Determining the Major Genetic Regulators of *Mycobacterium tuberculosis*-induced Cytokine Expression in Monocytes and Macrophages with a Cellular GWAS

Joshua Ivie

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Reading Committee:

Thomas Hawn, Chair

Kevin Urdahl

Marshall Horwitz

Program Authorized to Offer Degree:

Pathobiology

University of Washington

Abstract

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Joshua Ivie

Chair of the Supervisory Committee:
Thomas Hawn
Department of Medicine

Tuberculosis (TB), largely due to infection with the causative pathogen *Mycobacterium tuberculosis* (Mtb), has claimed over 1 billion lives in the past 200 years, including 1.3 million lives in 2022 alone. Efforts to eradicate TB have been complicated by heterogeneity in infection outcomes after Mtb exposure. Host genetics may contribute as much as half of an individual's susceptibility to certain infection outcomes, however, the causal variants responsible remain unknown. 70,000 years of human infection has led to intricate pathogen and host adaptations that fight for control of crucial infection responses. In particular, the macrophage cytokine response is heavily modulated by both pathogen and host and requires a fine-tuned balance for successful control of infection. Prior studies attempting to define genetic regulators of Mtb infection have often focused on genome-wide association studies (GWAS) measuring clinical phenotypes. A

cellular GWAS, which measures genetic regulation of the cytokine response to live Mtb infection, *in vitro*, may identify important biology, and has not been assessed previously.

This dissertation aimed to assess if Mtb-induced human monocyte and macrophage cytokine responses are genetically controlled and if a cellular GWAS approach can be used to identify genetic regulators that are important in modulating immune and clinical TB response outcomes. Using Mtb-induced cytokine expression profiles from individuals in a Ugandan cohort, we performed WGCNA, heritability, and cellular GWAS analyses. These findings were further assessed in independent cohorts from South Africa and Seattle, and in subsequent *in vitro* validation and mechanistic investigation. Initial expression profiling identified 35 hub genes which were central to the Mtb-induced cytokine response, and determined a heritable component of Mtb-induced IL1B and IL6 expression. Our cellular GWAS in Ugandan individuals identified several SNPs surpassing suggestive significance and a significant enrichment of Mtb-induced TNF association in the genes of two pathways. Further investigation revealed that one of these pathways, alpha-linolenic acid metabolism, was validated *in vitro* using PLA2 inhibitors. We further identified multiple SNPs which showed population spanning effect when assessed in the Seattle population and validated cytokine effect for two of these SNPs which mapped to the genes SLIT3 and SLC1A1. SLIT3 was additionally found to enhance Mtb intracellular replication. Finally, SNPs from both of our *in vitro* validated loci, associated with SLC1A1 and SLIT3, were associated with the clinical TB phenotypes, susceptibility to tuberculous meningitis (TBM), and TBM survival, respectively. Taken together, this research identified multiple genetic regulators of the Mtb-induced myeloid cell cytokine response which were validated *in vitro*, and shows the promise of the cellular GWAS approach for identifying novel host response factors with clinical relevance.

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DEDICATION

I would like to dedicate this dissertation to my wife, Nikki who has always supported me.

Chapter 1. INTRODUCTION

1.1 Overview of Tuberculosis

1.1.1 *Burden of Tuberculosis*

Tuberculosis (TB) has led to more deaths from an infectious agent than any other disease in human history including over 1 billion deaths within the last 200 years [1]. Although the rate of mortality has decreased in recent decades, TB remains the leading cause of death from an infectious agent worldwide, leading to 10.6 million illnesses and 1.3 million deaths in 2022 [2]. In the context of total global burden of disease, TB was estimated to lead to a loss of 122 million disability adjusted life years (DALYs) in 2019 which would place it as the fourth leading cause of DALY loss of all disease burden [3,4]. Recent efforts to combat TB globally have been complicated by the COVID19 pandemic which disrupted many TB response efforts, led to an increase in global TB deaths from 2019 to 2021, and increasing TB incidence throughout 2022 [2]. Although the WHO goals for its End TB strategy were a 75% decrease in TB deaths and a 50% decrease in TB incidence from 2015 to 2025, the current decreases as of 2022 have fallen far short with only a 19% and 8.7% decrease respectively [2]. As such, there is a clear need for increased TB response efforts and novel methods to counter the global burden of disease that it imposes.

1.1.2 *Causative pathogen*

TB is primarily caused by bacterial pathogens of the *Mycobacterium tuberculosis* complex (MTBC) which have infected humans for tens of thousands of years [5]. The *Mycobacterium* genus is composed of over 170 different species which predominately exist in environmental

niches [6]. Only a few of these mycobacterial species have evolved to infect humans and the majority of TB cases globally are due to *Mycobacterium tuberculosis (sensu stricto)* (Mtb) [6]. Mtb is unique among many of these species in that its infection and propagation throughout the population is entirely reliant on human transmission, and it has limited ability to infect other hosts outside of nonhuman primates [6]. Over the many millennia of this human transmission, Mtb has evolved substantially, dropping many non-essential genes, and acquiring hundreds of other genes through horizontal gene transfer [7]. This has led to Mtb developing numerous adaptations which allow it to manipulate the host to be a suitable environment for its continued replication and sustain the global prevalence that we continue to struggle with today.

1.1.3 *Mtb transmission and progression of infection*

The course of an infection after an individual is exposed to Mtb is complex, and progression of infection is characterized by enormous heterogeneity between individuals [8,9]. However, a classical understanding of the Mtb infection lifecycle can be summarized as follows. Mtb predominately exists as a respiratory pathogen and new infections are transmitted via respiratory droplets that are aerosolized during coughs of an individual with active TB [10]. After inhaling the aerosolized respiratory droplets, which may contain only a few bacilli, Mtb encounters and is phagocytosed by resident alveolar macrophages (AMs) within the lung [10]. Once internalized, Mtb survives within this intracellular niche, subverting endosomal maturation, and directing downstream macrophage response [10–13]. This leads to subsequent dissemination of Mtb in the lung interstitium and infection of numerous other innate immune cells which have been recruited [11]. Mtb has evolved mechanisms to survive and even utilize the defense mechanisms of responding innate cells such as monocyte derived macrophages (MDMs), neutrophils, and granulocytes allowing the infection to persist [14–18]. Infected phagocytes will

often migrate and aggregate together forming the basis of an organized granuloma structure [19]. Although Mtb actively acts to delay the host adaptive response, one is usually eventually generated and T cells can be recruited to the area of infection [20]. Within the first few weeks of infection, the resultant immune response will either continue to spread, progressing into active TB disease, or the generated T cell response will organize the immune cell response to allow for containment of infected cells within a granuloma [21]. This state of latent TB infection (LTBI), where Mtb remains in a dormant state within the granuloma, can be sustained for decades while leaving the individuals completely asymptomatic [22]. By some estimates, upwards of one quarter of the world's population may be infected latently and many may never progress to disease [23]. However, in cases of immune perturbation such as becoming immunocompromised, Mtb containment within the granuloma can fail, and lead to further dissemination of Mtb [22,24]. Once dissemination reaches the airways, Mtb can proceed to grow to substantial numbers, at which point, it can then be aerosolized by coughing and proceed to begin the infection cycle anew in nearby individuals [25].

1.1.4 *Heterogeneity in outcomes*

Although the above indicated Mtb infection lifecycle may be representative of many infections, individual outcomes after Mtb exposure are highly heterogeneous and may vary substantially. This spectrum of outcomes can be classically divided into four major categories: resistance to infection, establishment of an asymptomatic latent infection, development of pulmonary TB disease, or progression to various forms of severe TB disease [9]. The ability to efficiently test for Mtb in asymptomatic individuals is currently unavailable and requires reliance on an imperfect, indirect measure of immune response to Mtb antigens via the tuberculin skin test (TST) or interferon-γ release assay (IGRA) [26]. Additionally, increasing research indicates

diversity within these categories and the ability to advance or reverse positions depending on immune status [27]. In response to these difficulties, cohorts have been thoroughly characterized incorporating long term follow up to identify TST and IGRA conversion, changes in HIV status, level of Mtb exposure, and differences in TB disease progression [28]. Within these studies, approximately, 7-25% of the population have been identified to be able to clear or contain Mtb infection without developing a standard T-cell response [29–31]. These individuals, termed resisters (RSTRs), will remain TST (tuberculin skin test) and IGRA (interferon gamma release assay) negative for decades despite high exposure to individuals with infectious TB. This indicates that certain individuals can resist progression after exposed to Mtb using innate mechanisms or alternate non IFN-gamma dependent T-cell pathways [32]. The majority of individuals who are exposed to Mtb will develop LTBI as assessed by TST/IGRA positivity but not display symptomatic progression for decades and only around 5-10% of infected individuals will progress to develop pulmonary TB [9]. Due to reliance on the TST/IGRA as an indirect assessment of current infection, it is unclear if latent infection is a major contributor to new sources of active TB, however, there is substantial evidence that the majority of new active TB cases progress from recent infections[33]. Finally, a small portion of the population containing only around 1% of infected individuals, will progress to states of severe TB disease including tuberculous meningitis (TBM), miliary TB, and landouzy sepsis [34]. Within these severe clinical TB states, inability to contain the initial Mtb infection leads to dissemination throughout the body. Once dispersed, the immune response can create dramatic damage as it tries to contain the infection, as in the case of TBM, where inflammatory response to infection of the meninges leads to severe neurological disease [35].

1.1.5 *Current response options*

Major TB response efforts are currently centered around vaccination in endemic areas and antibiotic treatment of disease which are both beset by many difficulties. Vaccination is reliant on the decades old Bacillus Calmette-Guerin (BCG) vaccine, which remains the only licensed vaccine to date [36,37]. Although, BCG significantly reduces severe forms of extrapulmonary TB in children, it has had variable efficacy and often fails to protect older individuals [38]. For individuals that progress to infection, treatment relies on antibiotics which are highly burdensome, often lasting 6 months, and are often accompanied by side effects [39]. Substantial efforts to reduce this burden and recently have succeeded in developing a 4 month rifapentine and moxifloxacin regimen which is non inferior to the standard 6 month treatment [40]. However, resistance to antibiotics is prevalent, and drug-resistant TB led to 160,000 deaths 2022 [2]. Individuals with multi-drug resistant (MDR) TB are forced to take more substantial antibiotic regimens, such as the recently developed, bedaquiline, pretomanid, and linezolid regimen [41]. However, this regimen can often present toxic effects which can be difficult to manage. Due to these difficulties, individuals often fail to complete treatment regimens, which only further adds to the problem. In response, many countries are forced to place infrastructure to ensure treatment plans are followed which represents its own significant financial burden [9]. The ability to develop new antibiotics is further hampered by the complexity of the mycobacterial cell wall and the intracellular niche that Mtb employs [39]. However, new antibiotics are in development and continual optimization of drug combinations may increase our treatment efficacy in the future.

1.2 HOST RESPONSE TO MTB

1.2.1 *Initial response*

The host response necessary to control Mtb infection requires the action of many different cell types and response pathways to act in a fine-tuned and balanced fashion. Although many mechanisms of host protection are poorly understood, a more specific overview of the host response is as follows. The first immune cells to respond after inhalation of infecting Mtb are resident AMs [9]. In contrast to monocyte derived macrophages (MDMs), which are recruited from the blood in response to infection, a population of AMs is constantly present in the lungs, and acts to maintain the lung through many actions in addition to pathogen response [42]. Since its primary purpose is preservation, many studies have found that AMs are predominately anti-inflammatory cells and only switch to become inflammatory cells after prolonged infection with Mtb [43,44]. Since the AM is not highly specialized for pathogen destruction, Mtb can often overcome the cell's defense mechanisms and manipulate them for its own benefit.

Without interference from Mtb, a successful AM response would result in phagocytosis, and shuttling Mtb to the lysosome for subsequent destruction. After which, cytokine and chemokine signaling pathways would result in recruitment of monocytes to the area, which could differentiate into macrophages and dendritic cells that could clear the remaining infection and initiate downstream adaptive immune responses, respectively [45]. However, Mtb dramatically modulates this flow of events. After initial phagocytosis, Mtb secretes numerous effectors lipid and protein effectors which act to prevent phagosome maturation, acidification, and lysosomal targeting [12]. Mtb can then permeabilize the phagosomal membrane allowing Mtb and its secreted effectors to access the cytosol [12,13]. Although host autophagy pathways can usually

retarget these membranes for delivery to the lysosome, Mtb prevents these processes and continues to thrive intracellularly [10,46]. Since these cells cannot always overcome the pathogen, recruitment of a greater immune response, and targeted cell death of infected cells become crucial. Mtb has many pathogen-associated molecular patterns (PAMPs) that can be recognized by host TLR, C-type lectin, scavenger, and complement receptors, that should activate inflammatory pathways such as NF-kB signaling, and lead to pro-inflammatory Tumor Necrosis factor (TNF), Interleukin 6 (IL6), and Interleukin 1 beta (IL1B) cytokine production, all of which are associated with host protection from initial infection [10,47]. However, Mtb also interferes with these responses, and multiple secreted factors have been identified to decrease inflammatory cytokine production [48,49]. As a last resort, these cells can choose to undergo apoptosis, which is a controlled form of cell death in which plasma membrane integrity is maintained [50]. However, Mtb interferes with this protective apoptosis pathway, and instead directs the cells towards necrosis in which plasma membrane integrity is lost [16,51,52]. Rather than leading to control, this leads to further dissemination of the infection.

1.2.2 *Recruitment of subsequent response*

Although the AM is initially the primary infected cell within the host airways, it largely serves as a vehicle of dissemination to other immune cells. Mtb infection induces AM travel to the lung interstitium in a manner that is both dependent on Mtb secretion systems and host Interleukin-1 signaling [11]. Once in the interstitium, continued replication of the infection within the AM can occur, resulting in initial macrophage aggregation and granuloma formation, and eventual dispersion to other cells drawn to the site of infection [10]. Although these recruited cells are more specialized for inflammatory infection control, Mtb is often able to overcome their defense mechanisms as well. One of these cell types is the MDM, which eventually becomes the

most infected cell in the course of Mtb infection [53]. Even though the MDM is more inflammatory and associated with increased ability to clear Mtb infection, Mtb is often able to overcome its defense mechanisms using the same strategies it uses to overcome AMs [54]. Additionally, some evidence suggests that differing states of MDM polarization exist after differentiation, and infection of certain MDMs may be the basis of increased Mtb survival and form the core of persistence within the granuloma [55,56].

Neutrophils and granulocytes recruited to cites of infection are often equally as unsuccessful at infection elimination. Mtb is more easily able to avoid oxidative killing by neutrophils and can bypass neutrophil extracellular traps (NETs) which are a major neutrophil defense mechanism [15,57]. Instead of promoting infection resolution, neutrophil infection is often associated with further dissemination of infection due to necrotic cell death, promotion of nutrient availability, and a damaging host response due to induction of a type 1 IFN [14,17,58]. In summary, rather than resolve infection, neutrophil and granulocyte recruitment often acts to further it.

Although subsequent recruitment of the adaptive response is a central element of the initial innate response, Mtb response factors substantially delay initiation of the adaptive response and mitigate its impact. Initiation of the adaptive response requires antigen presentation within the lymph node, however, Mtb directly interferes with antigen presentation, and decreases in inflammatory cytokine production limit subsequent lymph node egress which can delay the adaptive response for weeks [10,20]. The adaptive response, largely focused on the actions of recruited CD4+ T cells, is often essential in successful containment of Mtb infection. CD4+ T cells, through direct macrophage interaction and production of IFN-gamma, are able to greatly further macrophage activation, increase their microbicidal capabilities, and organize granuloma

structure [21,45]. The importance of CD4+ T cells can be seen in cases of AIDS, in which HIV depletes this population, and predisposes those individuals to disseminated pulmonary TB infection [22]. Finally, CD8+ T cells and antibodies have been discovered to play beneficial roles in Mtb response and may be important in the design of future therapeutic interventions [59–61].

1.2.3 *Progression of infection*

Although the causal mechanisms are poorly understood, after a few weeks of infection, or decades of latent infection, some individuals will fail to contain the infecting Mtb and progress towards active TB [22,24]. As Mtb replicates freely outside of the granuloma, it can then spread to other sites of the body. Initially, this may result in sub clinical TB, in which the individual can test sputum positive but not have any symptoms [27]. However, the increasing burden of infection will often quickly lead to active TB disease with symptomatic cough, fever, and weight loss [9]. Depending on the extent of the immune response, tissue damage can be severe and lead to a variety of different TB disease phenotypes according to site of dissemination. Once Mtb reaches the airways, a poor ability to contain the infection will result in relatively unhindered replication and high numbers of Mtb can then be aerosolized by coughing [25]. Dispersion can proceed to infect many areas of the lung or other organs as is seen in the case of miliary TB [34]. Additionally, Mtb can spread via the blood stream and infect the meninges. The substantial inflammatory response to a blood infection can result in landouzy sepsis and extensive neurological inflammation can result in TBM [62]. Quick, responsive, antibiotic treatments are often essential in individuals that have progressed to these states of TB disease or death will likely ensue.

1.2.4 *The cytokine response*

Although only a small snapshot of the multitudinous complexities of Mtb infection, the above summarized features highlight the numerous adaptations that both pathogen and host have coevolved over millennia of infection. An effective Mtb response often requires the host to hit a perfect middle ground, where either an over or under response can quickly benefit Mtb or lead to host damage [47]. The cytokine response shows this evidently in cases where this balance is perturbed. In studies of mice with IL1B and TNF knockout, mice are unable to control Mtb infection [63,64]. Furthermore, TNF inhibitors commonly used for autoimmune diseases can render individuals highly susceptible to developing TB [65]. However, at the other end of the spectrum, Mtb has learned to benefit from these responses even when they are present. For IL1B this is shown in AM studies which previously indicated the importance of IL-1 signaling in AMs transmitting Mtb infection to the lung interstitium [11]. For TNF, Mtb is able to utilize the TNF response within the granuloma, shifting its role from pro-apoptotic to pro-necrotic, and thereby allow it to infect new cells [51]. Lastly, in cases of TB disease, under response of IL1B and TNF can quickly allow the infection to spread, but over response can quickly lead to death [47].

Other myeloid cytokines such as IL6, also play substantial roles in Mtb response, by increasing ability to generate T cell responses and respond to high bacterial burden. Mice deficient in IL6 cannot control high dose Mtb infection and have reduce ability to clear active TB infections, but IL6 is also heavily implicated in severe cytokine storm which can occur in response to active TB [66–68]. Finally, not all Mtb-induced cytokines are beneficial to the host. Type 1 interferons, such as IFNB, are often associated with negative infection outcomes and are utilized by Mtb infection to compromise host immune efficacy [69,70]. One of the major outcomes of Mtb permeabilization of phagosomal membrane is release of DNA into the cytosol

which will then trigger an interferon response [71,72]. Type 1 interferons are associated with counteracting beneficial aspects of IL1B and promoting the less efficacious and highly damaging neutrophil response [14,73]. These negative effects are highlighted in mouse studies where IFN receptor knockout mice are less susceptible to Mtb infection and in human studies showing that a type 1 IFN response can often proceed the onset of severe TB disease [58,74]. In all of these cases, the balance of the cytokine response is intensely fought over by both pathogen and host to control infection outcome. Further understanding of how this balance modulates beneficial infection outcomes is needed to allow for the potential design of host directed therapies which can decrease the burden of TB disease.

1.2.5 *Predispositions to exposure outcomes*

Many of the factors which predispose an exposed individual to a particular outcome remain unknown. However, variation in environment, Mtb strain, and host factors including age, sex, comorbid conditions and genetics have all been indicated to cause significant differences in predisposition [9,75]. From an environmental perspective, changes in predisposition are often due to factors modulating the health of the lungs. Smoking and inhalation of silica particulate have both been significantly associated with predisposition to TB disease [76,77].

Diversity of the infecting strain of Mtb can also play a major role in infection outcome. Constant evolution has led to substantial strain diversity, resulting in several different lineages which are dispersed throughout different geographic regions. Different strains can often have dramatic variation in host infectivity, drug susceptibility, and disease severity, including differences in extra-pulmonary TB, and TBM disease [78–84]. Confident assessment of the mechanism of effect has been complicated by host differences with *in vivo* models, however,

there is a consistent indication of strain differences in cytokine modulation being a major factor in subsequent progression.

Lastly, many host factors have also been associated. Host age has been identified to play a major role, especially in the context of infant infection, which has high rates of disease progression [9]. Interestingly, sex also plays a major role as incidence of active TB has been found to be almost two times as high in men than women [9]. Numerous comorbidities are significantly associated with disease progression, including HIV coinfection, diabetes, silicosis, and malnutrition [75,76]. Differences in host genetics have been heavily associated with differences in Mtb infection outcome. Over the 70,000-year history of Mtb infection, it is likely that humans have coevolved allowing for the accumulation of numerous genetic adaptations at the global and population specific level [5]. As far back as 1978, twin studies identified that differences in susceptibility to Tuberculosis were highly heritable [85]. This was complimented with early research on TST conversion as far back as 1994 using sibling studies which additionally indicated a heritable component [86]. Since then, many individuals who were identified with mendelian susceptibility disorders have been discovered to be due to genetic mutations in essential host response pathways such as the IL12 and IFNg response [87]. In recent studies, the contribution of genetics to predisposition of infection outcome has continually been identified. One recent estimate obtained using SNP based relatedness found that upwards of 55% and 50.4% may be heritable for TB susceptibility and TST/IGRA conversion resistance, respectively [29].

1.3 Previous Genetic studies

1.3.1 *Clinical GWAS*

With the high indicated heritability involved in these previous studies, researchers have long sought to identify the major causal variants responsible. Many initial studies focused on candidate gene studies, and have identified significant associations within crucial pathways [88]. With increased availability to large scale genotyping, researchers have switched to using genome wide association studies (GWAS) which assess single nucleotide polymorphisms (SNPs) across the genome. Multiple studies have employed this approach and have identified significant associations [89–95]. However, throughout these studies, the identified causal loci only explain a small portion of the genetic effect indicated in heritability calculations [29]. Additionally, many of the identified loci fail to validate across populations and are rarely mechanistically defined [88]. One of the reasons prior studies may miss major variants explaining missing heritability could be due to a focus on using clinical cohorts measuring complex, endpoint phenotypes, which incorporate significant environmental and pathogen strain variation that may hinder ability to identify a significant genetic signal [96,97]. In response, a few clinical GWAS have begun to try and overcome these difficulties by accounting for strain variation and incorporating more thorough epidemiological characterization of cohorts. However, the increased burden required to collect this data can limit cohort sample sizes which are already underpowered compared to GWAS in other fields.

1.3.2 *Cellular GWAS*

In response to these difficulties, some researchers have begun to employ a cellular GWAS approach, which experimentally determines the effects of individual SNP variation on specific,

in vitro, intermediate phenotypes [96]. Using this approach, the researcher can focus on specific cellular traits which are known to be essential in clinical disease progression, but with the added benefit of being directly measurable and more clearly defined. Furthermore, by characterizing the genetic regulation of these important response pathways *in vitro*, researchers can often reduce the influence of traditional confounding variables. Cellular GWAS results can then be used to identify novel host Mtb response factors and provide mechanistic insight into clinical phenotype associations. This approach has been employed in numerous cellular GWAS efforts previously, including assessment of cytokine production after multiple microbial stimuli, cell death, and intracellular replication of *Salmonella* and *Chlamydia* [98–104]. However, responses to live Mtb infection have not been previously explored.

1.4 DISSERTATION AIMS

1.4.1 *Dissertation overview*

Further characterization of the underlying biology responsible for the heterogeneity in Mtb exposure outcomes is crucial to increase our ability to respond to the global burden of Mtb. Efforts to decrease new infections and TB deaths have fallen far short of end TB strategy goals. If we can identify elements of the host response that are critical in modulating TB progression, we may be able to utilize these to increase our ability to respond to this global burden. Utilizing a cellular GWAS approach, we can unravel host genetic regulation of crucial response elements, using *in vitro*, intermediate phenotypes. Throughout this review, we have noted the central importance of the cytokine response and MDM cell type in Mtb response. A greater understanding of how this essential immune response is regulated may yield important insight

into predisposition to Mtb exposure outcomes and identify novel response elements for subsequent research.

1.4.2 *Dissertation specific aims*

This dissertation aims to characterize the major genetic regulators of the myeloid cell cytokine response to live Mtb infection. We hypothesized that Mtb-induced cytokine expression in myeloid cells is genetically regulated by variants that are also associated with other Mtb-response phenotypes and clinical outcomes. Few genetic variants identified to date have shown consistent effect across populations and have rarely been validated or mechanistically defined. Using a cellular GWAS approach, including multiple populations and *in vitro* validation, we intend to characterize the Mtb-induced cytokine response in human myeloid cells and how it is genetically regulated, which may subsequently fill some of these crucial gaps in knowledge. This will be accomplished through a multi-step workflow as outline in figure 1.1.

