that CD11b cellular populations, specifically neutrophils or monocytes expressing S100a8/a9, are the likely cellular population mediating increased susceptibility during chronic TB. Using *Mtb* infected CD11b deficient mice will allow for the assessment of whether CD11b promotes *Mtb* susceptibility. Since this thesis main focus the impact of neutrophils on Mtb infection, it would be ideal to have a CD11b floxed mouse under a MPR8 cre promoter, to specifically determine how CD11b deficiency in the neutrophil impacts TB infection and disease. Additionally, it is of considerable interest that while *S100a9* KO mice are protected from infection with HN878 and HN563 (hypervirulent strains), *S100a9* KO mice do not show any protection when infected with H37Rv (a lab adapted strain). It would be informative to assess how S100a9 deficiency promotes *Mtb* control or susceptibility in other *Mtb* strains. Establishing the impact of S100A8/A9 on other *Mtb* strains, especially clinical or multidrug resistant strains, would be clinically relevant.

The data presented in this thesis demonstrates there is a defect in lung neutrophil accumulation, which may be dependent on CD11b integrin surface expression. These data garners the question
of whether S100A8/A9 impacts neutrophil migration within our *Mtb* model. To determine whether S100A8/A9 impacts neutrophil migration, transwell experiments with infected neutrophils migrating to a neutrophil chemoattractant (e.g. CXCL-1) may be used. Neutrophil chemotaxis has been associated with cytoskeletal reorganization and actin polymerization and regulation. *S100a9* deficient neutrophils had actin defects when responding to IL-8 stimulation, suggesting that S100a9 has a role in cytoskeletal dynamics and reorganization (217). Functional actin coordinates cell surface integrin regulation (e.g. CD11b/CD18), which could impact neutrophil migration and adhesion. Together, this suggests that S100a8/a9 expression in the lung may induce chemokines that mediate neutrophil accumulation, potentially directly act as a chemoattractant for neutrophils (229), but also by regulating the expression of CD11b to regulate additional neutrophil migration into the lung.

S100A8/A9 is known to interact with TLR4 and RAGE, while CD11b expression can be driven by interactions with TLR4 on neutrophils (212, 235). Thus, S100A8/A9 may engage TLR4 and RAGE to upregulate CD11b expression on neutrophils. RAGE inhibitor, FPS-ZM1, has been shown to be protective emphysema and Alzheimer's disease animal models (224, 236). During chronic TB, we show that transient use of RAGE inhibitors is protective and is sufficient to decrease lung *Mtb* burden, but not lung inflammation. Whether use of RAGE inhibitor limits neutrophil accumulation and thus reduces the *Mtb* niche is not fully explored and should be the focus of future studies. Excitingly, our data showing that S100a8/a9 deficiency delays TB reactivation suggests that RAGE inhibitors may be potentially used as host directed therapeutics in combination with current antibiotic regimens to improve *Mtb* control and should be further studied. In addition to the RAGE inhibitor, this thesis has shown that inhibition of TGF-β signaling via CWHM 12, also suggesting that mediating TGF-β signaling as a host directed therapy has the
potential of enhancing *Mtb* control. If RAGE and TGF-β inhibitors provide synergistic effects promoting *Mtb* control, it is also important to also contemplate the potential off target effects that it may have in addition to being protective.

Currently, the diagnostic method for TB is an automated PCR assay that detects mycobacteria in sputum expectorated by patients. Due to limited access, the most common method used is a sputum-based microscopy to identify *Mtb*. Although this is a good method it solely identifies individuals with ATB. Therefore, new non-sputum based screening tool for identifying individuals with ATB is required so that they can be prioritized for clinical investigation and treatment. The human data presented in this thesis demonstrate that S100A8/A9, CXCL-1 and CXCL-10 serum protein levels are able to differentiate between ATB and HC, and although less effective can also discriminate between ATB and QFT+ LTBI. Thus, further validation of the use of S100A8/A9, CXCL-1, and CXCL-10 in different geographical cohorts and in treatment failure and relapse studies will be useful and timely. In addition to assessing geographical cohorts, it is necessary to determine the specificity and sensitivity is amongst other acute bacterial infections that are prevalent in those areas. It also would be beneficial to determine whether S100A8/A9, CXCL-1 and CXCL-10 is a valid biomarker for individual who are HIV+.

In summary, the studies within this thesis have suggested and described a pivotal role for S100A8/A9 proteins and TGF-β in mediating TB pathogenesis and its association with lung neutrophil accumulation. Additionally, experimental studies targeting the S100A8/A9 and TGF-β signaling pathways provide novel opportunities to explore host directed therapeutics for TB.
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