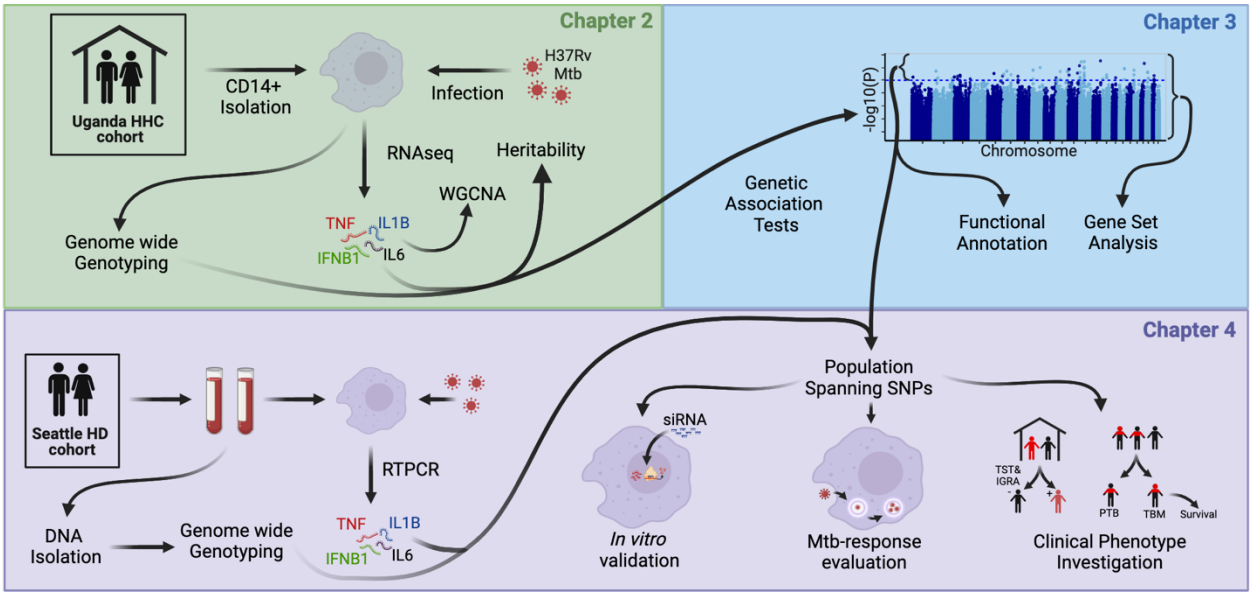

Figure 1.1. Progression of analyses and workflow of dissertation chapters.
Summary of discussed topics within dissertation chapters 2-4. Chapter 2 includes evaluation of the Mtb-induced cytokine response within the Uganda cohort, including induction analyses, heritability assessments, and WGCNA profiling within Uganda and validated in a

related South Africa cohort. Chapter 3 includes cellular GWAS analyses from the Uganda cohort including subsequent loci functional annotation, gene set analysis (GSA), and *in vitro* validation of GSA results. Chapter 4 includes profiling an independent Seattle cohort to determine variant transferability, including subsequent *in vitro* validation, Mtb-response, and clinical phenotype evaluation. Created in biorender.com.

Chapter 1 has summarized the many complexities of Mtb infection and the central importance of the host myeloid cell, cytokine response, and genetic variation in determining infection outcome. The subsequent chapters of this dissertation will investigate the myeloid cytokine response, define the major genetic variants regulating it, and confirm their effect in an independent population, *in vitro*, and in relation to other clinical TB phenotypes. Chapter 2 will perform initial characterization of the Mtb-induced cytokine response, including identifying how cytokines are induced, how that induction is related to the overall Mtb response, and if the Mtb-induced cytokine response is genetically regulated. Chapter 3 will identify the major genetic variants and pathways responsible for differential Mtb-induced cytokine expression within individuals from Uganda, including functional annotation and *in vitro* validation. Chapter 4 will identify which of the variants identified in chapter 3 show transferability in an independent Seattle cohort, validate them *in vitro*, and investigate their effect on other macrophage response and clinical phenotypes. Finally, chapter 5 will summarize the work performed in this dissertation and discuss project limitations and potential future directions.

Chapter 2. PROFILING THE MTB-INDUCED CYTOKINE RESPONSE

2.1 CHAPTER SUMMARY

The mechanisms behind heterogeneity in human immune responses to *Mycobacterium tuberculosis* (Mtb) are poorly defined necessitating further research. In particular, the Mtb-induced cytokine response in human monocytes shows promise due to its central roles throughout all stages of Mtb infection. We characterized and identified Mtb-induced cytokine expression in four important myeloid cytokines, IL1B, IL6, TNF, and IFNB1 using a Ugandan cohort RNAseq dataset. We subsequently performed WGCNA to identify central pathways and genes related to Mtb-induced cytokine expression. Lastly, we performed SNP based heritability assessments of Mtb-induced cytokine expression. As a result, we identified 35 hub genes related to the Mtb-induced cytokine response which were centered around STAT3 and genes of the NFkB pathway and found that Mtb-induced IL1B and IL6 expression had some indication of genetic regulation. Overall, these results identify central elements of the Mtb-induced cytokine response and indicate that genetic study of their regulation in future studies is warranted.

2.2 INTRODUCTION

Despite, having access to effective antibiotics and a vaccine for over 100 years, Tuberculosis (TB) remains the leading cause of infectious death and was responsible for 1.3 million deaths and 10.6 million infection related illnesses in 2022 alone [2]. Efforts to decrease the burden of TB death and illness have fallen far short of goals outlined in the WHO end TB strategy. As such, there is a substantial need for new insights and novel methods to respond to

the global toll of TB. One area of focus is on obtaining greater understanding of the host responses necessary to successfully contain a *Mycobacterium tuberculosis* (Mtb) infection [8]. Although the causal mechanisms have not been fully defined, factors predisposing individuals to certain infection outcomes have been identified, including environmental, comorbidities, and genetic makeup [9,76,77,86]. In particular, genetics has had a long established role in regulating infection outcomes with recent estimates indicating a 50.6 and 55% heritable component of TST/IGRA conversion resistance and TB disease susceptibility, respectively [29]. However, despite significant effort using candidate gene, linkage analyses, and genome-wide association studies (GWAS), the major causal mechanisms behind this heritability have not been identified.

In an effort to define the underlying biology responsible for the heterogeneity of infection outcomes, a few studies have begun to evaluate inter-individual variation using *in vitro* assays [96]. By assessing variation of intermediate traits in controlled *in vitro* experiments, researchers can eliminate confounding variables, and uncover biological differences in measurable, infection relevant traits. In particular, understanding the underlying reasons for resistance to TST/IGRA conversion has garnered great interest [29–31]. Cohorts for this clinical phenotype involve individuals who are heavily exposed to individuals with active TB, and yet do not show signs of infection based upon years of TST/IGRA tests (RSTRs). Previous efforts by Simmons et. al, have attempted to define differences between RSTRs and those who develop latent TB infection (LTBI) at the Mtb-induced expression response level [105,106]. Specifically, monocytes isolated from donor samples of two cohorts of Ugandan household contacts and South African coal miners, were characterized by RNAseq after 6-hour *in vitro* Mtb infection. Although, these studies defined general expression related differences between RSTR and LTBI individuals, they

did not focus on specific host response elements, and performed limited assessment of genetic contribution underlying expression differences.

In order to further characterize the Mtb-induced response in human monocytes, we sought to define the major factors related to Mtb-induced cytokine expression and define the genetic contribution to inter-individual variation in cytokine induction. Effective response to Mtb infection requires a macrophage capable of recognition, phagocytosis, and pathogen destruction [107]. Responses that are insufficiently inflammatory can instead result in intracellular persistence, non-apoptotic cell death, and improper recruitment of downstream responses [10]. In particular, the macrophage secreted cytokines, TNF, IL1B, IL6, and IFNB1, have previously been found to play crucial roles in regulating susceptibility, Mtb clearance, cell death, and recruitment of the adaptive response [35,58,63,67,68,80,108–111]. As such, understanding the major elements underlying differences in these cytokine responses is crucial.

One way to define the underlying Mtb response is using weighted gene correlation network analysis (WGCNA) [112]. By evaluating the overall expression response, and clustering genes with correlated expression, different modules of the overall response can be identified. Highly correlated genes within a module are often influenced by the same factors and are enriched in similar response pathways. Although WGCNA has been used previously to profile baseline monocyte and whole blood expression differences between RSTR and LTBI individuals within a Uganda and South Africa cohorts, it did not evaluate Mtb-induced expression [106]. By using WGCNA to identify correlated gene networks of the monocyte expression response to Mtb, we can then identify which modules, pathways, and genes are central to the cytokine response. In cases of complex disease etiologies which are often not easy to understand, as in the

case of bipolar disorder, this approach has shown promise in defining relevant underlying biology [113].

Furthermore, since genetic variation contributes so heavily to inter-individual variation in clinical phenotypes, it would be interesting to assess the heritability of the Mtb-induced cytokine response. Previous estimates of the heritability of the *in vitro* cytokine response have been performed in response to numerous microbial stimuli such as LPS, TB whole cell lysate, TB culture media antigens, and *Salmonella*, and *Chlamydia* infections in various cell types [98,114,115]. The genetic contribution to inter-individual variation in cytokine production within these studies has often been found to be substantial. The heritability of cytokine expression in response to live Mtb has not been performed.

In the current study, we characterized the human monocyte cytokine expression response to live Mtb infection and determined its genetic regulation. We identified four myeloid cytokines, IL1B, IL6, TNF, and IFNB1, which were strongly induced upon Mtb infection. We then identified the major correlated gene networks of the Mtb-induced expression response and how they related to these Mtb-induced cytokines. This resulted in identification of 35 hub genes and multiple signaling pathways which were central to the cytokine related Mtb-induced response. Lastly, we identified that a proportion of inter-individual variation in Mtb-induced IL1B and IL6 expression may be due to genetic contribution. Proper regulation of the Mtb-induced cytokine response has consistently been shown to be a central element of Mtb infection progression. As such, further elucidation of Mtb-induced cytokine response regulation is vital, and may lead to important insights which can support future efforts to combat TB globally.

2.3 METHODS

2.3.1 *Human donor and clinical cohorts*

Preliminary profiling of the host cytokine response was performed using previously generated datasets which performed ex vivo characterization of monocytes from two cohorts of human donors from Kampala, Uganda and North West Providence, South Africa [105,106]. Individuals from Uganda were part of a highly Mtb-exposed Ugandan household contact cohort. Subjects were recruited between 2002 and 2012 and followed for serial TST and IGRA testing over an 8-to-10-year period after which PBMCs were collected. Individuals from South Africa who were collected as part of a longitudinal study of gold miners in South Africa who had worked for at least 15 years in close proximity work conditions. Individuals were admitted for study from August 2015 to December 2016, if HIV-negative, age 33-60, and no history of symptomatic or prior treatment. Upon enrollment, individuals were screened for TB positivity using Quantiferon-TB (QFT) and TST evaluations and PBMCs were collected.

Cohort characteristics are included (Table S2.3). For specific cohort information, including clinical definitions, please refer to original publication [106].

2.3.2 *Cell culture, reagents and CD14+ isolation*

Monocyte preparation from the Uganda and South Africa cohorts were previously described [105,106]. Briefly, PBMCs were thawed and rested for 1 day in culture media composed of RPMI-1640 (Gibco) supplemented with FBS (Atlas Biologicals) at a final concentration of 10% and Macrophage colony-stimulating factor (M-CSF, Peprotech) at a final concentration of 50ng/mL. Subsequently, PBMCs were processed by negative CD14 isolation

(Miltenyi, Monocyte Isolation kit II) to enrich for monocytes which were plated and rested in previously indicated culture media for an additional day before infection.

2.3.3 *RNA extraction for assessment of Mtb-induced cytokine expression*

After 24 hour rest, plated monocytes were infected with Mtb H37Rv Mtb thawed from cultures previously grown to mid log phase and 2 times Sauton's washed, at an MOI of 1 or a mock infection media only condition. Cytokine expression timepoints were assessed after 6 hours of infection after which cells were lysed in trizol and RNA was isolated using a modified miRNeasy protocol (Qiagen).

2.3.4 *RNAseq Processing and Determination of Mtb-induced cytokine expression*

Mtb-induced cytokine expression was generated from previously generated and processed RNAseq data [105,106]. Briefly, RNAseq was performed using Illumin HiSeq 2500 at a read depth of 30 million paired-end 50 base pair reads. For the Uganda dataset, RNAseq was performed in two separate batches and combined into a single dataset using combat normalization of batch effect [116]. Sequences for both Uganda and South Africa were then aligned to the GRCh38 reference genome and gene counts were normalized according to library size using voom [117]. Mtb-induced cytokine expression was obtained by subtracting mock-infected media only RNAseq log2 voom normalized cytokine counts from their corresponding Mtb-infected counts per individual. Individuals with Mtb and media RNAseq samples that were split between batch were not included in further analyses.

2.3.5 *WGCNA*

WGCNA was performed using the WGCNA package in R [112]. All analysis was performed on total RNAseq expression values of more than 14 thousand genes which surpassed

rare gene filtering. IL1B was subtracted from analysis prior to module determination to avoid confounding effects of perfect gene to trait significance within a module. Module determination was performed using signed analysis with a power of 12 and a deepsplit of 3 to minimize number of genes per module. Initial module enrichment analyses were performed using WGCNA in package and the top 3 significant results per module were reported. Hub genes were selected from modules with a moderate module to trait and module membership to gene significance correlation ($R > 0.4$). Hub genes from these modules were selected based on top 10% connectivity within each module established in STRING using a default, 0.4 medium confidence setting, as well as demonstrated a high module membership and at least a low gene significance score (0.8 and |0.2| respectively) [118]. Consensus hub genes from both Uganda and South Africa were combined across modules and enrichment analysis was performed within STRING. Selected results with the highest significance were selected from the KEGG (Kyoto Encyclopedia of Genes and Genomes) and Reactome pathway databases for discussion [119,120]. Finally, results from STRING network analysis were visualized via Cytoscape and individual genes were colored and organized according to number of network interactions within the final network of hub genes [121].

2.3.6 *Genotyping*

Genotyping for the Uganda cohort was performed using the Illumina MEGA^{EX} and Omni5 genotyping chips as previously indicated [29,122]. Briefly, two subsets of the larger Ugandan household contact cohort were genotyped. Within the MEGA^{EX} subset, 82 RSTR and 195 LTBI individuals were profiled. Within the Omni5 subset, 15 RSTR and 33 LTBI were profiled. In total, 121 individuals HIV- individuals with either MEGA^{EX} or Omni5 genotyping were found and 91 of these had overlapping RNAseq information. SNPs that were assessed in both chip

panels were extracted and all individuals were combined into a common dataset. SNP quality control was then performed filtering for < 1 x 10^{-6} Hardy-Weinberg Equilibrium (HWE), > 5% minor allele frequency (MAF), > 95% call rate.

2.3.7 *Heritability analysis*

Heritability analysis was performed using the genome-wide complex trait analysis (GCTA) software [123]. Briefly, common SNPs (>5% MAF, < 1x10^{-6} HWE, > 95% call rate) were extracted for 91 individuals which had overlapping Mtb-induced monocyte RNAseq expression values. A genetic relatedness matrix (GRM) for the 91 individual dataset was created within GCTA using the above indicated autosomal SNPs and REML analysis was performed for each cytokine while adjusting for genetic relatedness and the covariates, age, sex, and RNAseq batch.

2.3.8 *Statistics*

Significant induction of Mtb-induced cytokine expression and correlation of Mtb-induced cytokine expression was assessed using a simple unadjusted linear model. Thresholds of correlation were determined as 0.3-0.5, 0.5-0.8, and > 0.8 as weakly, moderately, and highly correlated respectively. WGCNA and GCTA analysis were performed using baseline setting unless otherwise specified. Gene ontology (GO) term enrichment analysis for modules was performed using in package WGCNA hypergeometric enrichment. Briefly, GO terms for cellular compartment (CC), biological process (BP), and molecular function (MF) were all tested and FDR correction was performed across all terms per module tested. Hub gene, gene set analysis was performed using STRING database hyper geometric enrichment analysis and FDR corrected across results within each pathway.

2.4 RESULTS

2.4.1 *Characterization of the Mtb-induced cytokine response*

In order to characterize the Mtb-induced cytokine response, we examined cytokine expression in 100 individuals of a Ugandan household contact cohort with a previously generated RNASeq dataset of CD14+ monocytes infected for 6 hours with H37Rv Mtb or a media only control (Table S2.3). We initially focused analysis on the myeloid cytokines, IL1B, IL6, TNF, and IFNB1. For all four cytokines, we saw a highly significant increase in expression from the media only to TB infection stimulation condition (Figure 2.1, $P < 0.0001$).

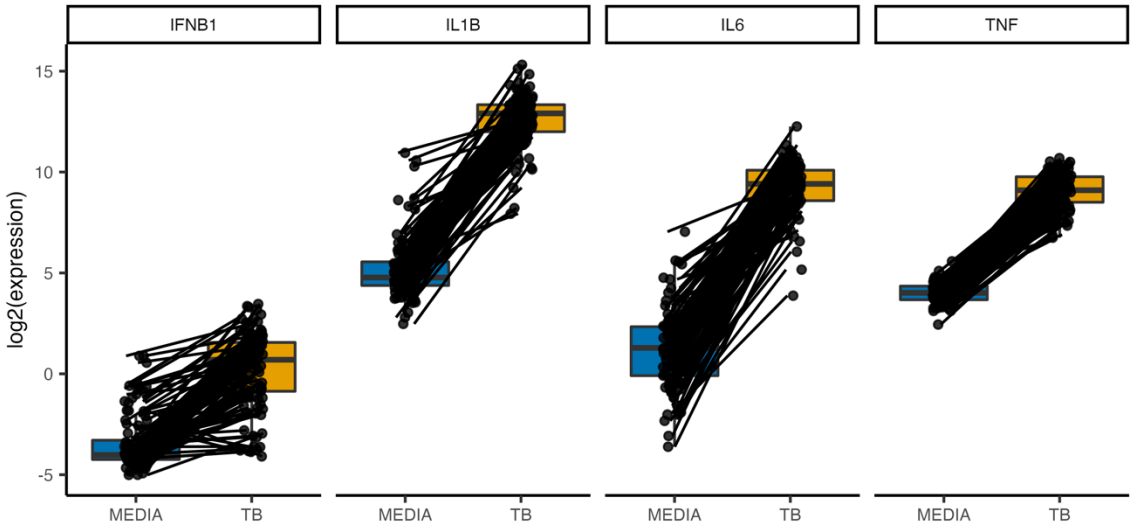

Figure 2.1. Cytokine expression is strongly induced upon Mtb infection.
CD14+ monocyte expression of the cytokines IFNB1, IL1B, IL6, and TNF is shown for the media only, mock infection and Mtb infection conditions. Log2 expression of voom normalized counts are shown for 100 individuals with lines connecting individuals between conditions.

Variability in cytokine expression distributions often spanned between 8- and 256-fold between individuals. In order to characterize inter-individual variation in cytokine expression that is specific to Mtb-induction, we performed further analyses on baseline subtracted Mtb-media

expression values. Since many cytokine induction pathways share common elements and induction of expression may be coregulated, we further determined if cytokine expression values were independent or correlated among individuals. We found that Mtb-induced cytokine expression of the more traditional inflammatory cytokines IL1B, IL6, and TNF, was highly correlated (Figure 2.2, R = 0.51-0.8). Whereas IFNB1 was only moderately correlated with one cytokine, TNF (Figure 2.2, R = 0.35).

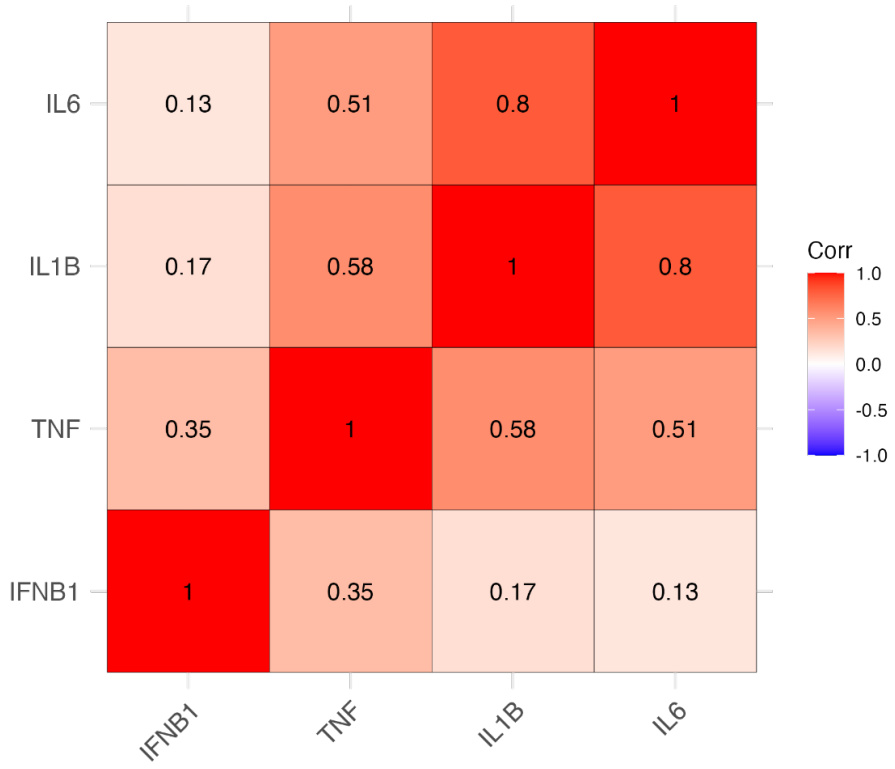

Figure 2.2. Correlation of Mtb-induced cytokine expression
The correlation of Mtb-induced cytokine expression values of IFNB1, TNF, IL1B, and IL6 within individuals of the Uganda cohort

Together, these data show that expression of the myeloid cytokines, IL1B, IL6, TNF, and IFNB1 is highly induced by Mtb-infection and that the level of cytokine induction often

correlates with the induction of other cytokines, especially among the pro-inflammatory cytokines IL1B, IL6, and TNF.

2.4.2 *Characterization of gene correlation networks*

Since substantial correlation of expression was observed among cytokines, we decided to further evaluate the co-expression network using WGCNA [112]. We performed initial clustering of Mtb-induced gene expression values within our RNAseq dataset (13,972 genes) and identified 20 separate modules of correlated gene networks. Module size ranged from 33 to 1727, and a majority of genes were grouped into modules with 4,789 genes which were not clustered into any module. Modules relevant to Mtb-induced cytokine expression were identified by performing module to trait correlation analyses initially using Mtb-induced IL1B expression as a phenotypic trait. Of the 20 identified modules, 2 were found to have a moderate positive correlation with Mtb-induced IL1B (R 0.59-0.66) and 4 were found to have a moderate negative correlation (R -0.51- -0.65) (Figure 2.3A). To further define the contents of each module, we performed go term enrichment of each of the modules that were moderately correlated with Mtb-induced IL1B expression and had a moderate gene significance to module membership score. We found significant go term enrichment for each of our modules, with the blue module capturing genes associated with the mitochondria, brown associated with response to a stimulus, red associated with vesicle transport, turquoise associated with transcription factor activity, and royal blue associated with heme binding (Table S2.4).

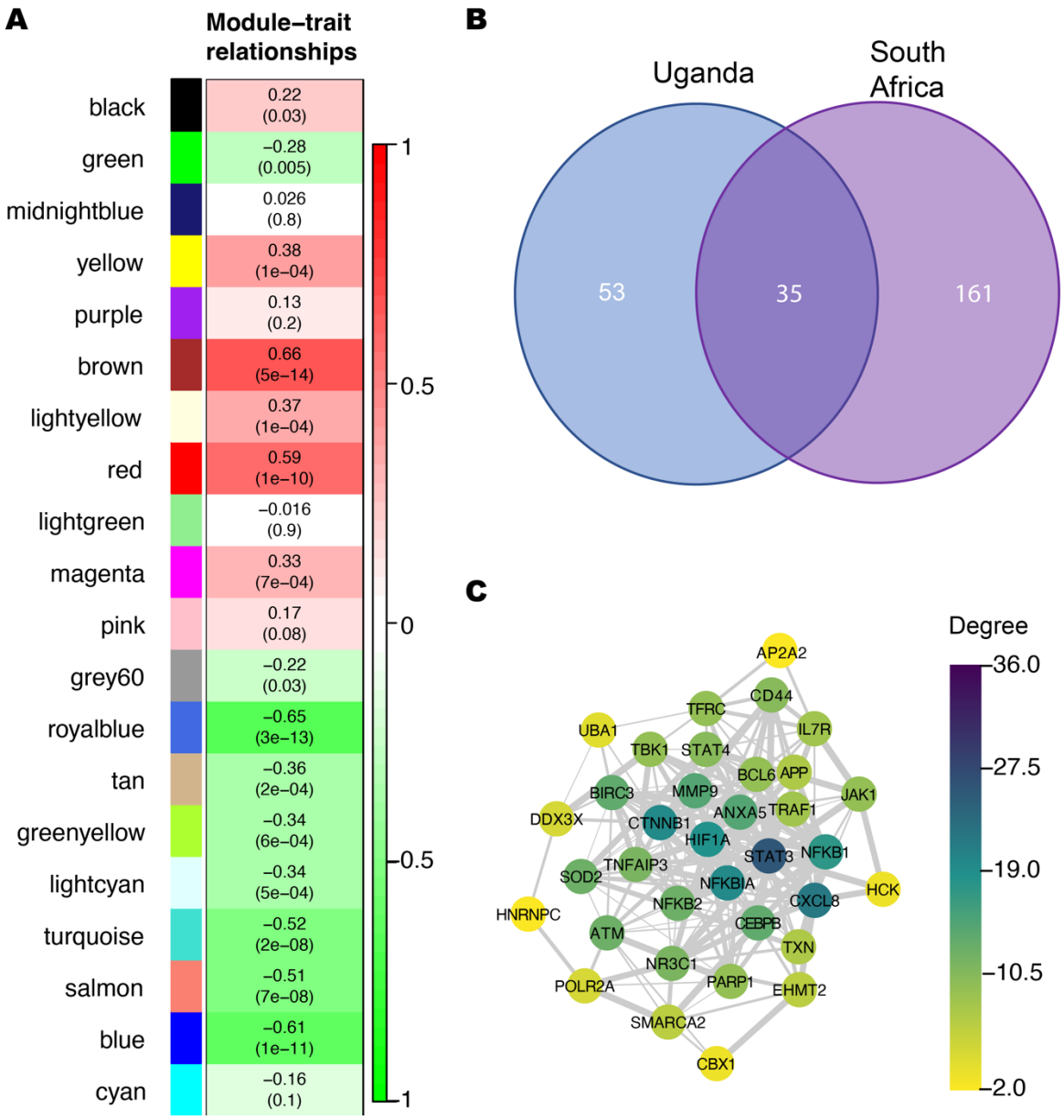

Figure 2.3. WGCNA reveals coexpressed gene modules and central hub genes related to Mtb-induced IL1B expression

WGCNA analysis of Mtb-induced RNAseq expression values within the Uganda and South Africa cohorts. (A) The 20 gene network modules after clustering analysis were correlated with Mtb-induced cytokine expression showing 2 modules with moderate postitive correlation and 4 modules with moderate negative correlation. (B) Hub gene analysis identified 35 conserved hub genes central to cytokine correlated modules within the Uganda and South Africa cohorts. (C) Network visualization of the 35 genes involved identifies genes central to Mtb-induced cytokine response with STAT3 being the most central gene.

2.4.3 *Determining the hub genes of Mtb-induced gene correlation networks*

Due to the substantial number of genes in the majority of modules, term sizes were often large limiting ability to discern meaningful biology. As such, we further performed a hub gene analysis to determine the central genes within each of these modules. All genes within each module were analyzed for network interactions using STRING and highly correlated genes with the top 10% connectivity were extracted as potential hub genes. We found 53 distinct hub genes across all modules that fulfilled this category. In order to further validate these hub genes, we additionally profiled a secondary RNAseq dataset of CD14+ monocytes from 48 individuals of a South Africa coal miner cohort profiled with identical infection conditions. We identified 161 hub genes within this cohort, and 35 consensus hub genes were found to be shared within both populations (Figure 2.3B). Further network analysis revealed the most interconnected of these hub genes was STAT3 and enrichment analysis identified a highly significant signal for the KEGG: NF-kappa B signaling pathway as well as expected pathways within reactome (signaling by interleukin and cytokine signaling in the immune system pathways) (Figure 2.3C, Table 2.1). In summary, WGCNA analysis of Mtb-induced IL1B expression identified 5 correlated coexpression modules and the consensus hub genes of these modules across two populations were found to be highly linked to STAT3 and the NF-kappa B signaling pathway.

Table 2.1. STRING identifies significant enrichment of hub genes in NF-kappa B and inflammatory signaling pathways

Pathway Database	Term description	Genes in Pathway	Pathway term size	Strength	False discovery rate (FDR)
KEGG	NF-kappa B signaling pathway	9	101	1.7	7.82E-11
KEGG	Pathways in cancer	13	515	1.15	4.50E-10
KEGG	Hepatitis B	9	158	1.51	1.17E-09
KEGG	Kaposi sarcoma-associated herpesvirus infection	9	187	1.43	3.71E-09
KEGG	IL-17 signaling pathway	7	91	1.64	2.42E-08
REACTOME	Cytokine Signaling in Immune system	17	706	1.13	1.62E-12
REACTOME	Signaling by Interleukins	15	453	1.27	1.62E-12
REACTOME	Immune System	23	1979	0.82	2.35E-12
REACTOME	Disease	18	1702	0.77	4.73E-08
REACTOME	Infectious disease	14	917	0.93	1.03E-07

- The top 5 most significantly enriched KEGG and Reactome pathways associated with the 35 hub genes using STRING

2.4.4 *Determination of heritability of Mtb-induced cytokine expression*

In order to profile the genetic contribution of Mtb-induced cytokine response regulation, we initially wanted to determine if Mtb-induced cytokine expression was heritable between individuals. We identified 91 individuals in the Uganda cohort who had simultaneous genotyping information and Mtb-media RNAseq cytokine expression data. Since individuals were profiled by either Illumina MEGA^{EX} or OMNI-5 genotyping chips, SNPs shared between platforms were extracted, and quality filtered yielding 430,604 SNPs to assess heritability. For Mtb-induced

IL1B expression among these 91 individuals, we obtained an indicated heritability (variance in cytokine expression explained by SNPs on the genotyping chip) of 32.2% (Table 2.2). Mtb-induced IL6 expression also yielded detectable heritability at 9.2%. Meanwhile, TNF and IFNB1 yielded very little if any detectable heritability. Although, GCTA was able to detect heritability, estimates often included large standard error estimates that limit our ability to interpret results. These results suggest that there may be a heritable component of Mtb-induced cytokine expression for the cytokines IL1B and IL6.

Table 2.2. Heritability estimates of Mtb-induced cytokine expression

Cytokine	Heritability (H2SNP)	Standard Deviation
IL1B	0.322	0.469
IL6	0.092	0.552
TNF	< 0.001	0.671
IFNB1	< 0.001	0.598

2.5 DISCUSSION

In order to evaluate the Mtb-induced cytokine response, we assessed cytokine induction levels, determined correlated gene expression networks, and estimated SNP-based heritability. The four myeloid cytokines, IL1B, IL6, TNF, and IFNB1, were all strongly induced upon Mtb infection and display substantial inter-individual variation in Media, Mtb, and Mtb-media cytokine expression values often spanning 64-fold differences. As such, these four cytokines would likely be viable for further study as an outcome phenotype. These four cytokines have previously been found to be induced upon Mtb-infection and are similar to RNAseq induction values found after 6-hour infection in murine bone-marrow derived macrophages [124].

Although other cytokines may be of value to study, such as other interleukins and interferons, we have focused on these four myeloid cytokines which have substantial literature established importance and limits the need for multiple correction due to a plurality of phenotypes tested.

We additionally found that there was a high degree of correlation of Mtb-induced expression among the cytokines. IL6 and IL1B had the highest degree of correlation followed by TNF with IFNB1 being the most distinct. This is likely expected due to differences in the pathways that lead to expression of these cytokines with IL6 and IL1B sharing activation pathways, whereas TNF requires the interaction of numerous elements, and the interferon response is regulated by distinct pathways [125–127]. Due to the correlation between these cytokine responses, it is likely that changes that cause differences in one cytokine could likely also be affecting other cytokine responses as well.

In order to further characterize the Mtb-induced cytokine response we performed WGCNA to identify clusters of highly correlated genes after Mtb infection. These correlated gene modules could then be analyzed for relation to Mtb-induced cytokine expression allowing for determination of important networks of genes that may be involved in the cytokine response. We identified 20 modules of genes, 5 of which had a moderate correlation with Mtb-induced IL1B expression ($R > |0.4|$). Initial enrichment analyses revealed that modules positively correlated were involved in general stimulus response pathways and vesicle-transport pathways whereas negative correlated pathways were involved with mitochondrial, and transcriptional elements. Although these enrichment analyses reveal that some expected gene expression networks are being identified, a majority of modules and pathways identified were broad, encompassing more than 1000 different genes, which complicated our ability to make more specific claims of important elements of the host response.

In order to focus our analysis, we extracted hub genes which have the highest degree of module membership, degree of connectivity to other genes within the network, and correlation with Mtb-induced IL1B expression. This approach has previously been used to characterize the hub genes of WGCNA from other important outcome phenotypes such as bipolar disorder [113]. We identified 53 hub genes within the Uganda cohort and 35 of these were found to be maintained using identical processing in a similarly profiled South African coal miner dataset. These 35 hub genes should serve as high confidence genes that are heavily connected to networks related to Mtb-induced IL1B expression. The most interconnected gene within these 35 hub genes was STAT3, followed by CTNNB1, NFKBIA, and HIF1A. Overall enrichment analysis of these 35 genes identified significant enrichment of numerous pathways. Expected pathways such as cytokine signaling in the immune system were again found, however, we were also able to find identify smaller pathways such as the NF-kappa B signaling pathway as the most significantly enriched KEGG pathway. These hub genes and pathways may represent some of the more essential elements of the host response related to the Mtb-induced cytokine response. As such, genetic variation leading to modifications of these central genes and pathways may substantially affect numerous other network connected genes which are important in the host response to Mtb.

To characterize the extent to which Mtb-induced cytokine expression was determined by genetic variation, we utilized a SNP-based heritability assessment to determine what proportion of the total variation in cytokine expression was attributable to SNPs for which we had genotyping information for. We were only able to ascertain heritability estimates for IL1B and IL6 which showed 32.3% and 9.2% respectively. However, for each of our heritability estimates, we obtained large standard deviations upwards of 60%, which limits our ability to confidently

say we defined the heritability of any cytokine phenotype. Previous studies attempting to assess heritability of quantitative in vitro phenotypes have reported similar issues, and have compensated by showing a strong correlation with other non-SNP based heritability assessments [98]. We did not employ other assessments of heritability, however, we do find that our assessment of heritability does show similarities with previous estimates of cytokine induced by Mycobacterium tuberculosis whole cell lysate (TBWCL) in human PBMCs [114]. This study reported a high level of IL1B heritability nearing 100% with IL6 near 50% while TNF was undetectable. IFNB1 was not assessed in this previous study, however, even with only three shared values, heritability estimates between our studies are highly correlated ($R2 > 0.8$). Interestingly, our heritability estimate for TNF was found to be highly different than a previous estimate within the Uganda population which found nearly 70% of TNF induced by Mtb-culture media stim of whole blood was genetically regulated [115]. TNF was the only shared analyzed between studies limiting comparison, however, this may suggest differences in methodology or potentially that the particular cell types which lead to heritability of TNF may require more than monocytes alone. Potential reasons for our inability to determine heritability with confidence could be due to limited sample size and assessment of a quantitative phenotype with an imperfect distribution. Within this analysis, we adjusted for sex, age, RNAseq batch, and genetic relatedness using a GCTA generated genetic relatedness matrix. Potential inclusion of principal components (PCs) of population structure could increase ability to determine heritability with higher accuracy, however, previous clinical phenotype heritability estimates within this population have indicated no effect of PC inclusion [29]. Regardless, we believe that these heritability estimates suggest that the extent of an individual's Mtb-induced cytokine expression may be somewhat determined by heritable genetic factors.

In summary, the above indicated data have demonstrated that IL1B, IL6, TNF, and IFNB1 cytokine expression is strongly induced in human CD14+ monocytes upon *in vitro* Mtb infection. This cytokine response is part of an overall host response composed of numerous related coexpressed gene networks which may center around a few essential genes and pathways. Lastly, there may be some heritable component of Mtb-induced cytokine expression. Further understanding the genetic variants leading to inter-individual variation in the Mtb-induced cytokine response may reveal important insights into the mechanisms of individual predisposition to Mtb infection outcomes and should be a focus of subsequent research.

2.6 SUPPLEMENTAL TABLES

Table S2.3. Demographic data for the Uganda and South Africa RSTR monocyte RNAseq cohorts

	Uganda HHC (n=100)	South Africa CM (n=55)
Gender		
F (%)	38 (51.4)	0 (0)
M (%)	36 (48.6)	55 (100)
Age		
Mean (SD)	22.5 (8.8)	48.3 (5.3)
Median	Not reported	49
[Min, Max]	[14, 66]	[36, 60]
Race		
Black (%)	100 (100)	48 (87.3)
White (%)	0 (0)	6 (10.9)
Other	0 (0)	1 (1.8)
TB status		
LTBI (TST+)	51 (51)	29 (52.7)
RSTR (TST-)	49 (49)	26 (47.3)

Table S2.4. WGCNA module go term enrichment analysis

Module	Module Size	Padjust	Module Genes in Term	Term Size	Term Ontology	Term Name
blue	1411	4.77E-04	201	1318	CC	mitochondrion
brown	1413	2.07E-27	872	6241	BP	response to stimulus
brown	1413	3.05E-23	423	2479	BP	response to organic substance
brown	1413	1.34E-22	367	2063	BP	cellular response to organic substance
brown	1413	3.80E-21	213	989	BP	response to cytokine
brown	1413	6.64E-21	655	4480	BP	cell communication
red	618	5.09E-14	172	1921	CC	vesicle
red	618	6.38E-14	156	1669	BP	vesicle-mediated transport
red	618	8.31E-13	165	1860	CC	cytoplasmic vesicle
red	618	9.72E-13	165	1863	CC	intracellular vesicle
red	618	1.35E-11	274	3863	BP	transport
turquoise	1667	2.85E-28	392	1814	MF	DNA binding
turquoise	1667	5.41E-25	277	1165	MF	DNA-binding transcription factor activity, RNA polymerase II-specific
turquoise	1667	1.70E-24	290	1250	MF	DNA-binding transcription factor activity
turquoise	1667	4.00E-22	497	2638	MF	nucleic acid binding
turquoise	1667	1.51E-18	750	4575	BP	heterocycle metabolic process

Chapter 3. MTB-INDUCED CYTOKINE GWAS REVEALS NUMEROUS GENOMIC LOCI WITH SUGGESTIVE ASSOCIATIONS AND SIGNIFICANT ENRICHMENT OF PATHWAYS ASSOCIATED WITH MTB-INDUCED TNF

This chapter has been adapted from a previously generated manuscript currently under review at the Journal of Clinical Investigation and is included here with permission.

3.1 CHAPTER SUMMARY

Although immune *Mycobacterium tuberculosis* (Mtb) response phenotypes are indicated to be genetically regulated, the causal mechanisms responsible have not been identified. Using a cellular genome-wide association study (GWAS) approach, we identified genetic variants associated with Mtb-induced monocyte cytokine expression in a cohort of Ugandan individuals. Genetic results were subsequently functionally annotated and assessed for enrichment in biologically relevant pathways. We identified 77 loci suggestively associated with Mtb-induced IL1B, IL6, TNF, and IFNB1 expression, which showed plausible mechanisms of effect after functional annotation. SNPs with Mtb-induced TNF signal were enriched within genes of the alpha-linolenic acid metabolism pathway which was validated *in vitro* using macrophage PLA2 inhibitor assays. These data suggest the cellular GWAS approach may identify genetic variants important in the Mtb-induced cytokine response and require further investigation.

3.2 INTRODUCTION

Mycobacterium tuberculosis (Mtb), the causative agent of Tuberculosis (TB), has caused more deaths than any other pathogen over the course of human history [1]. Over 70,000-years of infections, humans and Mtb have had a long coevolutionary history allowing for the accumulation of numerous genetic adaptations in both pathogen and host at the global and population specific level [5,7]. Currently, TB remains the leading infectious killer worldwide and efforts to reduce global burden have fallen far short of WHO goals [2]. This is likely due to a variety of reasons including antibiotic resistance, lack of a consistently efficacious vaccine, and an incomplete knowledge of the host immune response required to resist infection and control disease progression [36,37,128]. After an individual is exposed to Mtb, a spectrum of outcomes is observed which are classically divided into three major categories: resistance to infection, establishment of an asymptomatic latent infection, and development of active TB disease [9]. Although parts of the heterogeneity of clinical outcomes are attributable to known clinical risk factors, the majority of mechanisms are poorly understood [129]. One hypothesis is that genetic variation in the host immune response regulates the pleiotropic outcomes after Mtb exposure. Defining the genetic variants responsible for controlling the Mtb-induced host response may yield important insights into differences in host progression and identify novel areas of Mtb-response biology for further research.

Several lines of evidence suggest that genetic factors regulate susceptibility to Mtb infection and TB disease [29,85,86,88–95,115,130,131]. Mendelian studies in pediatric populations support an important role of IL-12-IFNγ mediated pathways for controlling mycobacterial infection [87]. Several variants have been associated with pulmonary TB across a number of genome-wide association studies [89–95,130,131]. However, these findings are often

inconsistent in follow up studies and the mechanistic reasons responsible for their association is seldom investigated. Furthermore, the variants previously identified only explain a fraction of the genetic effect indicated by heritability estimates [29,88,130]. Potential reasons for this missing heritability include cohort sample size, heterogeneity of clinical phenotypes, lack of adjustment for non-genetic risk factors, and Mtb strain diversity. A cellular GWAS approach addresses these limitations by examining genetic variants associated with inter-individual intermediate traits measured by *in vitro* assays [96]. Unencumbered by many of the same limitations of clinical studies, variants regulating essential pathogen-induced cellular traits can be identified. This technique has been used to characterize the genetic regulation of multiple cellular phenotypes including cytokine production in response to a variety of microbial stimuli, cell death, and intracellular replication of infections such as *Salmonella* and *Chlamydia,* however, the effect of genetic variation on live Mtb infection remains unexplored [96,98–104].

Macrophages are pivotal innate immune cells in TB pathogenesis due to their early roles in initial detection of Mtb, serving as a cellular home for persistent infection, and involvement in clearance of infection [8,107]. Macrophage signaling pathways are important at each of these steps to modulate pathogenesis. In particular, the host cytokine response plays a large role in determining infection outcomes, requiring a sufficient response to control infection without over responding and causing tissue pathology [47]. Interleukin-1 beta (IL1B), Interleukin-6 (IL6), Tumor Necrosis Factor (TNF), and Interferon beta (IFNB1), as key components of this response, have important functions in initial detection of Mtb, controlling infection, initiating effective T cell responses, and modulating immunopathology [35,58,63,67,68,80,108–111,132]. Understanding the genetic regulation of the Mtb-induced cytokine response in the human

macrophage could fill important gaps in our understanding of the heterogenous host response to Mtb.

In the current study, we characterized the *in vitro* Mtb-induced cytokine expression profile of monocytes isolated from individuals of a Ugandan cohort and performed genome-wide assessment of the major genetic variants and genetically regulated pathways associated with differential Mtb-induced cytokine response. We identified 77 loci and two pathways which were associated with Mtb-induced cytokine expression. Subsequent functional annotation of these loci and *in vitro* validation of the alpha-linolenic acid metabolism pathway supports the use of the cellular GWAS approach to identify genetic regulators of the Mtb-induced cytokine response with promise for further investigation.

3.3 METHODS

3.3.1 *Human donor and clinical cohorts*

Individuals from the Uganda cohort were part of a highly Mtb-exposed Ugandan household contact cohort described previously [29]. Briefly, index cases with culture confirmed TB and their highly exposed household contacts were recruited between 2002 and 2012. High exposure was defined as having lived with the index TB case for at least 7 consecutive days within the last 3 months. If household contacts met this criteria, they were followed for serial TST and IGRA testing over an 8-to-10-year period after which PBMCs were collected. Many of these individuals were further assessed during a follow up study from 2014-2017. If individuals remained TST/IGRA negative throughout the entire follow up period, they were termed (RSTR). Latent TB infection (LTBI) was determined as persistent TST/IGRA positive tests from initial enrollment throughout follow up. Lastly, some individuals were not available during follow up study and were termed no active TB. High-quality genotyping passing QC was obtained for 917

individuals within this cohort and used for subsequent genetic studies. Our GWAS involved ex vivo characterization of monocytes via RNAseq for 100 individuals within this cohort who were either RSTR or LTBI and had simultaneously available genotyping data. Details, including clinical definitions, are previously published and clinical cohort tables are included [29,122] (Table S3.2).

3.3.2 *Cell culture, reagents and CD14+ isolation.*

Monocyte preparation from the Uganda cohorts was previously described [105,106]. Briefly, PBMCs were thawed and rested for 1 day in culture media composed of RPMI-1640 (Gibco) supplemented with FBS (Atlas Biologicals) at a final concentration of 10% and Macrophage colony-stimulating factor (M-CSF, Peprotech) at a final concentration of 50ng/mL. Subsequently, PBMCs were processed by negative CD14 isolation (Miltenyi, Monocyte Isolation kit II) to enrich for monocytes which were plated and rested in previously indicated culture media for an additional day before infection. For *in vitro* validation of PLA2 inhibitors, monocyte derived macrophages were similarly obtained from cryopreserved PBMCs which were thawed, resuspended at 2 million cells/mL, and differentiated for 5 days in above indicated culture media. Monocyte derived macrophages were then isolated using magnetic bead column purification via negative selection (Miltenyi, pan monocyte isolation kit) and rested in culture media for 24 hours before infection or stimulation.

3.3.3 *RNA extraction and analysis for assessment of Mtb-induced cytokine expression*

Mtb-induced cytokine expression was generated from previously generated and processed RNAseq data [105,106]. After 24 hour rest, plated monocytes were infected with Mtb H37Rv Mtb thawed from cultures previously grown to mid log phase and 2 times Sauton's washed, at an

MOI of 1 or a mock infection media only condition. Cytokine expression timepoints were assessed after 6 hours of infection after which cells were lysed in trizol and RNA was isolated using a modified miRNeasy protocol (Qiagen). RNAseq was performed using Illumin HiSeq 2500 at a read depth of 30 million paired-end 50 base pair reads. RNAseq was performed in two separate batches and combined into a single dataset using combat normalization of batch effect [116]. Sequences were then aligned to the GRCh38 reference genome and gene counts were normalized according to library size using voom [117]. Mtb-induced cytokine expression was obtained by subtracting mock-infected media only RNAseq log2 voom normalized cytokine counts from their corresponding Mtb-infected counts per individual.

3.3.4 *Genotyping and Imputation*

For the Uganda cohort, genotyping was performed using Illumina MEGA^{EX} and Omni5 genotyping chips as previously indicated [29,122]. Briefly, from the larger Ugandan household contact cohort, 149 Active TB, 82 RSTR and 195 LTBI individuals were genotyped with the MEGA^{EX} panel and 201 Active TB, 15 RSTR, 33 LTBI, and 233 No Active TB individuals were genotyped with the Omni5 panel. To maximize overlap of individuals who had previously generated Mtb-induced monocyte RNAseq data, an additional 9 individuals who had previously failed genotyping QC were genotyped again using the MEGA^{EX} genotyping platform and combined in with the larger MEGA^{EX} dataset. The MEGA^{EX} and Omni5 datasets were independently processed for imputation. SNPs with > 99% call rate, < $1x10^{-6}$ HWE, and > 1% MAF, were aligned to a reference genome according to TOPMed Freeze 8 variants and imputed using the TOPMed server [133]. Overlapping SNPs within both imputations were combined and filtered for imputation quality score > 0.5, > 5% MAF, and < $1x10^{-6}$ HWE prior to testing.

3.3.5 *GWAS analysis*

The cellular GWAS in the Uganda cohort was performed using the GENESIS package in R [134]. We used a linear model adjusted for age, sex, experiment (RNAseq batch), pc1, pc2, and kinship to examine whether SNPs were associated with Mtb-induced cytokine expression (Mtb-media value). Covariates were included in final model analysis if found to significantly effect the distribution of any of the four Mtb-induced cytokines assessed using a linear model (Figure 3.1A). The number of PCs included in the analysis was determined using an elbow plot to select number of PCs where variation with additional PCs added was minimal (Figure 3.1B). Genotyped SNPs were used to generate PCs and kinship matrix using KING kinship estimation. Genomic inflation factor was assessed for each cytokine to detect significant result inflation and reported (Figure 3.1A).

3.3.6 *Functional annotation of genomic loci and eQTL analysis*

Individual SNP results were clustered into genomic loci using a PLINK clustering protocol in which SNP summary p-values were used to group each loci with a SNP surpassing $P < 1x10^{-5}$ into a lead SNP and group all less significant linked SNPs within PLINK default 250kb, at an LD threshold of 0.1 [135]. All SNPs surpassing $P < 1x10^{-3}$ within each locus were then annotated using Ensembl VEP for, proximal gene mapping, CADD score, and global MAFs [136]. Assessment of eQTL effect was similarly performed using all SNPs surpassing $P < 1x10^{-3}$ tested for associated differences in cis gene expression (+/- 250kb) using a linear model. All SNPs within each locus $P < 0.01$ were additionally annotated for previously reported associations in GWAS catalog to account for potential population differences in LD patterns [137].

3.3.7 *MAGMA gene set analysis*

MAGMA-v1.07 was used to map SNP locations to strict gene coordinates with 0 kb flanks [138]. Gene level *P*-values were calculated using previously calculated GWAS level *P*-values for each cytokine with a multi=all model. C2-canonical pathways (subset to KEGG, REACTOME, PID, and BIOCARTA) and C5-GO Gene sets were obtained from MSigDB [139]. Gene sets with greater than 10 genes with gene level *P*-values were evaluated for significantly enriched signal after adjusting for multiple-correction using default MAGMA settings. Gene sets were further analyzed for single gene biasing of significance via early QQ plot deviation.

3.3.8 *PLA2 inhibitor assay*

AACOCF3 (Tocris biosciences) was purchased and applied extracellularly to human MDMs as previously indicated for a 3-hour pre-treatment in comparison to a EtOH vehicle control. TB whole cell lysate (25ug/mL, BEI resources), or live Mtb infection as described above, was used for 24 hours, after which, supernatants were extracted and assessed for TNF, IL1B, and IL6 secretion using ELISA (R&D, Duoset)

3.3.9 *Statistics*

Genetic tests were performed as previously indicated using GENESIS R package, assocTestSingle, accounting for necessary covariates, and genetic relatedness as a polygenic random effect [134]. GENESIS uses a penalized quasi-likelihood approximation of the generalized linear mixed model as previously developed in GMMAT. A commonly used threshold *P*-value of 1×10^{-5} was used for determining variants with suggestive association. eQTL analysis was performed using a linear model of each loci SNP association with differences in mock infected RNAseq gene expression values located within +/- 250 kilobases (kb) of the

SNP. Final eQTL results were FDR adjusted across all results. AACOCF3 inhibitor treatment significance was assessed using a linear mixed model incorporating donor as a random effect. Treatment was then assessed for significant group differences via ANOVA, and pairwise comparisons were made and adjusted for multiple comparisons using Tukey method.

3.4 RESULTS

3.4.1 *Ugandan cohort yields 100 individuals and 8.3 million SNPs for cellular GWAS analysis*

To discover genetic variants associated with inter individual variation in Mtb-induced cytokine expression, we extracted genotyping information from an imputed dataset of the Ugandan household contact cohort. All 100 individuals with previously extracted Mtb-media cytokine expression values were found to be present in the imputed dataset. In order to extract high quality, common SNPs, we filtered for Imputation quality score ($R2 > 0.5$), Hardy Weinberg equilibrium ($HWE < 1e\text{-}6$), minor allele frequency ($MAF > 0.05$), and less than 1% individual or SNP missingness. Overall, this yielded 8.3 million high quality SNPs within our 100 individual cohort that were suitable for genome wide analysis.

3.4.2 *Cohort analysis identifies necessary covariates for genome wide analysis*

In order to identify necessary covariates to control for type I error in our genome wide analysis, we assessed if any Mtb-induced cytokine expression distribution was significantly different according to covariate condition. We assessed for sex, age, RNAseq batch (experiment), and TST/IGRA positivity (sample group) using a linear model (Figure 3.1A). Mtb-induced IL6 and TNF were found to be significantly different according to donor age and Mtb-

induced IFNB1 was highly significant according to RNAseq batch. No significant difference was found between individuals who were TST/IGRA+ or TST/IGRA- for any Mtb-induced cytokine. In order to standardize analyses between cytokines, age, sex, and experimental batch were included for subsequent analyses. In addition to controlling for genetic relatedness using a genetic relatedness matrix (GRM), we further assessed population structure using a principal component analysis (PCA) of SNP based relatedness within our cohort. An elbow plot of the output PCs was utilized to identify a break point where additional PCs yielded little additional added variance explained and justified inclusion of the first 2 PCs for subsequent analyses (Figure 3.1B). In sum, these analyses justify sex, age, experiment, pc1, pc2, and GRM inclusion in subsequent genome wide analysis.

A

Covariate	IL1B	IL6	TNF	IFNB1
AGE	0.1927	0.0460*	0.00748**	0.7672
SEX	0.7049	0.6940	0.1263	0.7757
Experiment	0.9407	0.8971	0.1831	0.0001***
Sample Group	0.2160	0.3167	0.4144	0.2140
Final Inflation factor (λ)	**1.0371**	**1.0163**	**0.9866**	**0.9319**

B

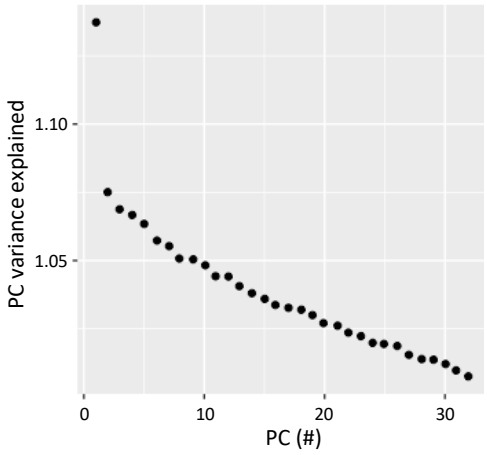

Figure 3.1. Results of covariate inclusion analyses and final effect on lambda inflation factor.
(A) Significance of differences in TB-Media cytokine expression distribution according to each of the covariates, age, sex, RNAseq batch (experiment), and LTBI or RSTR clinical status (sample group) using a linear model. Significance determined as (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Final lambda scores indicating inflation of GWAS results shown for each cytokine showing negligible inflation of results. (B) Elbow plot of variance explained for each pc showing little additional variability added with each pc past pc2.

3.4.3 *Mtb-induced cytokine GWAS yields 77 genomic loci with suggestive associations*

Using an additive, linear mixed model, score-based assessment within GENESIS, we did not identify any signal at a genome-wide level of significance ($P < 5 \times 10^{-8}$) (Figure 3.2). However, a total 77 genomic loci reached suggestive significance level ($P < 1 \times 10^{-5}$), including 51 for Mtb-induced IL1B, 24 for IL6, 6 for TNF, and 6 for IFNB1 (Table S3.3). Overall result distributions for each cytokine showed no strong inflation, with lambda values ranging from 1.04 to 0.93 suggesting there were no unaccounted for factors strongly influencing result significance (Figure 3.1A). As a whole, these 77 suggestive loci were identified as initial candidates for genetic regulation of the Mtb-induced cytokine response within the Ugandan population.

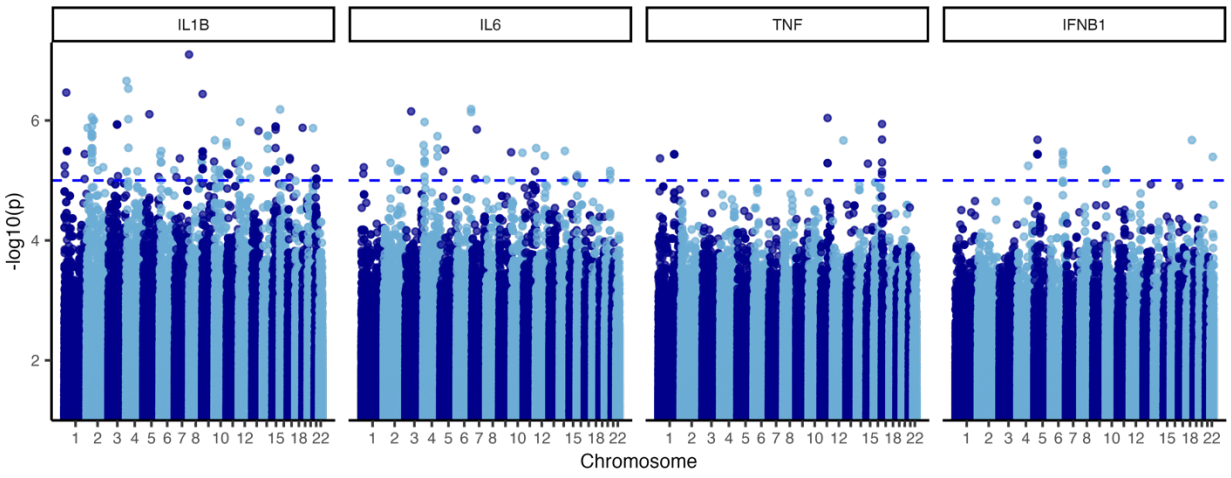

Figure 3.2. GWAS revealed 77 distinct genomic loci suggestively associated with Mtb-induced cytokine induction.
Manhattan plot shows GWAS results of all four cytokines plotted for -log10(*P*-values). Results were calculated in GENESIS to account for population structure, genetic relatedness, sex, age, and RNAseq batch. Horizontal line indicates suggestive threshold, $P < 1 \times 10^{-5}$.

3.4.4 *SNP associations with multiple cytokines*

Since we previously identified that Mtb-induced cytokine levels were correlated, we wanted to identify if SNP associations were cytokine specific or had significant effects on multiple cytokines. Of the 77 total loci, 10 were found to have a suggestive association on both Mtb-induced IL1B and IL6 simultaneously. To more comprehensively assess pleiotropic effects of the identified loci on multiple cytokines, we tested the lead SNP of each locus for any significant effect on the four cytokines with a nominal threshold ($P < 0.05$, Figure 3.3A), and at a more stringent Bonferroni corrected threshold (0.05/77 loci, $P < 6.49 \times 10^{-4}$, Figure 3.3B). Only 4 loci showed an association across all four cytokines, $P < 0.05$. However, at this threshold, there was significant overlap among the inflammatory cytokines, IL1B, IL6, and TNF, with 53 loci showing a shared association. Using the more stringent, Bonferroni corrected threshold, the shared association of TNF with IL1B and IL6 dropped off, but a substantial link between IL1B and IL6 was still observed with 41 loci maintaining shared significance. At both thresholds, IFNB1 was observed to have the largest proportion of its total suggestive loci that were specific to IFNB1 alone. Together, these data show that many of the loci associated with one Mtb-induced cytokine, often have some level of associated effect on other cytokines, especially among the pro-inflammatory cytokines IL1B, IL6, and TNF.

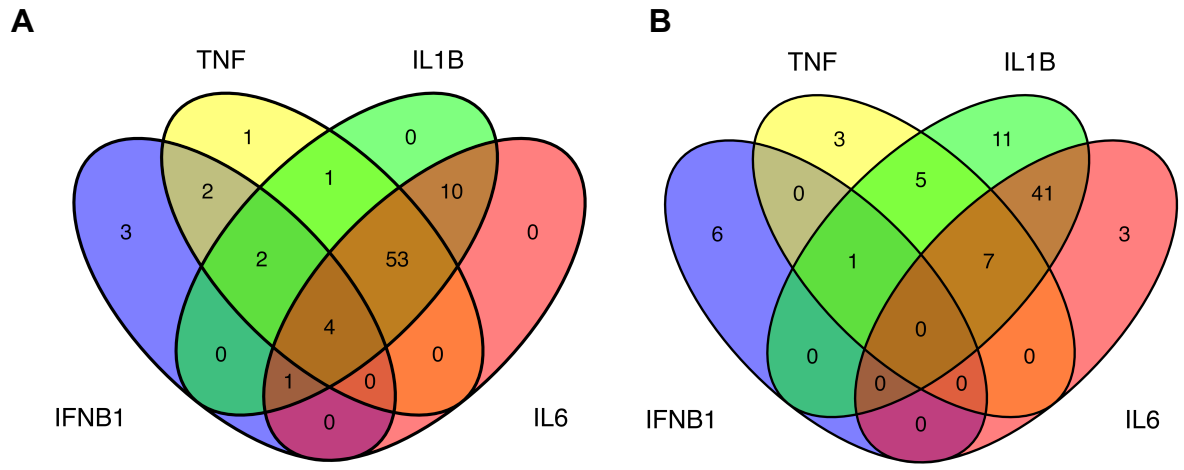

Figure 3.3. Candidate genetic loci often have shared association in multiple cytokines. (A) Pleiotropic effects among cytokines were assessed for each of the 77 suggestive loci at a nominal, $P < 0.05$ threshold, (B) and at a more stringent Bonferroni corrected threshold, $P < 6.49 \times 10^{-4}$.

3.4.5 *Functional annotation of candidate loci reveals potential mechanism and history of phenotypic effect*

To identify potential mechanisms of genetic effect, we mapped each of the 77 loci to proximal genes, assessed expression quantitative trait loci (eQTL) effects, predicted deleterious SNPs, and identified previous locus associations in the literature and GWAS catalog (Table 3.1, Table S3.3). Many of the mapped genes have previously been associated with cytokine regulation and Mtb response [70,140–147]. Additionally, many loci have SNPs with CADD (Combined Annotation Dependent Depletion) scores ranking them from the top 10% to 1% most deleterious SNPs (CADD = 10-20). One of the more severe CADD scores within these loci is due to a missense mutation within exon 7/28 of the canonical isoform of the gene CORO7. It results in an arginine to glutamine substitution and is predicted to have a deleterious (score = 0.01) and probably damaging (score = 0.988) effect on the gene CORO7 using the SIFT and PolyPhen prediction algorithms, respectively [148,149]. Several genes had significant cis-eQTL

effects on adjacent genes. Lastly, annotating SNPs with previous phenotype associations within GWAS catalog revealed many loci had previous COVID19, autoimmune disease, protein-QTL, and myeloid cell phenotype associations. Together, these analyses demonstrate that many of the genomic loci identified often have additional forms of evidence which support their potential to have an effect on Mtb-induced cytokine expression.

Table 3.1. Functionally Annotated Suggestive Genomic Loci Associated with Mtb-Induced Monocyte Cytokines

Chr	Band	Lead SNP	Cytokine	*P* value	Mapped Gene	CADD Score	eQTL	GWAS Catalog*
1	q42.12	rs73083530	IL1B	$3.64x10^{-6}$	H3F3A	20.2	-	
1	p36.32	rs116572061	IL1B	$5.71x10^{-6}$	PRDM16	19.28	-	-
1	p35.3	rs1448341	IL1B	$3.23x10^{-6}$	-	5.32	-	COVID-19
1	p36.11	rs35679058	IL6	$6.04x10^{-6}$	near PIGV	3.06	-	Granulocyte/ Monocyte count
2	p12	rs4852387	IL1B/IL6	$9.97x10^{-7}$	-	9.92	GCFC2	-
2	p25.1	rs7563835	IL1B	$1.33x10^{-6}$	HPCAL1	6.07	-	-
2	q32.1	rs6710680	IL1B/IL6	$6.04x10^{-6}$	-	21	-	-
5	q11.2	rs3111657	IL6	$3.1x10^{-6}$	LINC 01033	8.94	SNX18	-
5	q34	rs79380271	IL1B	$8.74x10^{-6}$	SLIT3	10.83	-	-
6	q23.3	rs6904731	IFNB1	$3.33x10^{-6}$	AHI1	9.75	AHI1	Systemic Lupus, Neutrophil Count
7	q36.3	rs78925109	IL1B	$7.96x10^{-8}$	PTPRN2	3.34	-	-
9	p24.2	rs10974620	IL1B	$3.63x10^{-7}$	SLC1A1	8.65	-	-
15	q21.3	rs12900655	IL1B	$1.26x10^{-6}$	NEDD4	19.48	-	
16	p13.3	rs9673241	IL1B	$6.56x10^{-7}$	C16orf96	19.02**	ROGDI	NMRAL1 Abundance
16	p13.3	rs76359705	IL1B/IL6	$4.88x10^{-6}$	RBFOX1	16.66	-	-
17	p13.3	rs62068280	TNF	$1.15x10^{-6}$	NXN	14.28	-	
20	q13.32	rs2180796	IL1B/IL6	$6.92x10^{-6}$	PIEZO1P2	15.56	NPEPL1	-
21	q22.11	rs58283029	IL1B	$9.39x10^{-6}$	near IFNAR2	7.982	-	IFNAR2 Abundance
22	q13.2	rs6003161	IFNB1	$4.05x10^{-6}$	near SCUBE1	18.1	-	-

- a subset of genetic variants with suggestive associations ($P < 1x10^{-5}$) with Mtb-induced cytokines in monocytes (full list in Supplemental Table 2).
* Relevant GWAS catalog traits summarized for brevity
** Locus contains deleterious CORO7 missense mutation

3.4.6 *Gene set analysis: MAGMA identifies pathways enriched for Mtb-induced TNF effect*

To further probe the genetic regulation of the Mtb-induced cytokine response, we performed gene set analysis (GSA) using MAGMA (Multi-marker Analysis of GenoMic Annotation), to determine if SNPs had a significant joint association with Mtb-induced cytokine response in particular gene sets within MSigDB [138,139]. Gene *P*-values were first determined from the aggregate signal of SNPs located within gene boundaries and adjusted for multiple correction while accounting for linkage disequilibrium (LD). GSA was then performed for each cytokine independently. Overall, across all gene sets and all four cytokines, only two significant associations were found, both of which were canonical pathways enriched for genes associated with Mtb-induced TNF (Figure 3.4A). One of these two significant pathways was alpha-linolenic acid metabolism, which has been implicated in direct effects on mycobacterial growth in addition to other macrophage response phenotypes [150–152]. Analysis of the 19 gene level p-values within the gene set identified early QQ plot deviation consistent with significance being a true gene set effect rather than a single outlier gene (Figure S3.5). Network visualization demonstrated that much of the alpha-linolenic acid metabolism pathway gene set is composed of a variety of PLA2 family members, which are known to regulate inflammation [153]. The more significant gene level p-values appear to be spread amongst multiple unrelated PLA2 isozymes within the pathway (Figure 3.4B). In sum, these data demonstrate that SNPs associated with differences in Mtb-induced TNF expression are significantly enriched in a variety of PLA2 genes within the alpha-linolenic acid metabolism pathway.

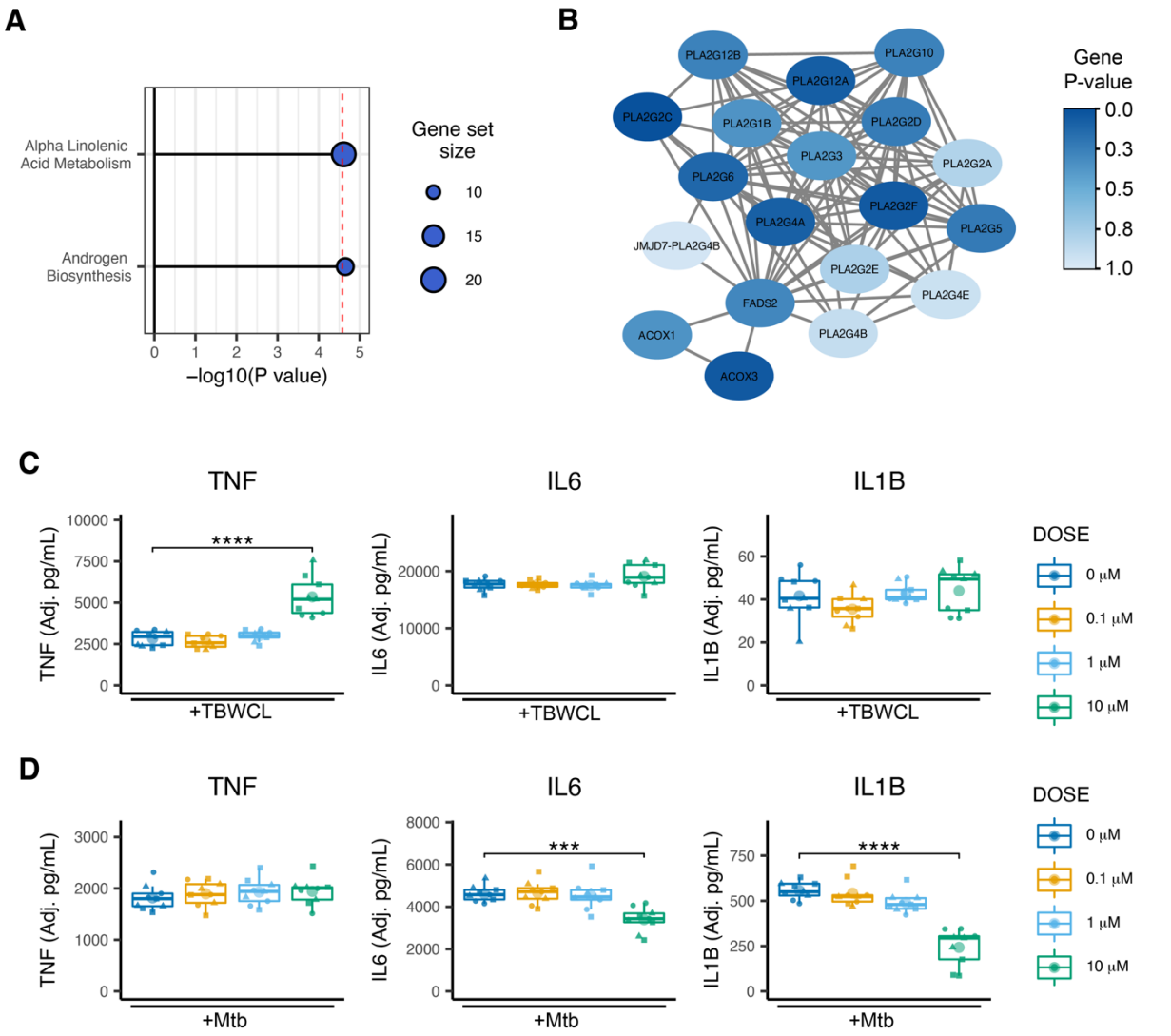

Figure 3.4. MAGMA enrichment identifies Mtb-induced TNF association with alpha-linolenic acid metabolism.

(A) Previously calculated SNP *P*-values for each cytokine were used to calculate gene level *P*-values, mapped to within each gene, accounting for SNP LD using MAGMA multi-model. Selected C2-canonical pathways (KEGG, Reactome, Biocarta, and PID) and C5-GO terms were assessed. Significant results after bonferroni multiple correction are shown. Dashed-vertical line indicates threshold for significance (*P*adjust < 0.05). (B) Network of genes within alpha-linolenic acid metabolism gene set was annotated for interaction using StringDB and plotted for MAGMA calculated gene *P*-value significance showing multiple PLA2 isoforms with increased significance C) TBWCL and D) Mtb-induced secretion of TNF, IL1B, and IL6 assessed by ELISA after increasing doses of cPLA2 inhibitor, AACOCF3, pretreatment in human macrophages shows PLA2 enzymes do significantly effect TBWCL-induced TNF response and Mtb-induced IL1B and IL6 response. Results shown are from 3 biological replicates in 3 human donors per treatment. Significance

assessed using linear mixed model adjusting for random effect of donor followed by ANOVA and pairwise comparisons. Individual data points plotted according to model calculated values adjusted for donor intercept. Significance determined as (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Individual donor values are presented in Figure S3.6

3.4.7 *Inhibition of PLA2 activity in vitro effects Mtb- and TBWCL-induced cytokine secretion*

In order to validate this finding, we next examined whether *in vitro* perturbation of PLA2 activity modulated the Mtb-induced cytokine response. We first compared the effects of the cytosolic PLA2 inhibitor, AACOCF3, on macrophage cytokine production in response to Mtb whole cell lysate (TBWCL) and identified a highly significant effect on Mtb-induced TNF secretion at the 10 μM dose (Figure 3.4C, $P < 0.0001$). No significant effect on IL1B or IL6 was observed. In contrast, upon live Mtb infection, we found that macrophage production of TNF was not significantly affected, whereas IL1B and IL6 were significantly decreased (Figure 3.4D, $P < 0.001$). In summary, these data show that the PLA2 enzymes identified as candidate regulators of the Mtb-induced cytokine response, showed a validated effect when perturbed *in vitro*.

3.5 DISCUSSION

The major genetic variants regulating the macrophage cytokine response to live Mtb have not been defined. Using a cellular GWAS, we identified candidate genetic regulators of the macrophage Mtb response at the SNP and pathway level within a Ugandan population which showed effect when investigated *in vitro*. We identified 77 candidate genomic loci with a suggestive association with Mtb-induced cytokine expression. Follow up macrophage assays confirmed Mtb-induced cytokine regulation by PLA2 inhibitors. This study suggests that a cellular GWAS is a promising approach to identify candidate genetic regulators with significant effect on Mtb response when recreated *in vitro*.

Although no variants passed a traditional significance threshold in the Uganda discovery cohort ($P < 5 \times 10^{-8}$), in depth annotation of each suggestively associated locus ($P < 1 \times 10^{-5}$) highlighted many loci of interest which could serve as a potential hypothesis generating resource for further *in vitro* validation. Multiple loci identified were mapped to genes previously associated with regulation of cytokine levels and response pathways and two of these genes, NEDD4 and IFNAR2, have already been shown to effect intracellular replication of Mtb [70,147]. This is consistent with previous cellular GWAS studies which have often been able to identify multiple important genetic insights controlling a phenotype within a single study [98,99,101–104]. Lead SNPs for 26 of the 77 identified loci were found to be at least 10 times more common in the AFR panel than any other 1000 Genome panel (AMR, EAS, EUR, or SAS, Table S3.3). This suggests that many of these variants which were specifically identified using a cohort of Ugandan individuals would not have been discovered if performed in other populations. This is similar to prior observations in other cellular GWAS studies and highlights the importance of evaluating populations which are often underrepresented in genetic studies [98].

In addition to annotation of individual genetic variants, we also performed gene and gene set analysis to determine if a joint effect of multiple SNPs was enriched in a biologically relevant way. SNPs associated with Mtb-induced TNF expression were enriched in two separate canonical pathways. To our knowledge, this is one of the first times that genetic regulators of cytokine induction have been identified using the aggregate effect of genome wide SNPs. One of the two pathways identified was in alpha-linolenic acid metabolism which was previously implicated in numerous facets of the host response to Mtb including cytokine induction, cell death, and effect on intracellular replication [150–152]. Much of the signal within the pathway

was concentrated among a variety of unrelated PLA2 isoforms which serve as an important source of arachidonic acid (AA) by catalyzing its release from membrane phospholipids [153]. AA and its downstream intermediates, have diverse pro- and anti-inflammatory effects on the host response to Mtb that can vary based on cell type, host species, and mycobacterial strain [154–157]. Interestingly, we found that PLA2 inhibition altered TNF levels in opposite directions when comparing TB whole cell lysate and live Mtb. The substantial difference in lysate vs live Mtb may be in line with prior research in which H37Rv was able to modulate the products of AA metabolism differently from the avirulent strain H37Ra so that the anti-inflammatory lipoxin, LXA_4, was favored over the inflammatory prostanoid, PGE2 [158]. Regulating lipoxin levels has previously been shown to be essential in Mtb response, and LTA4H mutations which lead to increases in LXA_4 have previously been shown to effect TNF production, and have clinical associations with pulmonary TB, multibacillary leprosy, and TB meningitis [159]. Although further research is necessary to ascertain the exact mechanism of effect, these data highlight the large and complex role that PLA2 inhibition plays in the human macrophage cytokine response and suggests that genetic variants causing differences in PLA2 activity could play a substantial role in controlling the extent of an individual's Mtb-induced cytokine response.

Our study has several limitations. First, although significant genetic effect was found at the gene set level, no individual variants in the Ugandan cohort passed a traditional genome wide significance threshold of $P < 5x10^{-8}$. By performing controlled, *in vitro* assays, a cellular GWAS approach can mitigate many confounding factors found in assessment of clinical phenotypes. However, each additional individual included represents a significant endeavor to profile and limits attainable sample sizes which may be necessary for determining variants surpassing

traditional genome-wide significance thresholds. We attempted to mitigate this limitation by profiling the maximum number of individuals who could have overlapping RNAseq and genotyping information. We identified 9 individuals that had failed previous genotyping QC due to low call rate and reperforming genotyping brought our total cohort size to 100 individuals. However, previous cellular GWAS efforts which obtained genome-wide significance have often included multiple hundreds of individuals [98,114]. We were also limited in our sample availability within the Uganda cohort. The Uganda cohort was ideal for initial studies because we are assessing a relevant population with endemic TB that has already been extensively profiled over decades. However, because of the effort to profile each individual donor, sample availability is limited. As such, we were unable to validate our candidate cellular GWAS loci within an identical population. Furthermore, we were limited in our ability to perform subsequent mechanistic studies using donor samples from the Uganda population. We compensated for these difficulties using functional annotation of candidate loci and *in vitro* validation of pathway results which may add weight to results in the absence of genetic validation.

In summary, we used a cellular GWAS approach to characterize the major genetic regulators of the Mtb-induced cytokine response in myeloid cells and identified candidate genetic loci, pathways, and genes which may have an important role in Mtb response biology within the Ugandan population. Further defining the mechanisms by which these genetically regulated genes control Mtb-induced cytokine infection could yield important insights in our understanding of differences in host disease progression and increase our ability to respond to the global burden of TB.

3.6 SUPPLEMENTAL FIGURES

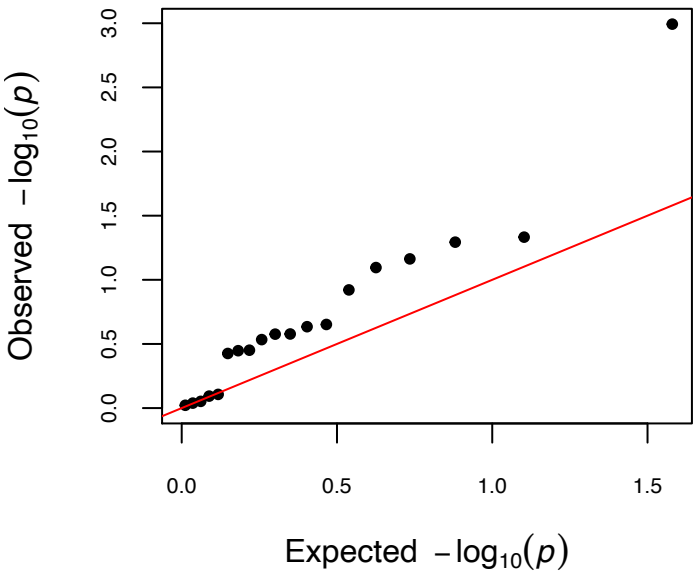

Figure S3.5. QQ plot of gene *P*-values of the alpha-linolenic acid metabolism gene set. MAGMA calculated gene *P*-values for association with Mtb-induced TNF expression were plotted for the 19 genes in the alpha-linolenic acid metabolism pathway and show an early deviation from expected trajectory suggesting a gene set rather than single gene effect.

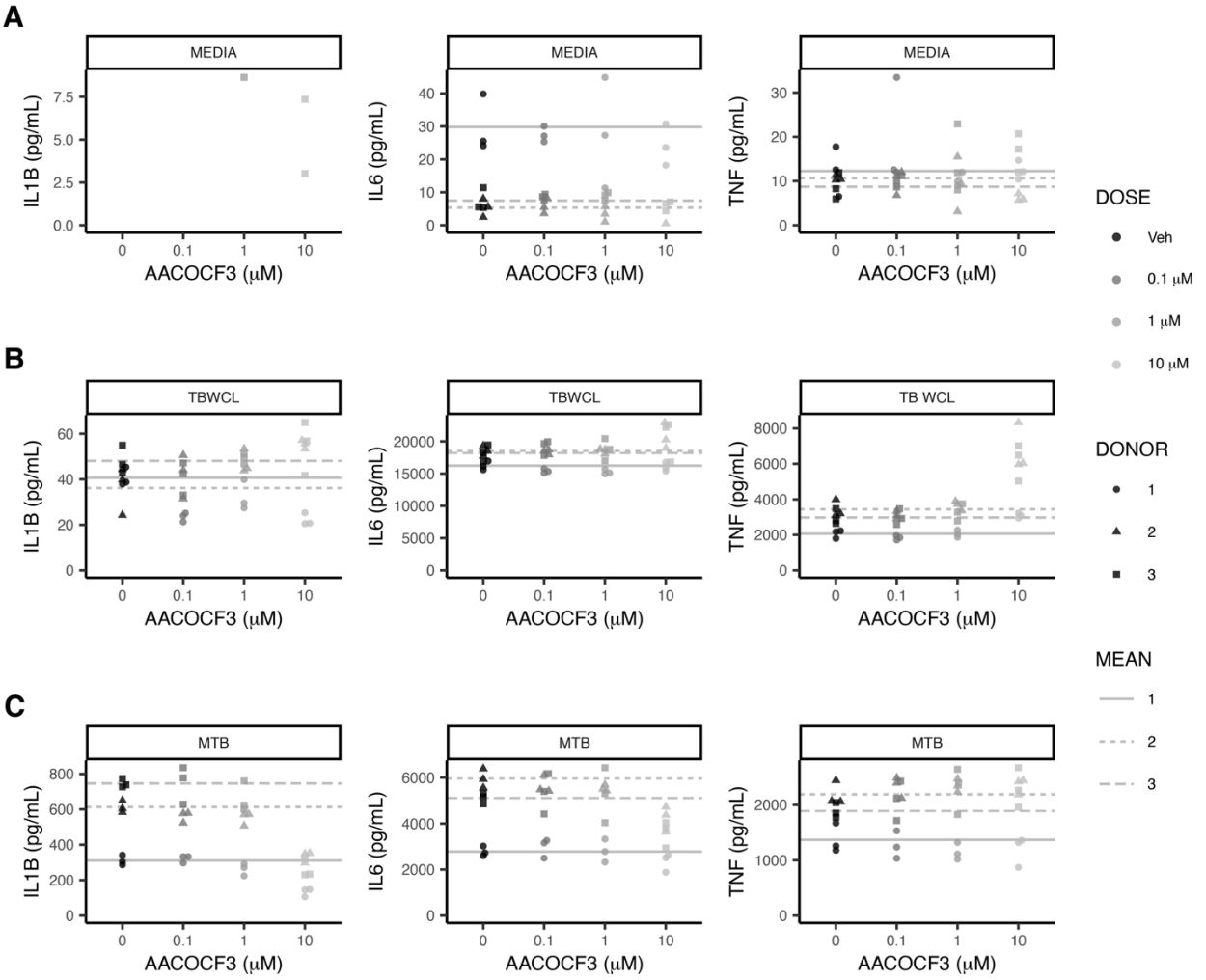

Figure S3.6. Unadjusted results of AACOCF3 treatment *in vitro* experiments.
Results for each *in vitro* experiment are plotted without adjustment for donor as a random effect. Dot shapes represent 3 different human donors and mean lines show the mean of 3 independent stimulations or infections per donor for the control condition. (A) Cytokine protein secretion for the media only (B) TB WCL, and (C) live Mtb stimulation conditions are plotted according to dose of AACOCF3 pretreatment. Results show consistent differences in cytokine production for significant outcomes within donor infections despite baseline differences in control means between donors. Adjustment of donor as a random effect allows for significance assessment and visualization of consistent biological effect across individual infections and donors despite baseline mean differences.

3.7 SUPPLEMENTAL TABLES

Table S3.2. Demographic data for the Uganda Cellular GWAS cohort

	Uganda HHC (n = 100)
Gender	
F (%)	43 (43)
M (%)	57 (57)
Age	
Mean (SD)	23.4 (9.5)
Median	21.5
[Min, Max]	[14,67]
TB status	
LTBI (TST+)	51 (51)
RSTR (TST-)	49 (49)
TB disease	0 (0)
HIV status	
HIV+	0 (0)
HIV-	100 (100)

Table S3.3. All Functionally Annotated Suggestive Genomic Loci Associated with Mtb-Induced Monocyte Cytokines

Chr	Band	rsID	Cytokine	P.value	Mapped Gene	CADD Score	eQTL	AFR AF	AMR AF	EAS AF	EUR AF	SAS AF	GWAS catalog
12	q23.1	rs10860639	IL6	3.9E-06	ANO4	7.1	-	0.54	0.56	0.49	0.67	0.60	-
9	p24.2	rs10974620	IL1B	3.6E-07	SLC1A1	8.7	-	0.21	0.30	0.45	0.25	0.20	-
1	p36.23	rs11120868	IL1B	7.8E-06	CAMTA1	4.2	-	0.16	0.35	0.34	0.28	0.24	-
10	q26.11	rs112029	IL6	3.5E-06	near TXNP1	9.4	-	0.43	0.29	0.29	0.19	0.24	body height, blood pressure traits, smoking initiation
2	q14.3	rs113338319	IL1B	6.8E-06	-	7.7	-	0.03	0.00	0.00	0.00	0.00	-
4	p15.2	rs114835412	IL1B	2.9E-07	-	4.5	-	0.05	0.00	0.00	0.00	0.00	-
14	q24.3	rs114919997	IL1B/IL6	1.8E-06	IFT43	18.2	-	0.04	0.00	0.00	0.00	0.00	-
1	p36.32	rs116572061	IL1B	5.7E-06	PRDM16	19.3	-	0.13	0.01	0.00	0.00	0.00	-
19	p13.3	rs11881732	IL1B	1.3E-06	PALM	6.9	-	0.27	0.24	0.12	0.36	0.18	-
4	p15.2	rs11938023	IL1B	2.9E-06	-	15.5	-	0.18	0.13	0.08	0.18	0.19	essential tremor
1	p36.13	rs12095353	TNF	4.3E-06	-	0.9	-	0.19	0.26	0.14	0.25	0.10	-
3	q11.2	rs12107024	IL1B	1.2E-06	-	17.9	-	0.44	0.29	0.42	0.49	0.54	-
10	q26.3	rs12263329	IL1B	2.3E-06	ADGRA1	3.0	-	0.13	0.02	0.00	0.00	0.00	-
11	p15.4	rs12276965	IL1B	7.6E-06	-	15.1	-	0.10	0.37	0.04	0.35	0.25	-
10	q21.1	rs12416448	IL1B	6.7E-06	-	10.9	-	0.44	0.30	0.11	0.10	0.33	-
15	q21.3	rs12900655	IL1B	1.3E-06	NEDD4	19.5	-	0.13	0.16	0.03	0.06	0.07	delta-5 desaturase measurement, body height
2	q24.1	rs13012138	IL6	6.3E-06	UPP2	5.5	-	0.17	0.04	0.02	0.08	0.04	-
5	p13.2	rs13159021	IFNB1	2.1E-06	near RXFP3	11.6	-	0.11	0.53	0.43	0.86	0.50	pigmentation traits, body height
7	p14.2	rs138302119	IL6	9.4E-06	-	10.2	-	0.02	0.00	0.00	0.00	0.00	-
4	q27	rs1383229	IFNB1	5.7E-06	-	4.7	-	0.24	0.03	0.00	0.00	0.02	-
1	p35.3	rs1448341	IL1B	3.2E-06	-	5.3	-	0.07	0.03	0.00	0.04	0.06	COVID-19
13	q31.1	rs146736603	IL1B	1.5E-06	-	9.6	-	0.01	0.00	0.00	0.00	0.00	-
1	p36.12	rs150485313	IL1B/IL6	3.4E-07	near ALPL	4.3	-	0.01	0.00	0.00	0.01	0.00	-
12	q24.22	rs16947739	TNF	2.2E-06	KSR2	1.8	-	0.25	0.22	0.08	0.06	0.03	-
12	q23.3	rs17034127	IL1B	5.8E-06	near STAB2	14.1	-	0.14	0.12	0.21	0.11	0.32	-
4	p16.3	rs182741934	IL1B	2.2E-07	-	5.1	-	0.04	0.01	0.06	0.03	0.03	-
15	q26.3	rs192165301	IL6	8.6E-06	-	2.6	-	0.02	0.00	0.00	0.00	0.00	-
4	q28.1	rs193254859	IL1B	7.1E-06	-	8.8	-	0.04	0.00	0.00	0.00	0.00	-
15	q14	rs1948650	TNF	5.3E-06	DPH6-DT	0.0	-	0.18	0.43	0.50	0.32	0.30	-
20	q13.32	rs2180796	IL1B/IL6	6.9E-06	PIEZO1P2	15.6	NPEPL1	0.52	0.25	0.18	0.43	0.34	-
10	p14	rs2497461	IFNB1	6.7E-06	-	6.0	-	0.10	0.21	0.49	0.13	0.17	-
5	p13.3	rs2877219	IL6	7.1E-06	PDZD2	1.0	-	0.16	0.34	0.32	0.36	0.35	-
8	p23.3	rs28892957	IL6	9.7E-06	DLGAP2	1.7	-	0.26	0.20	0.02	0.34	0.24	-
6	p25.1	rs3024358	IL1B	3.8E-06	F13A1	3.1	-	0.15	0.19	0.00	0.18	0.11	-
5	q11.2	rs3111657	IL6	3.1E-06	LINC01033	8.9	SNX18	0.13	0.01	0.06	0.02	0.06	-
1	p36.11	rs35679058	IL6	6.0E-06	near PIGV	3.1	-	-	-	-	-	-	granulocyte and monocyte

													percentages
6	p22.3	rs3793114	IL1B	3.2E-06	ATXN1	15.6	-	0.13	0.03	0.03	0.04	0.08	-
6	p22.3	rs4715975	IL1B	4.9E-06	JARID2	4.9	-	0.08	0.22	0.24	0.26	0.16	-
2	p12	rs4852387	IL1B/IL6	1.0E-06	-	9.9	GCFC2	0.92	0.63	0.75	0.66	0.53	-
17	p11.2	rs4986047	IL1B	4.2E-06	near KCNJ12	1.9	-	0.11	0.03	0.00	0.04	0.09	body mass index, body height
11	q24.2	rs523563	IL6	7.0E-06	-	8.3	-	0.42	0.40	0.40	0.35	0.42	-
1	q25.1	rs551501	TNF	3.7E-06	near LINC01657	4.8	-	0.11	0.01	0.00	0.00	0.00	-
5	q13.1	rs555659199	IL1B	7.9E-07	-	12.9	-	0.02	0.00	0.00	0.00	0.00	-
8	q24.3	rs56996464	IL1B	5.0E-06	-	1.1	-	0.06	0.00	0.00	0.00	0.00	-
7	p11.2	rs58200656	IL1B/IL6	1.4E-06	-	8.1	-	0.11	0.07	0.11	0.05	0.15	-
21	q22.11	rs58283029	IL1B	9.4E-06	near IFNAR2	8.0	-	0.08	0.28	0.25	0.15	0.26	cytokine measurement
11	q13.5	rs59477197	TNF	9.1E-07	-	8.9	-	0.05	0.00	0.00	0.00	0.00	-
22	q13.2	rs6003161	IFNB1	4.1E-06	near SCUBE1	18.1	-	0.31	0.47	0.40	0.48	0.56	-
10	q21.3	rs60344079	IL1B	7.2E-06	CTNNA3	5.9	-	0.09	0.01	0.00	0.00	0.00	-
8	q24.22	rs60372392	IL1B	4.8E-06	-	13.4	-	0.13	0.01	0.00	0.00	0.00	-
10	p15.3	rs61834188	IL1B	2.1E-06	near RNU6-576P	7.4	-	0.09	0.18	0.09	0.16	0.21	-
11	q21	rs61910441	IL1B	5.2E-06	-	2.6	-	0.05	0.02	0.00	0.02	0.02	-
17	p13.3	rs62068280	TNF	1.2E-06	NXN	14.3	-	0.28	0.08	0.07	0.08	0.07	body height
12	q24.32	rs6489151	IL1B	9.3E-06	-	5.9	-	0.37	0.29	0.01	0.34	0.19	-
2	q32.1	rs6710680	IL1B/IL6	6.0E-06	-	21.0	-	0.01	0.00	0.00	0.00	0.00	-
2	p16.1	rs6730202	IL1B	8.9E-07	-	12.8	-	0.10	0.35	0.28	0.40	0.33	-
3	p14.2	rs6778638	IL6	7.1E-07	LINC00698	2.5	-	0.12	0.01	0.00	0.00	0.00	-
6	q23.3	rs6904731	IFNB1	3.3E-06	AHI1	9.8	AHI1	0.31	0.02	0.00	0.01	0.00	Blood cell traits, systemic lupus, fatty acid measurement
1	q42.12	rs73083530	IL1B	3.6E-06	H3F3A	20.2	-	0.13	0.01	0.00	0.00	0.00	gut microbiome measurement
4	p15.33	rs73225332	IL6	1.1E-06	-	4.2	-	0.08	0.01	0.02	0.01	0.05	-
12	p13.32	rs73253395	IL6	2.9E-06	-	2.8	-	0.03	0.00	0.00	0.00	0.00	-
8	q23.1	rs73295798	IL1B	9.3E-06	-	13.3	-	0.07	0.00	0.00	0.00	0.00	-
9	q34.13	rs73559254	IL1B/IL6	3.4E-06	near NTNG2	0.7	-	0.07	0.01	0.00	0.00	0.00	-
3	q13.12	rs73858478	IL1B	8.5E-06	near RF00019	1.8	-	0.19	0.01	0.00	0.02	0.03	-
4	q32.1	rs73860519	IL6	1.8E-06	RXFP1	9.0	-	0.11	0.01	0.00	0.00	0.00	-
18	q21.32	rs73964708	IFNB1	2.1E-06	-	5.9	-	0.04	0.00	0.00	0.00	0.00	body height
2	p25.1	rs7563835	IL1B	1.3E-06	HPCAL1	6.1	-	0.95	0.73	0.95	0.64	0.61	-
16	p13.3	rs76359705	IL1B/IL6	4.9E-06	RBFOX1	16.7	-	0.06	0.00	0.00	0.00	0.00	-
11	p14.3	rs77722829	IL1B	7.9E-06	LUZP2	2.7	-	0.11	0.02	0.00	0.00	0.00	-
7	p21.1	rs7780446	IL1B	8.8E-06	TSPAN13	1.6	-	0.03	0.00	0.00	0.00	0.00	-
7	q36.3	rs78925109	IL1B	8.0E-08	PTPRN2	3.3	-	0.10	0.01	0.00	0.00	0.00	-
5	q34	rs79380271	IL1B	8.7E-06	SLIT3	10.8	-	0.09	0.03	0.00	0.05	0.01	-
12	p13.33	rs7967085	IL1B	5.1E-06	CACNA1C	9.6	-	0.10	0.01	0.00	0.00	0.00	-
6	q26	rs79924080	IL1B/IL6	6.5E-07	PRKN	8.0	-	0.07	0.00	0.00	0.00	0.00	-
21	q21.1	rs80328010	IL1B	6.3E-06	near RPS3AP1	3.6	-	0.08	0.04	0.04	0.02	0.06	-
12	p12.3	rs9332921	IL1B	1.1E-06	MGST1	9.2	-	0.14	0.02	0.00	0.00	0.00	-
16	p13.3	rs9673241	IL1B	6.6E-07	C16orf96	19.0	ROGDI	0.18	0.56	0.61	0.61	0.39	QRS duration, BMI traits,

													NMRAL1 abundance, testosterone measurement, milk allergy

- All 77 with a suggestive *P*-value (1 x 10^{-5}) across all cytokines shown with lead SNP *P*-values, mapped genes, significant cis-eQTL associations, annotated CADD scores, 1000 Genomes population allele frequencies, and full GWAS catalog associations

Chapter 4. THE SLIT3 AND SLC1A1 CANDIDATE GENOMIC LOCI HAVE POPULATION-SPANNING EFFECT WHICH VALIDATES *IN VITRO* AND ARE ALSO ASSOCIATED WITH DIFFERENTIAL MACROPHAGE RESPONSE AND CLINICAL PROGRESSION PHENOTYPES

This chapter has been adapted from a previously generated manuscript currently under review at the Journal of Clinical Investigation and is included here with permission.

4.1 CHAPTER SUMMARY

Immune and clinical outcomes to *Mycobacterium tuberculosis* (Mtb) infection vary greatly between individuals yet the underlying genetic and cellular mechanisms driving this heterogeneity remain poorly understood. We assessed results generated in our previously performed cellular GWAS for transferability in an independent Seattle cohort. We subsequently validated promising results *in vitro*, evaluated their effect on other Mtb-response phenotypes, and assessed for clinical phenotype associations. Four of the 77 suggestive loci identified in Uganda maintained significant associations in Seattle. We validated cytokine effect with siRNA knockdown for two of these loci which mapped to the genes SLIT3 and SLC1A1. Furthermore, exogenous treatment of macrophages with SLIT3 enhanced Mtb intracellular replication. Finally, SLC1A1 and SLIT3 variants were associated with susceptibility to tuberculous meningitis (TBM) and subsequent survival in a Vietnamese cohort, respectively. In sum, we identified multiple variants associated with Mtb-induced cytokine expression that validated *in vitro* and were associated with clinical TB susceptibility.

4.2 INTRODUCTION

Tuberculosis (TB) remains a leading cause of death worldwide, including 1.3 million deaths in 2022 [2]. Clinical outcomes after exposure to *Mycobacterium tuberculosis* (Mtb) are varied and include pathogen clearance, asymptomatic infection, pulmonary TB, and tuberculous meningitis (TBM) [8]. Individual predisposition to certain clinical outcomes after Mtb exposure may be attributed to a variety of clinical risk factors, many of which are poorly understood [129]. One hypothesis is that genetic variation acquired over millennia of Mtb infections may regulate crucial host responses which can modulate infection progression after Mtb exposure [5,7]. Defining the causal genetic variants and how they mechanistically alter the Mtb-induced host response may elucidate some of the underlying differences in host progression.

Although genetic variation is consistently indicated to be a major factor in predisposition to certain clinical outcomes after Mtb exposure, the causal mechanisms responsible have not been defined. In many previous GWAS, variants surpassing genome-wide significance often appear to be specific to that population and show little concordance between studies [88]. Additionally, many studies identifying an association often fail to identify plausible mechanisms of effect and rarely validate that effect using *in vitro* studies. A cellular GWAS, which assesses genetic regulation of clinically relevant, *in vitro*, intermediate traits may represent an attractive approach to overcome some of these difficulties for many reasons [96]. Foremost, utilization of *in vitro* assays may eliminate some confounding factors present in clinical studies which may lead to the observed inconsistency in results. Second, assessment of an intermediate trait negates the need for a clinical cohort for initial studies. As such, different populations can be assessed in which TB is not endemic and without the burdensome clinical and epidemiological profiling necessary in clinical studies. Third, assessment of intermediate traits which relate to crucial host

response pathways may more readily translate into subsequent mechanistic understanding [96]. Although variation in an intermediate trait may not always lead to variation in a clinical phenotype, it may be easier to understand one host response factor at a time than the multitude involved in determining an endpoint infection outcome.

The Mtb-induced cytokine response within the macrophage may be a promising first candidate as a cellular GWAS phenotype due to the central role that it plays in modulating all stages of an Mtb infection. Initial control of Mtb infection requires an effective macrophage response capable of recognition, phagocytosis, and pathogen destruction [107]. Responses that are insufficiently inflammatory can instead result in intracellular persistence, non-apoptotic cell death, and improper recruitment of downstream responses [10]. In particular, the macrophage secreted cytokines, Tumor Necrosis Factor (TNF), Interleukin-1 beta (IL1B), Interleukin-6 (IL6), and Interferon beta (IFNB1), have previously been found to play crucial roles in regulating susceptibility, Mtb clearance, cell death, and recruitment of the adaptive response [35,58,63,67,68,80,108–111,132]. To our knowledge, genome-wide assessment of genetic regulation of live virulent Mtb-induced cytokine responses in macrophages has not been assessed previously.

In the current study, we characterized the cytokine expression profile of monocytes and macrophages isolated from two distinct human population cohorts in response to live Mtb infection and identified the major genetic variants associated with population spanning, differential, Mtb-induced cytokine response. Four loci were identified which showed significant transferability between cohorts. Two of these loci mapped to the genes SLIT3 and SLC1A1, identifying them as candidate response regulators which was validated using *in vitro* macrophage assays. Lastly, we investigated if the SLIT3 and SLC1A1 variants effecting the Mtb-induced

cytokine response were further associated with clinical TB phenotypes and found significant differences in TBM susceptibility and subsequent survival.

4.3 METHODS

4.3.1 *Human donor and clinical cohorts*

Our independent Seattle cohort analysis involved healthy donors from Seattle who were recruited between 2020 and 2022, and PBMCs were collected at the time of recruitment. Individuals who reported no current or previous history of serious medical health issues were included. Our clinical phenotype association analyses involved characterization of two population cohorts from Uganda and Vietnam. Individuals from Uganda were part of a highly Mtb-exposed Ugandan household contact cohort. Subjects were recruited between 2002 and 2012 and followed for serial TST and IGRA testing over an 8-to-10-year period and then in a subsequent follow up from 2014-2017. Assessment of clinical differences in TST/IGRA conversion was performed using a larger 325 individual cohort (97 RSTR, 228 LTBI) previously described [29]. Subjects of the Vietnam clinical cohort were collected over several sites in Ho Chi Minh City and were enrolled with either PTB or TBM and individuals with TBM were followed for survival outcome [160–163]. Assessment of clinical differences in PTB and TBM susceptibility was performed in comparison to control individuals within the Vietnam population, which have been previously described [164]. Details, including clinical definitions, are published for the Ugandan and Vietnam cohorts and clinical cohort tables are included (Table S4.2).

4.3.2 *Cell culture, reagents and CD14+ isolation.*

Human monocytes and macrophages were cultured in RPMI-1640 (Gibco) supplemented with FBS (Atlas Biologicals) at a final concentration of 10% and Macrophage colony-stimulating factor (M-CSF, Peprotech) at a final concentration of 50ng/mL for all experiments. For Seattle Healthy Donors and *in vitro* validation experiments, monocyte derived macrophages were obtained from cryopreserved PBMCs which were thawed, resuspended at 2 million cells/mL, and differentiated for 5 days in indicated culture media. The differentiated cell population was then subjected to magnetic bead column purification via negative selection (Miltenyi, pan monocyte isolation kit) to purify for Monocyte derived macrophages.

4.3.3 *Mtb-induced cytokine expression and secretion.*

Monocyte derived macrophages were infected with Mtb H37Rv Mtb thawed from cultures previously grown to mid log phase and 2 times Sauton's washed, at an MOI of 1 or a mock infection media only condition. For Mtb-induced cytokine expression, cells were lysed in trizol after 6 hours, after which RNA was isolated using a modified miRNeasy protocol (Qiagen). RNA was subsequently processed by cDNA synthesis and assessed via RTPCR in comparison to GAPDH control. Cytokine qPCR assays were purchased from Integrated DNA Technologies (IDT), (IL1B, Hs.PT.58.1518186; IL6, Hs.PT.58.40226675; TNF, Hs.PT.42.1656119; and IFNB1, hs.PT.58.39481063.g) and endogenous control GAPDH from applied biosystems (4310884E). Cytokine secretion was characterized using similar infection conditions for 24 hours, after which supernatants were extracted, filtered, and analyzed via ELISA for IL1B, TNF, and IL6 (R&D, Duoset).

4.3.4 *Genotyping and Imputation*

Individuals within the independent Seattle cohort analysis were part of a larger profiling effort of 256 individuals who were genotyped using an Illumina MEGA^{EX} panel. Genotyping data for all samples was subjected to pre-imputation QC. Samples who's SNP indicated sex matched metadata indicated sex and who's genotyping missingness was less than 1% were included. Additionally, SNPs were filtered to have greater than 99% call rate, 1 x 10^{-6} Hardy-Weinberg Equilibrium (HWE), and 1% minor allele frequency (MAF). All SNPs were then mapped to a Human Resource Compendium (HRC) variant in the reference genome and imputed using the Michigan imputation server and an HRC reference panel [165]. SNPs were subsequently filtered for > 5% MAF, > 1x10^{-6} HWE, for any overlapping SNP analysis. For the Uganda cohort clinical analyses, genotyping was performed using MEGA^{EX} and Omni5 genotyping chips as previously indicated [29,122]. Briefly, from the larger Ugandan household contact cohort, 149 Active TB, 86 RSTR and 198 LTBI individuals were genotyped with the MEGA^{EX} panel and 201 Active TB, 15 RSTR, 33 LTBI, and 233 No Active TB individuals were genotyped with the Omni5 panel. The MEGA^{EX} and Omni5 datasets were independently processed for imputation. SNPs with greater than 99% call rate, 1 x 10^{-6} Hardy-Weinberg Equilibrium (HWE), and 1% minor allele frequency (MAF), were subsequently aligned to a reference genome according to TOPMed Freeze 8 variants and imputed using the TOPMed server [133]. Overlapping SNPs within both imputations were combined and filtered for imputation quality score > 0.5, > 5% MAF, and > 1 x 10^{-6} HWE prior to testing.

4.3.5 *Seattle cohort population spanning SNP analysis.*

The independent Seattle cohort analysis was performed using the GENESIS package in R [134]. We used a linear model adjusted for, sex, pc1, pc2, and kinship to examine whether SNPs

were associated with Mtb-induced cytokine expression. Expression values were determined via -ΔΔCt RTPCR values (GAPDH normalized within sample and baseline media expression subtracted by donor). Covariates were included in final model analysis if found to significantly effect the distribution of any of the four Mtb-induced cytokines assessed using a linear model and the number of PCs included in the analysis was determined using an elbow plot to select number of PCs where variation with additional PCs added was minimal. Genotyped SNPs were used to generate PCs and kinship matrix using KING kinship estimation. SNP association was tested genome-wide and specifically queried SNPs were extracted for analysis.

4.3.6 *siRNA knockdown in human macrophages*

siRNA was performed in human macrophages isolated after 5 days of differentiation in indicated culture media. Lonza P3 primary cell nucleofector kit was used for nucleofections of 700,000 cells using 30pmol of siRNA. Cells were rested for 48 hours before experimentation. Three separate siRNAs were tested for each gene, and the siRNA with the greatest knockdown efficacy of at least 70% in comparison to Negative control siRNA was used for future experiments (Fig S3). DsiRNA sequences were purchased from IDT for SLIT3 primary (hs.Ri.SLIT3.13.1) and validation (hs.Ri.SLIT3.13.7) as well as for SLC1A1 primary (hs.Ri.SLC1A1.13.1) and validation (hs.Ri.SLC1A1.13.2) and assessed using IDT qPCR assays for SLIT3 (Hs.PT.58.25920874) and SLC1A1 (Hs.PT.58.19942121). All siRNA knockdown was performed in comparison to NC-1 negative control siRNA (IDT, 51-01-14-03), in technical triplicate, using three distinct human donors.

4.3.7 *hSLIT3 N- and C-terminal treatments*

Recombinant human SLIT3 N-terminal fragment, AA 34-1116 (#9255-SL) and C-terminal fragment (#9067-SL) was purchased from R&D systems. Cell fragments were resuspended according to manufacturer protocol and kept frozen at -20^{o}C until application, thawed, and resuspended in RPMI with 10% FBS. Cell fragments were applied 30 minutes prior to infection with Mtb as compared to a vehicle control with equal volume resuspension buffer (DPBS, Gibco). Similar SLIT3 fragment treatment was performed after the wash step of the initial 4-hr infection for intracellular replication assay.

4.3.8 *Intracellular replication of Mtb in infected macrophages*

H37Rv Mtb expressing mCherry was grown to mid log phase (OD < 0.6), single cell suspended using filtration through a 5uM filter, and diluted in RPMI to give final infection MOI of 1. Human monocyte derived macrophages were cultured in previously indicated media and rested 24 hours after column isolation. Infection was performed for 4 hours, after which, supernatant was removed and cells were washed in HBSS 2 times before adding fresh culture media. Intracellular replication was measured using total surface area of mCherry signal within a 96 well (Biotek Cytation 5, Agilent). 6 total infections per condition per individual were measured. Any initial differences in phagocytosis were similarly determined by surface area at this 4-hour timepoint. Subsequent intracellular replication was measured daily until end point at 72 hours.

4.3.9 *Statistics*

Genetic tests were performed as previously indicated using Genesis R package, assocTestSingle, accounting for necessary covariates, and genetic relatedness as a polygenic

random effect. Genesis uses a penalized quasi-likelihood approximation of the generalized linear mixed model as developed in GMMAT. Significance testing in the Seattle cohort was performed using GENESIS results extracted to test lead SNPs evaluated for one way significance to account for maintained directionality at a nominal, $P < 0.05$. The strict multiple correction threshold was determined by Bonferroni correction of this nominal *P*-value by number of lead SNPs tested within each cytokine. In vitro assay significance was assessed using a linear mixed model incorporating donor as a random effect. Differences in siRNA, and SLIT3 treatment were then assessed for significant group differences via ANOVA, and pairwise comparisons were made and adjusted for multiple comparisons using Tukey method.

4.4 RESULTS

4.4.1 *Multiple candidate loci have a population-spanning effect in an independent cohort*

Since genetic effects can be population specific, we next examined whether any of the Uganda suggestive loci were associated with Mtb-induced cytokine production in an independent cohort. Using a cohort of 40 Seattle healthy donors, we stimulated monocyte derived macrophages with Mtb or media alone and measured cytokine expression after 6 hours. The lead SNP of each locus suggestively associated with Mtb-induced cytokine expression in the Uganda cohort was examined in the Seattle cohort for association with Mtb-induced cytokine expression. Of the 77 SNPs in the original Uganda population, over half were unavailable to assess due to differences in SNP imputation and allele frequency between the Uganda and Seattle cohorts. However, of the remaining SNPs, 3 SNPs (rs10974620, rs79380271, and rs11881732) were found to be significantly associated with Mtb-induced IL1B expression and 1 SNP (rs16947739)

significantly associated with Mtb-induced TNF expression at a nominal, $P < 0.05$ threshold. Due to differences in number of loci tested per cytokine, only the SNP for Mtb-induced TNF (rs16947739) surpassed the significance threshold for multiple correction of number of SNPs tested. To account for differences between populations, all SNPs mapping within the four associated loci were assessed for linkage disequilibrium and association with cytokine expression. Two of the four loci, located at chromosome (chr) 9p24.2 and chr 5q34, showed a number of SNPs in LD which were significantly associated with reduced Mtb-induced IL1B in both populations (Figures 4.1A-D, S4.7, S4.8). The lead SNPs of the two loci (chr 9p24.2 and chr 5q34) were different in the two populations; rs10974620 and rs10815020 are the sentinel SNPs for the chr9 p24.2 loci, and rs79380271 and rs62378623 are the sentinel SNPs for the chr 5q34 loci, for Uganda and Seattle, respectively. The sentinel SNPs for Uganda are present in the majority of populations globally with the exception of some parts of Asia for rs79380271 (Figures 4.1E-F). Functional annotation revealed that the sentinel SNPs for the chr 9p24.2 and chr 5q34 loci were located within introns of the genes SLC1A1 and SLIT3 which have previously been linked to cytokine regulation in the literature [142,143]. Together, these data suggest that SLIT3 and SLC1A1 are lead candidate genes as major regulators of Mtb-induced IL1B in humans.

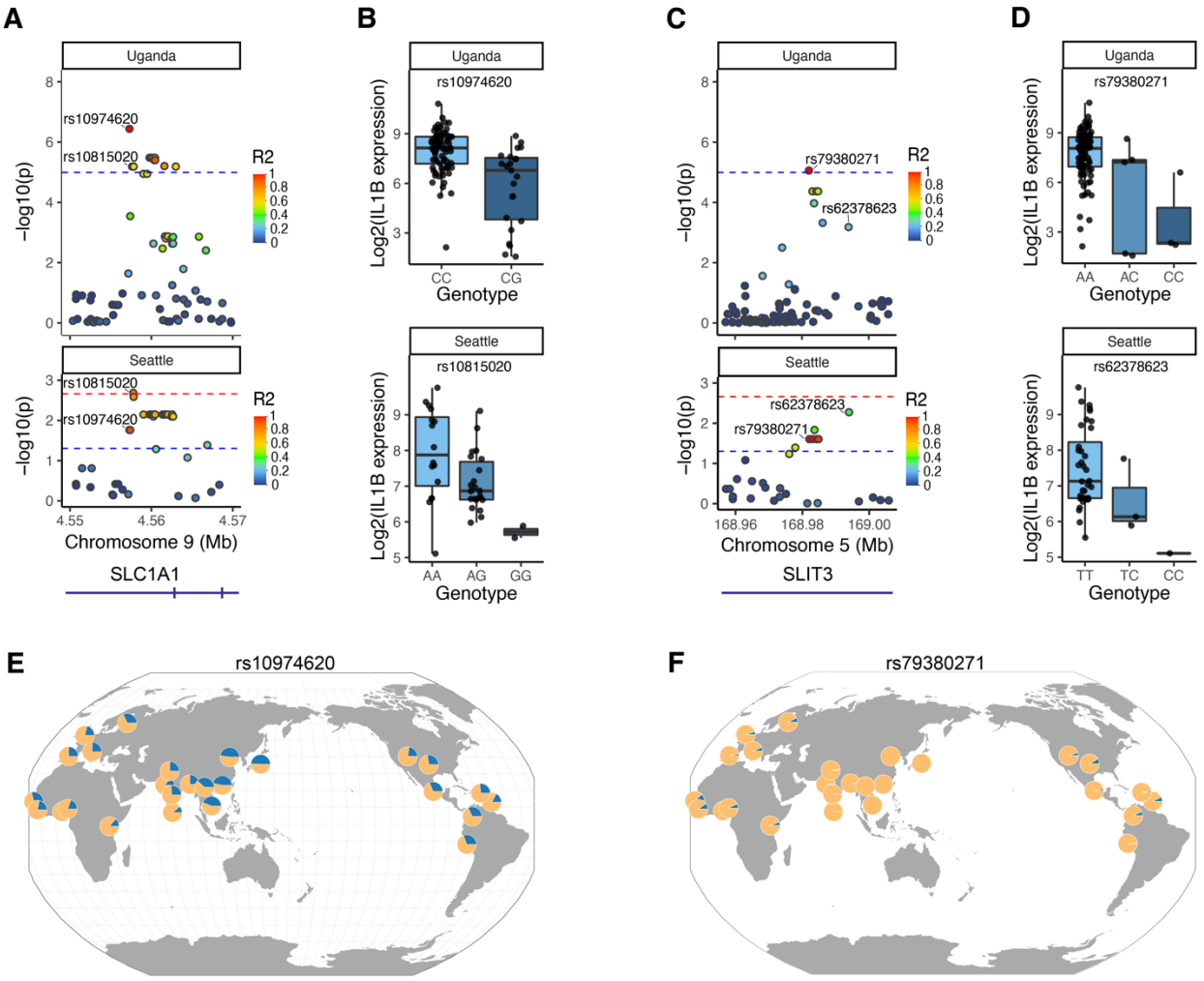

Figure 4.1. Multiple SNPs associated with Mtb-induced cytokine expression in the Uganda cohort show association with cytokine expression in a Seattle population cohort.

Lead SNPs that passed the suggestive threshold for a particular cytokine within the Uganda population were tested for association with the corresponding cytokine in the Seattle population. Four loci were associated with cytokine expression in the 2 populations ($P < 0.05$). To account for differences between populations, all SNPs within the two loci were assessed for linkage disequilibrium and association with corresponding cytokine expression. (A) *P*-values of SNPs of the chr 9p24.2 locus, within the gene SLC1A1 with coloration of individual SNP points indicating LD with rs10974620 within each population (B) Differences in Mtb-induced IL1B expression are plotted according to genotype for the chr 9p24.2 locus lead SNPs of the Uganda (rs10974620) and Seattle (rs10815020) population. (C) *P*-values of SNPs of the chr 5q34 locus, within the gene SLIT3 with coloration of individual SNP points indicating LD with rs79380271 within each population (D) Differences in Mtb-induced IL1B expression are plotted according to genotype for the chr 5q34 locus lead SNPs of the Uganda (rs79380271) and Seattle (rs62378623) population. Threshold lines indicate nominal *P*-value threshold (blue, $P < 0.05$ unadjusted), and multiple

corrected threshold (red, 0.05/number of SNPs tested for each cytokine). For both loci, multiple SNPs are significantly associated with decreased Mtb-induced IL1B expression in both populations. Global minor allele frequency of the (E) SLC1A1 locus Uganda sentinel SNP, rs10974620, and (F) SLIT3 locus Uganda sentinel SNP, rs79380271, plotted using Geography of Genetics Variants Browser

4.4.2 *SLC1A1 and SLIT3 affect Mtb-induced cytokine induction*

To experimentally validate whether the identified candidate genes had an effect *in vitro*, we used siRNA in MDMs and examined the effects of SLC1A1 and SLIT3 on Mtb-induced cytokine induction. Three or more siRNA sequences per gene were used to select an optimal targeting siRNA resulting in a more than 70% decrease in target gene expression (Figure 4.2).

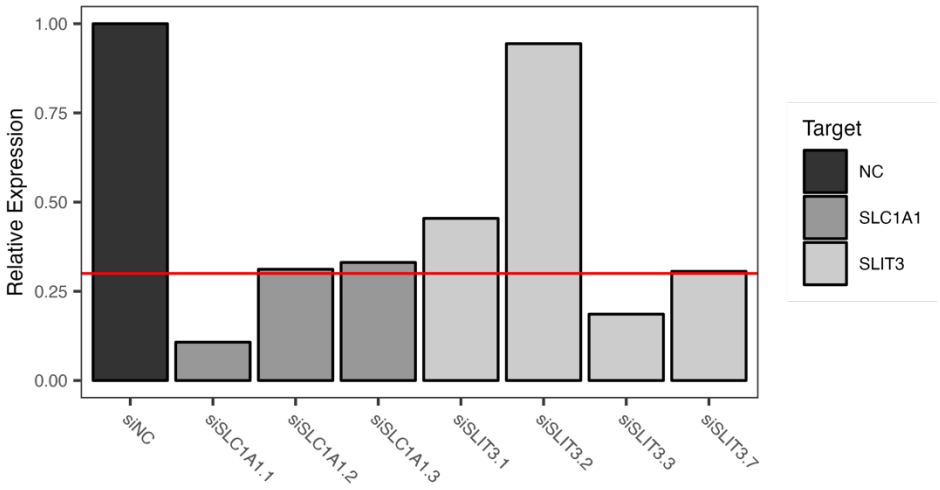

Figure 4.2. Knockdown of gene expression relative to Negative Control (NC) siRNA. Three siRNA per gene originally screened were tested for sufficient gene knockdown (>70% indicated by red line) for *in* vitro studies. Shows SLC1A1 siRNA 1 and SLIT3 siRNA 3 should be selected for initial studies.

RNAi knockdown of both SLC1A1 and SLIT3 led to a significant increase and decrease, respectively, in Mtb-induced IL1B protein secretion and mRNA expression that was robust across multiple human donors and siRNA target sequence (Figures 4.3A, 4.3B, 4.4, S4.9, S4.10, $P < 0.01$). SLIT3 knockdown also resulted in a highly significant decrease in IL1B expression in

the media only condition (Figure 4.4B, $P < 0.0001$). Since IL1B, IL6, and TNF expression was correlated in our initial GWAS screen in samples from Uganda, we further assessed for effect on these cytokines. SLIT3 knockdown was associated with decreased Mtb-induced IL6 and TNF protein secretion (Figures 4.4A $P < 0.05$) as well as mock infected TNF mRNA expression (Figure 4.4B, $P < 0.01$). In summary, these *in vitro* data indicate that expression of the candidate genes SLC1A1 and SLIT3 are associated with Mtb-induced cytokine responses in macrophages.

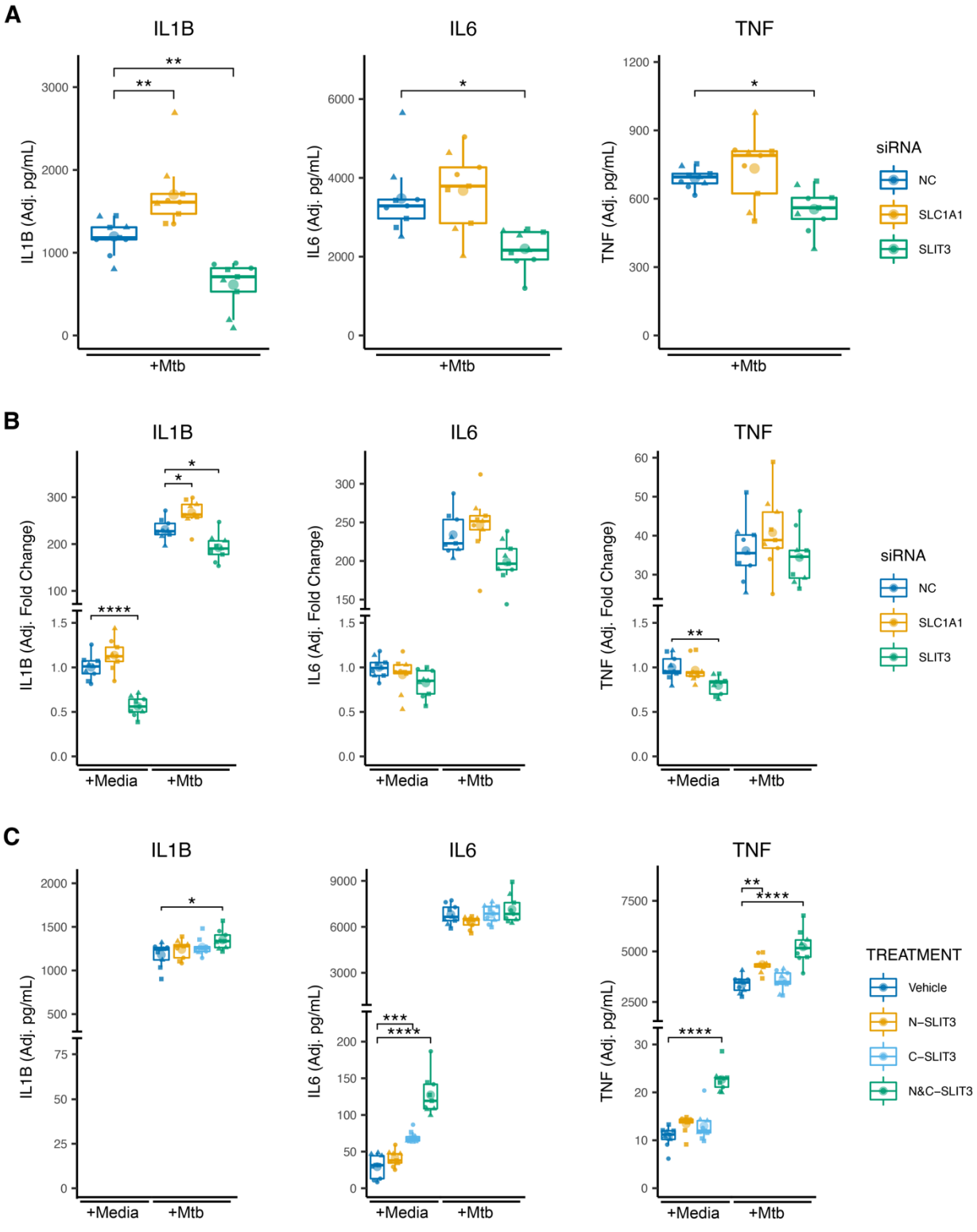

Figure 4.3. In vitro investigation of SNP-associated genes identifies SLC1A1 and SLIT3 as major regulators of the Mtb-induced cytokine response.

siRNA knockdown of SLC1A1 and SLIT3 expression was utilized to determine effect on (A) IL1B, IL6, and TNF secretion measured by ELISA after 24 hours of Mtb infection (MOI=1) and (B) IL1B, IL6, and TNF mRNA expression after 6-hour Mock and Mtb-infection. (C) Multiple doses of purified hSLIT3 N and C-terminal fragments were tested for effect on IL1B, IL6, and TNF secretion measured by ELISA after 24hr Mock and Mtb infection in macrophages. Results shown are from 3 biological replicates in 3 human donors per siRNA or treatment. Significance assessed using linear mixed model adjusting for random effect of donor followed by ANOVA and pairwise comparisons. Individual data points plotted according to model calculated values adjusted for donor intercept. Significance determined as (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Individual donor values are presented in Figures S4.9, S4.10, and S4.12.

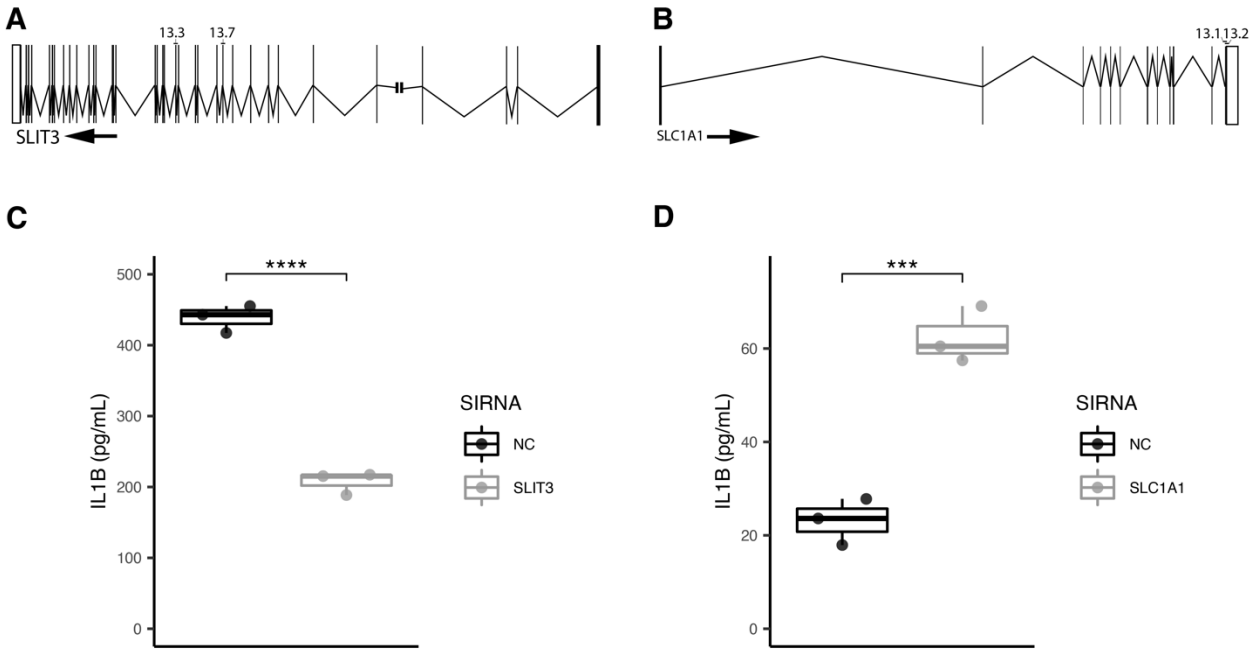

Figure 4.4. Targeted siRNA locations and validation of additional siRNA effect on Mtb-induced IL1B protein secretion.
(A) The targeted location of both the SLIT3 initial (siSLIT3.13.3) and validation (siSLIT3.13.7), and the (B) SLC1A1 initial (siSLC1A1.13.1) and validation (siSLC1A1.13.2) siRNA sequences are shown with their exonic location in their corresponding gene. (C) The effect of the SLIT3 validation and (D) SLC1A1 validation siRNA show consistent effect on Mtb-induced IL1B protein secretion when repeated with a unique siRNA sequence. Results shown are from 3 biological replicates in 1 human donor per siRNA or treatment. Significance assessed using simple linear model. Significance determined as (*** $P < 0.001$, **** $P < 0.0001$).

4.4.3 *Exogenous application of N- and C- terminal hSLIT3 increases the Mtb-induced cytokine response*

We next assessed if extracellular application of purified SLIT3 regulated cytokine induction. SLIT3 is cleaved into an N and C-terminal component which bind to the ROBO and PLEXIN A receptors respectively [166–169]. Two of the four ROBO family receptors, ROBO1 and ROBO3, as well as four PLEXIN A family receptors were found to be expressed in human monocytes (Figure 4.5). When N- and C-terminal SLIT3 were co-applied, Mtb-induced IL1B and TNF protein secretion was significantly increased (Figure 4.3C, $P < 0.05$). For Mtb-induced TNF, a significant effect is seen with N-SLIT3 pretreatment alone, and this was amplified when both fragments were applied. In the uninfected conditions, SLIT3 pretreatment induced IL6 and TNF, but not IL1B secretion, with an effect that was largest with co-treatment of both the N- and C-terminal fragments (Figure 4.3C, $P < 0.0001$). However, in contrast to Mtb-induced TNF, only the C-SLIT3 fragment led to a significant increase in IL6 secretion baseline with the N-terminal having no significant effect. Taken together, these results indicate that receptors for both the N- and C-terminal of SLIT3 are likely to be present in human MDMs and that N- and C-terminal SLIT3 treatment creates distinct effects on the cytokine response which are amplified when applied simultaneously.

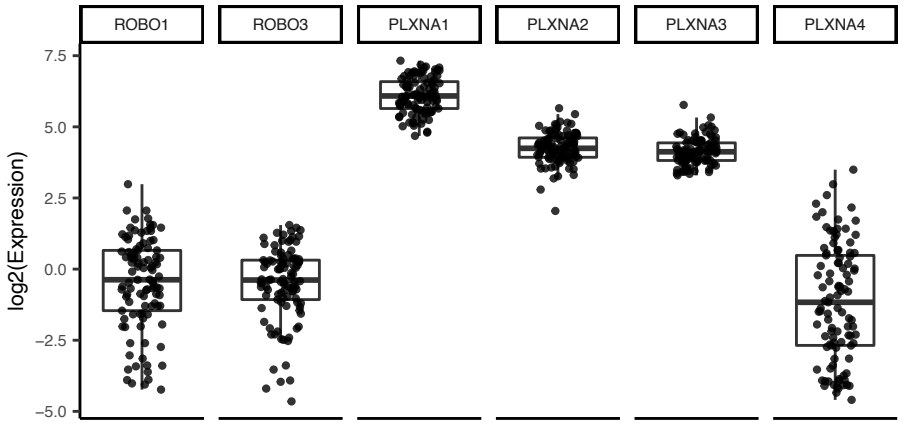

Figure 4.5. Expression of queried SLIT3 receptors.
Expression of queried genes with detectable expression in RNAseq for the ROBO and PLEXIN A family members. Receptors with observed expression were plotted as log2(counts per million). ROBO2 and ROBO4 expression was removed in rare gene filtering due to low expression values across samples.

4.4.4 *SLIT3 N- and C-terminal fragments induce increase in Mtb replication in MDMs*

We next assessed if SLIT3 had an effect on other Mtb-response macrophage phenotypes including phagocytosis and intracellular replication. Utilizing a microscopy-based assay measuring surface area of fluorescently labeled Mtb, we quantified relative abundance of macrophage associated Mtb at four hours post infection as well as growth over a 72-hour period. No significant differences in phagocytic uptake were seen between any SLIT3 treatment or vehicle treated control after 4 hours (Figure 4.6, Figure S4.12). However, the Mtb fluorescence surface area was increased in the N- and C-terminal co-treated samples at both the 48- and 72-hour timepoint (Figure 4.6, $P < 0.05$). The N-terminal only pretreatment had a trending but non-significant effect on growth at 72 hours whereas the C-terminal only pretreatment had no significant effect on Mtb growth at any timepoint. Overall, these results reveal that co-application of SLIT3 N- and C-terminal fragments has a significant effect on intracellular replication of Mtb that is not due to initial differences in phagocytic uptake.

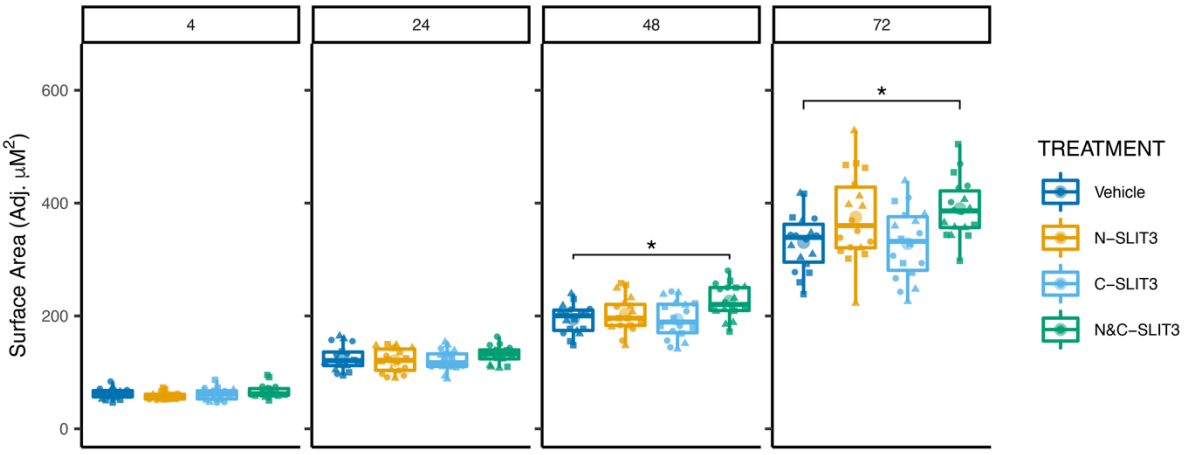

Figure 4.6. Evaluation of SNP-associated gene effect on other Mtb-response phenotypes reveals SLIT3 effect on intracellular Mtb
Intracellular Mtb replication in human macrophages assessed after hSLIT3 N- and C-terminal and co-treatment using fluorescence microscopy to measure Mtb-mCherry surface area within each well over 72 hours. 4-hour timepoint quantifies differences in initial phagocytic uptake and shows no significant differences between groups. Results shown are from 6 biological replicates in 3 human donors. Significant differences were evaluated at each timepoint and show a significant increase in intracellular Mtb replication with co-treatment of N and C terminal SLIT3 at 48 and 72 hours. Significance was assessed using a linear mixed model adjusting for random effect of donor followed by ANOVA and pairwise comparisons for each timepoint. Individual data points plotted according to model calculated values adjusted for donor intercept. Significance determined as *(* $P < 0.05$)*. Individual donor values are presented in Figure S4.12.

4.4.5 *Association of genetic variants with clinical TB phenotypes*

Since the Mtb-induced cytokine response plays diverse roles in modulating the spectrum of clinical TB progression, we next explored if our SLC1A1 and SLIT3 loci were associated with clinical TB phenotypes. We assessed four clinical phenotypes ranging in severity including TST/IGRA conversion, pulmonary TB (PTB), tuberculous meningitis (TBM), and TBM survival. Association with TST/IGRA conversion was tested using the SLC1A1 lead SNP (rs10974620) and SLIT3 lead SNP (rs79380271) in the previously discussed Ugandan cohort. PTB, TBM, and TBM survival association was similarly tested in the previously discussed Vietnam cohort using the SLIT3 Seattle lead SNP (rs62378623) due to lack of the rs79380271

genotype in the Vietnam population. Although no significant association was found for TST/IGRA conversion or PTB, both SNPs were associated with TBM phenotypes. The SLC1A1 SNP, rs10974620, was associated with higher susceptibility to TBM (odds ratio [OR], 1.238; 95% CI, 1.037-1.478; $P = 0.018$) (Table 4.1). Additionally, TBM mortality was higher among individuals with the SLIT3 SNP, rs62378623, T/C genotype (6/9, 66.6%) versus the T/T genotype (57/360, 15.8%; $P = 1.05 \times 10^{-4}$). Furthermore, evaluation of surrounding SNPs within each locus identified many SNPs in high LD that also showed significant association (Figure S4.13). Together, these data indicate that the genomic loci within SLC1A1 and SLIT3 that are associated with Mtb-induced cytokine expression are also associated with the severe clinical TB phenotypes, TBM and TBM survival.

Table 4.1. Lead SLC1A1 and SLIT3 genetic variant clinical phenotype association

Pulmonary TB:

		Genotype Frequency			
SNP	**Genotype**	**Case (PTB)**	**Control**	**OR (95% CI)**	***P* value**
rs10974620	C/C	0.336	0.377	1.083 (0.959, 1.224)	0.199
	C/G	0.484	0.451		
	G/G	0.180	0.172		
rs62378623	T/T	0.977	0.981	0.987 (0.579, 1.684)	0.963
	T/C	0.029	0.019		
	C/C	-	-		

TBM:

		Genotype Frequency			
SNP	**Genotype**	**Case (TBM)**	**Control**	**OR (95% CI)**	***P* value**
rs10974620	C/C	0.313	0.378	1.238 (1.037, 1.478)	0.018
	C/G	0.492	0.455		
	G/G	0.194	0.167		
rs62378623	T/T	0.980	0.985	1.525 (0.649, 3.584)	0.333
	T/C	0.020	0.015		
	C/C	-	-		

TBM survival:

		Genotype Frequency			
SNP	**Genotype**	**Survival (No)**	**Survival (Yes)**	**OR (95% CI)**	***P* value**
rs10974620	C/C	0.318	0.325	-	0.758
	C/G	0.485	0.481		
	G/G	0.197	0.193		
rs62378623	T/T	0.905	0.990	-	1.05×10^{-4}
	T/C	0.095	0.010		
	C/C	-	-		

4.5 DISCUSSION

Few genetic variants identified in previous GWAS have shown transferability in independent populations. Using the variants identified in our previous cellular GWAS approach, we identified genetic regulators of the macrophage Mtb response with population spanning effect, validated two of these *in vitro*, and linked them to other macrophage Mtb response and

clinical phenotypes. Our findings support the use of a cellular GWAS to identify novel host regulators of TB pathogenesis with clinical consequences.

In order to characterize SNPs with a more global impact, we examined an independent Seattle population cohort and identified four loci associated with Mtb-induced cytokine expression with consistent directionality of effect in both populations. The effect on Mtb-induced IL1B levels was validated for two of these loci, SLC1A1 and SLIT3, with SLIT3 showing an additional effect on IL6 and TNF secretion. SLIT3 has been implicated in cytokine regulation previously where it showed a similar proinflammatory effect in human gestational tissue [142]. However, another SLIT family member, SLIT2 has also been implicated in anti-inflammatory actions through its interactions with the ROBO4 receptor in endothelial cells [170]. This suggests that SLIT family members may have different functions in the cell or that a combination of receptors present on each cell type can result in a distinct response. We detected expression of ROBO1 and ROBO3 in monocytes while ROBO4 was absent. As such, SLIT3-ROBO interaction could have a distinctly proinflammatory effect in human macrophages. Although the SLIT3 N-terminal fragment interaction with the ROBO receptor has been previously described, the C-terminal fragment was more recently discovered to bind the PLEXIN A receptor family [169]. PLEXIN A4 has previously been implicated in regulation of TLR-induced cytokine signaling in myeloid cells, and in a prior cellular GWAS association of TNF response to Mtb purified protein derivative (PPD) [101,171]. To our knowledge, this is the first time that SLIT3 regulation of the cytokine response in human macrophages has been investigated, and the first to identify simultaneous effects of SLIT3 N- and C-terminal on cytokine production. ROBO1 receptor binding is largely thought to act through downstream RHOA GTPase activation whereas PLEXIN A receptor binding often acts through RAC1 GTPase activation [168,171]. Although

the mechanistic details have not been clarified in this study, it is intriguing to postulate that by targeting both of these pathways simultaneously, SLIT3 can exert an increased response, and further adds to the complexity that SLIT3 could have on a cell-to-cell basis depending on the receptors it expresses. We additionally identified that SLIT3 was also associated with increasing intracellular Mtb replication. Interestingly, SLIT2, was recently found to be actively upregulated during Mtb infection and lead to differences in host oxidative responses [172]. In contrast, we found that SLIT3 was extensively downregulated during Mtb infection (Figure S4.11). Lastly, we identified a genetic link between SLIT3 variants that regulate the Mtb-induced cytokine response and differences in TBM survival. In addition to the currently identified role in cytokine regulation, SLIT3 has also been implicated in modulating monocyte chemotaxis [168]. As such, SLIT3 could modulate TBM survival through differential cytokine response, control of Mtb replication, immune cell recruitment, or a combination of the three. Overall, this data suggests that SLIT ligands may regulate important steps in TB pathogenesis.

We additionally identified SLC1A1 as a major regulator of Mtb-induced IL1B levels. SLC1A1 is a glutamate and aspartate transporter and modulates glutamate levels in neuronal tissue. Amino acid transporters have been reported to have major effects on cytokine production and previous cytokine cellular GWAS studies identified an amino acid transporter, SLC36A4, in *Staphylococcus aureus*-induced IL-22 levels [99]. Modulation of aspartate transport and glutaminolysis through glutamate transport has previously been implicated to have major effects on the macrophage response [173,174]. Mice with heterozygous SLC1A1 deletion have different cytokine profiles and neuroinflammation compared to wild type [143]. The lead SNP identified in our study, rs109746720, has been identified in previous genetic studies to have a significant association with posttraumatic seizure and a SNP in high LD, rs7858819, was found to be an

eQTL increasing SLC1A1 expression [175,176]. Although we were unable to confirm rs109746720 as a monocyte eQTL in our cohort, (possibly limited by sample size with no homozygotes in Uganda), SLC1A1 expression changes associated with this SNP could play a role in its observed phenotypes. We also found that rs10974620 was associated with susceptibility to TBM in a Vietnam cohort. Given SLC1A1's effect on neuroinflammation in mice, it is possible that SLC1A1 regulation of the cytokine response could play a significant role in meningeal inflammation in TBM. Importantly, the identification of significant clinical associations with variants identified in this cellular GWAS approach highlights its ability to lead to variants with clinical impact.

The approach we utilized has several limitations. First, similar to our initial cellular GWAS approach, the sample size of our Seattle cohort was limited which limited our ability to validate transferability within this population. Although we were able to find multiple SNPs which surpassed significance at a nominal threshold of significance, $P < 0.05$, only one SNP was found to surpass multiple correction. Often, SNP significance was limited due to lack of individuals who were homozygous for the uncommon allele. As such, SNPs falling into this category could not demonstrate the same effect sizes as were initially identified in the Uganda cohort. We sought to overcome this limitation by performing *in vitro* validation of genes associated with our transferrable loci. Second, since our aim was to capture the early Mtb-induced myeloid cell response, we chose to only focus on monocyte and macrophage expression of four cytokines at an early 6-hour timepoint for our initial GWAS and subsequent Seattle cohort. Future studies could benefit from additional cytokines, time points, cell types, and assessment of cytokine protein levels. We attempted to mitigate this impact by also assessing 24 hour cytokine protein secretion levels in follow up *in vitro* studies and incorporating clinical

phenotypes which would likely be due to many different factors. Lastly, we could not fully identify the mechanism by which SNPs within a locus were creating causal effects. We performed thorough functional annotation, eQTL, and mQTL analyses, however, we were unable to decisively determine the causal SNPs within a loci. Due to the intronic location of the SNPs within the SLIT3 and SLC1A1 gene, we were able to propose these as lead candidate regulated genes and validate their effect *in vitro*. However, further *in vitro* analysis specifically recreating candidate variants would be necessary to make definitive genetic claims and specify if they specifically effect SLC1A1, SLIT3, or adjacent genes.

In summary, we used an independent Seattle cohort to characterize the major population spanning genetic regulators of the Mtb-induced cytokine response in myeloid cells and identified genes which may have an important role in Mtb response biology. Further defining the mechanisms by which these genetically regulated genes control Mtb-induced cytokine infection could yield important insights in our understanding of differences in host disease progression and increase our ability to respond to the global burden of TB.

4.6 SUPPLEMENTAL FIGURES

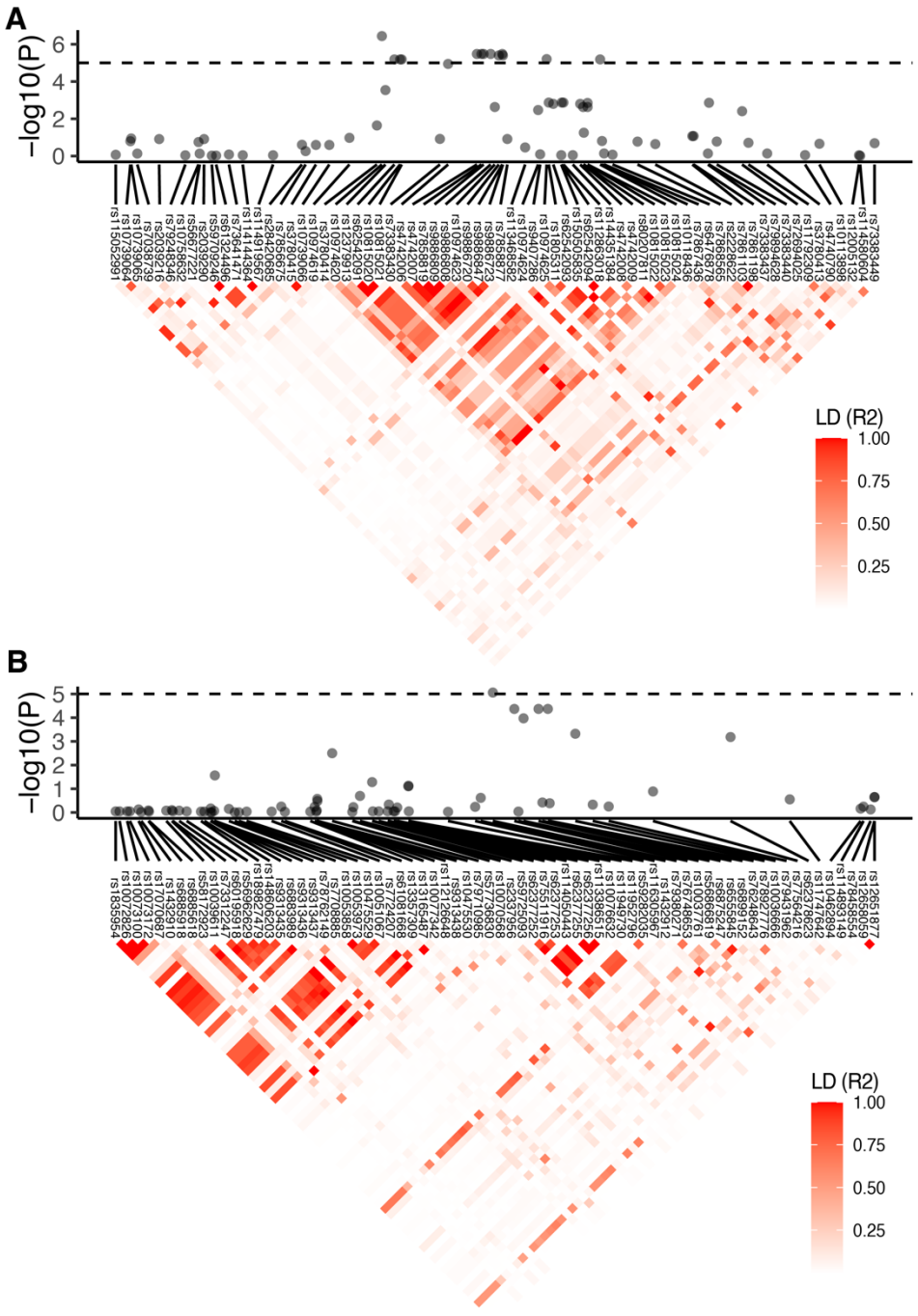

Figure S4.7. LD plots of the SLC1A1 and SLIT3 loci in Uganda.
P-values of SNP associations with Mtb-induced IL1B expression are plotted with corresponding LD heatmap for the (A) SLC1A1 and (B) SLIT3, (C) SLIT3 loci in the Uganda population. Multiple SNPs in linkage with the lead SNPs (rs10974620, rs79380271) show some association with many SLC1A1 loci SNPs showing suggestive significance ($P < 1x10^{-5}$).

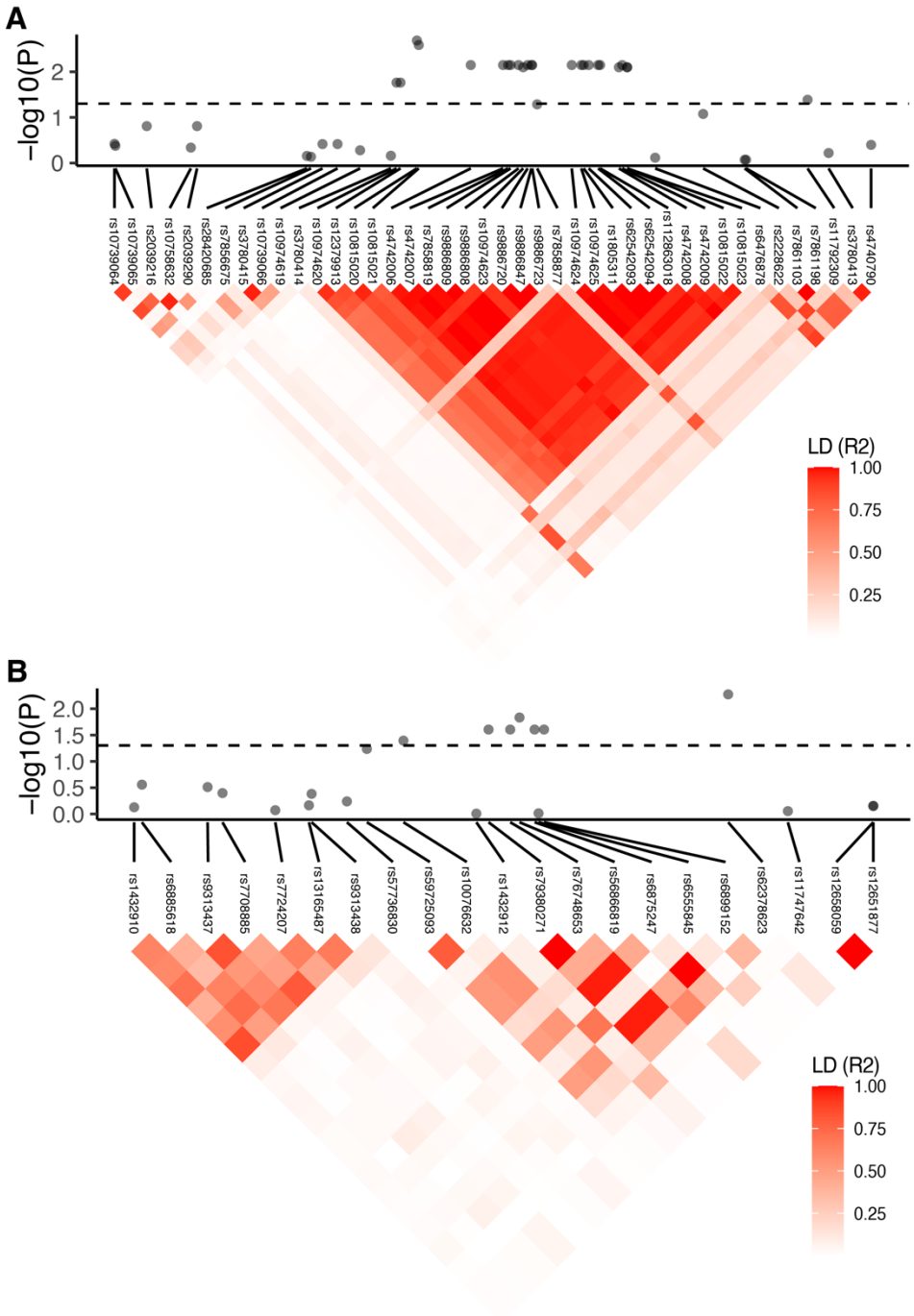

Figure S4.8. LD plots of the SLC1A1 and SLIT3 loci in Seattle.
P-values of SNP associations with Mtb-induced IL1B expression are plotted with corresponding LD heatmap for the (A) SLC1A1 and (B) SLIT3, (C) SLIT3 loci in the Seattle population. Despite differences in LD patterns between populations, multiple SNPs in linkage with the lead SNPs of each locus show significant associations in both populations.

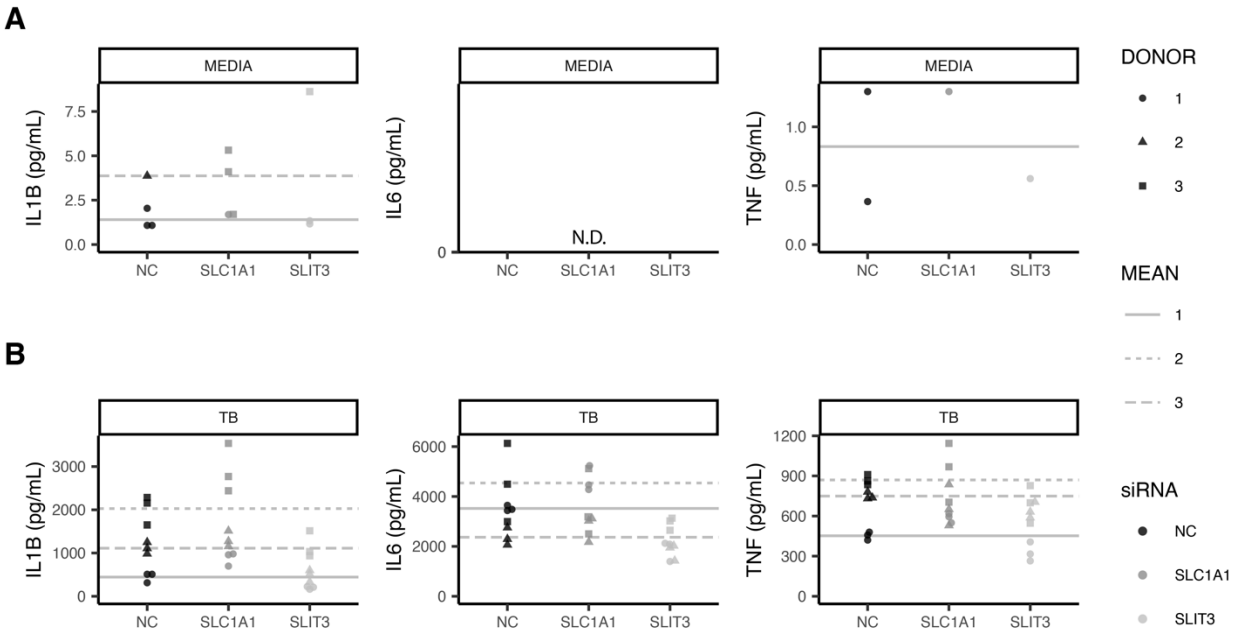

Figure S4.9. Unadjusted results of Mtb-induced cytokine secretion in the SLIT3 and SLC1A1 siRNA *in vitro* experiments.

Results for each *in vitro* experiment are plotted without adjustment for donor as a random effect. Dot shapes represent 3 different human donors and mean lines show the mean of 3 independent stimulations or infections per donor for the control condition. (A) Cytokine protein secretion for the media only, and (B) live Mtb stimulation conditions for the SLC1A1 and SLIT3 siRNA sequences compared to Negative control siRNA. N.D. indicates cytokine concentration was below determinable range by ELISA. Results show consistent differences in cytokine production for significant outcomes within donor infections despite baseline differences in control means between donors. Media values are undetectable for many samples and cannot be analyzed for significance in main analysis. Adjustment of donor as a random effect allows for significance assessment and visualization of consistent biological effect across individual infections and donors despite baseline mean differences.

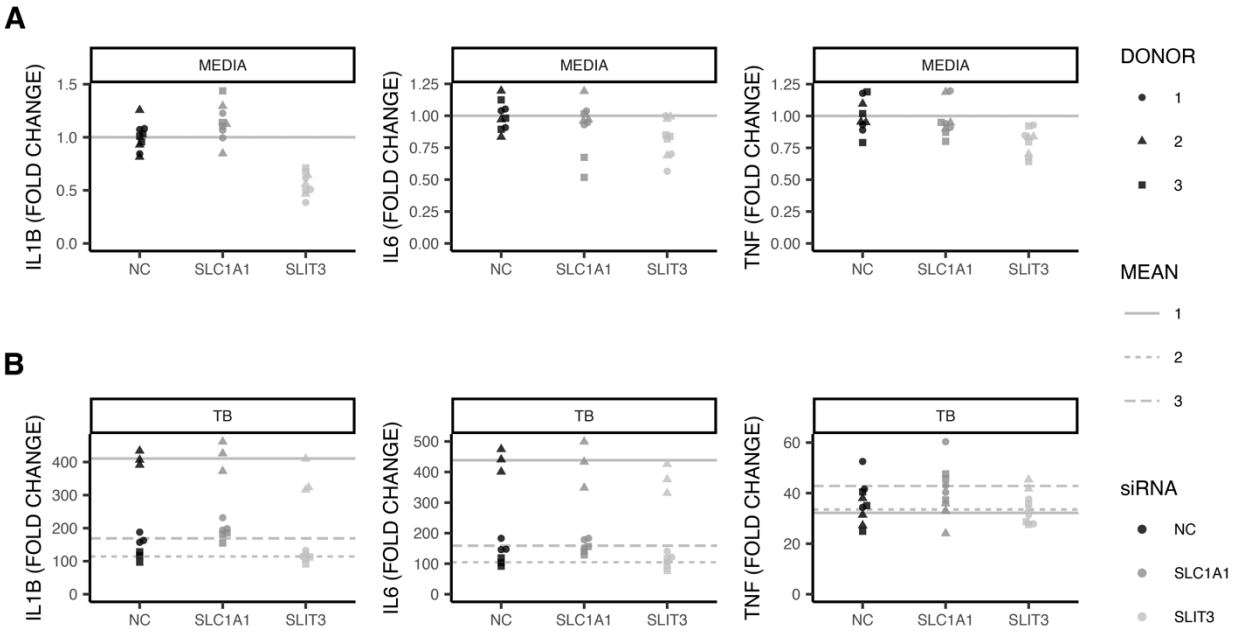

Figure S4.10. Unadjusted results of Mtb-induced cytokine expression in the SLIT3 and SLC1A1 siRNA *in vitro* experiments.

Results for each *in vitro* experiment are plotted without adjustment for donor as a random effect. Dot shapes represent 3 different human donors and mean lines show the mean of 3 independent stimulations or infections per donor for the control condition. (A) Cytokine expression for the media only, and (B) live Mtb stimulation conditions for the SLC1A1 and SLIT3 siRNA sequences compared to Negative control siRNA. Expression indicated by fold change of expression from mean of negative control samples per donor after normalized to GAPDH within sample. Results show consistent differences in cytokine production for significant outcomes within donor stim conditions despite baseline differences in control means between donors. Adjustment of donor as a random effect allows for significance assessment and visualization of consistent biological effect across individual infections and donors despite baseline mean differences.

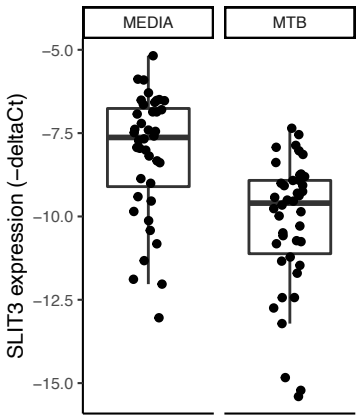

Figure S4.11. Expression of SLIT3 in monocyte derived macrophages according to stim condition.

Expression of SLIT3 with Mtb and Media stimulation assessed by qPCR shows significant decrease in SLIT3 expression after Mtb stimulation after samples have been normalized to GAPDH.

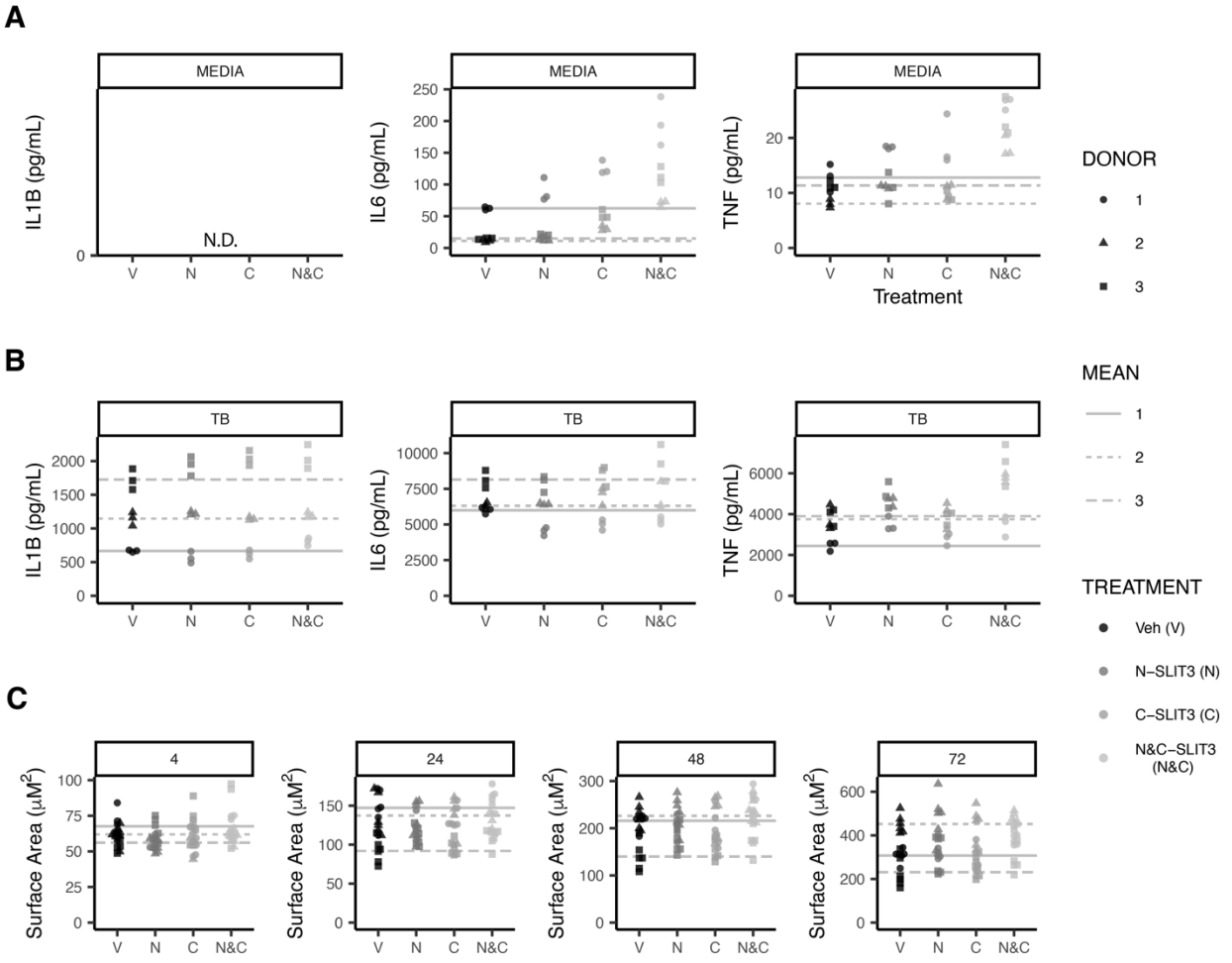

Figure S4.12 Unadjusted results of Mtb-induced cytokine secretion and intracellular replication in the exogenous SLIT3 pretreatment *in vitro* experiments.
Results for each *in vitro* experiment are plotted without adjustment for donor as a random effect. Dot shapes represent 3 different human donors and mean lines show the mean of 3 independent stimulations or infections per donor for the control condition. (A) Cytokine protein secretion for the media only and (B) live Mtb stimulation conditions are plotted after N-SLIT3 (N), C-SLIT3 (C), and N&C-SLIT3 (N&C) pretreatment compared to Vehicle only (V) control. (C) Mtb-mCherry surface area within infected macrophages at 4, 24, 48, and 72 hour post infection timepoints after N-SLIT3 (N), C-SLIT3 (C), and N&C-SLIT3 (N&C) pretreatment compared to Vehicle only (V) control. Results show consistent differences in cytokine production and control of intracellular Mtb replication for significant outcomes within donor infections despite baseline differences in control means between donors. Adjustment of donor as a random effect allows for significance assessment and visualization of consistent biological effect across individual infections and donors despite baseline mean differences.

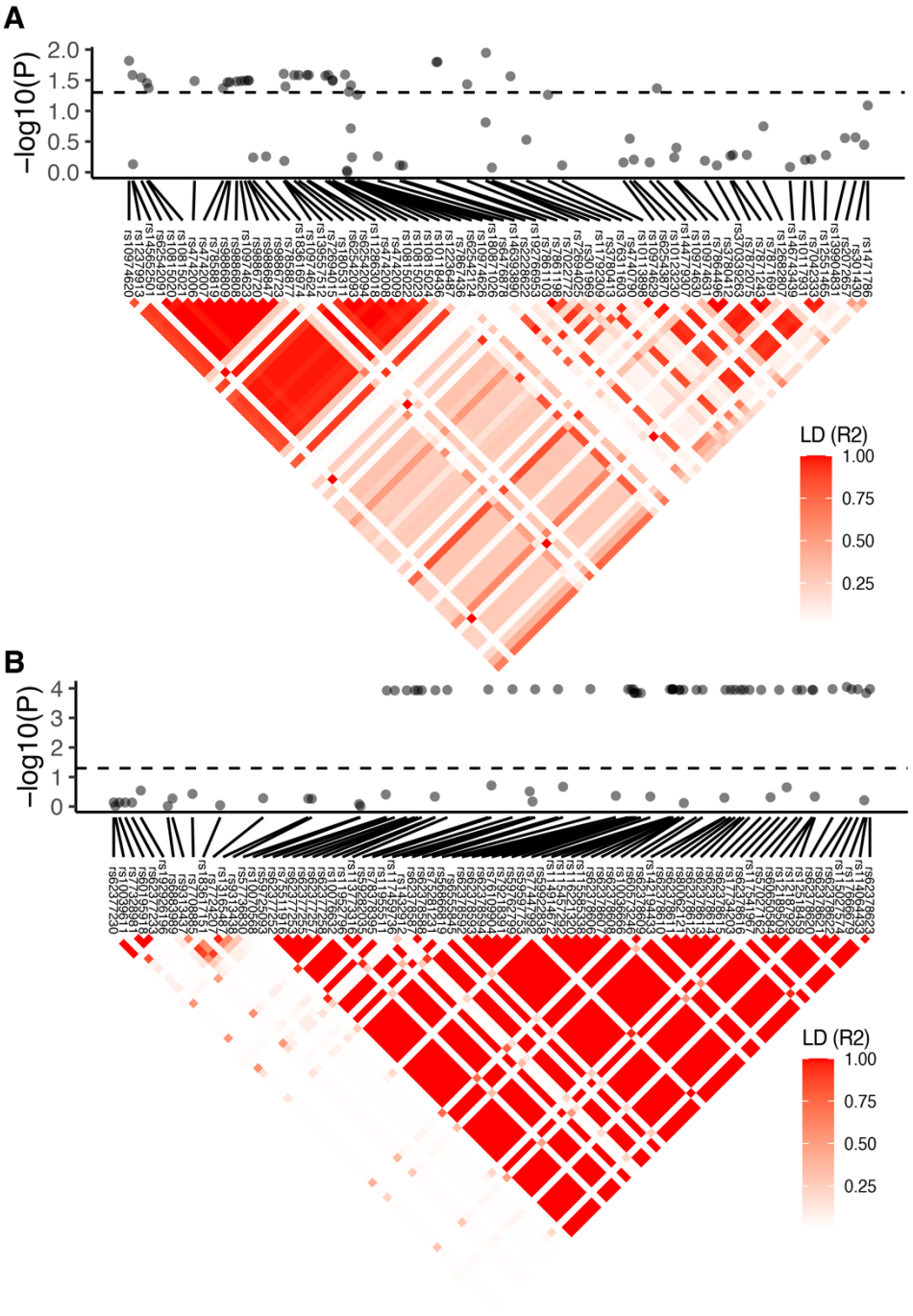

Figure S4.13. LD plots of the SLC1A1 and SLIT3 loci in Vietnam.
P-values of SNP associations with traits are plotted with corresponding LD heatmap for (A) SLC1A1 TBM association and, (B) SLIT3 TBM survival association within the Vietnam population. Significant SNPs for the SLC1A1 and SLIT3 loci are in high LD with many adjacent SNPs which also show similar levels of significance.

4.7 SUPPLEMENTAL TABLES

Table S4.2. Demographic tables for the Seattle *in vitro* and Uganda and Vietnam clinical cohorts

	Seattle HD (n = 40)	Uganda HHC cohort (n = 263)		Vietnam PTB Cohort (n = 2005)	
	Healthy (n = 40)	RSTR (n = 74)	LTBI (n = 189)	PTB (n = 1,598)	TBM (n = 407)
Gender					
F (%)	15 (40)	38 (51.4)	87 (46)	417 (26%)	160 (44%)
M (%)	25 (60)	36 (48.6)	102 (54)	1,181 (74%)	200 (56%)
Age					
Mean (SD)	36.9 (13.5)	22.5 (8.8)	24.0 (8.9)	39.3 (13.8)	42.5 (17.8)
Median	31.5	Not reported	Not reported	39	40
[Min, Max]	[20, 67]	[14, 66]	[14, 66]	[12, 92]	[15, 93]
Race					
Asian (%)	6 (15)	0 (0)	0 (0)	1594 (99.7)	-
Black (%)	0 (0)	100 (100)	100 (100)	0 (0)	-
Pacific Islander (%)	1 (2.5)	0 (0)	0 (0)	0 (0)	-
White (%)	26 (60)	0 (0)	0 (0)	0 (0)	-
Mixed (%)	3 (7.5)	0 (0)	0 (0)	0 (0)	-
Other	4 (10)	0 (0)	0 (0)	4 (0.3)	-

Chapter 5. Conclusions

5.1 SUMMARY AND IMPLICATIONS

The global burden of TB remains at unacceptable levels despite decades of significant efforts [2]. Response options are currently limited to a vaccine with variable efficacy and multi-antibiotic regimen dosing over multiple months [9]. Levels of antibiotic resistance and lost progress during the COVID19 pandemic indicate that future reduction in TB burden are not guaranteed [2]. As such, it is important that we investigate novel methods to help us combat TB globally. In particular, developing a greater understanding of the host response is of crucial importance. Certain individuals appear to be predisposed to preventing disease despite heavy exposure to Mtb, yet the mechanisms behind this remain undefined. Understanding which host response pathways lead to beneficial and detrimental outcomes can facilitate all TB approaches, allowing us to increase vaccine efficacy, and design host directed therapies which can assist current antibiotic regimens. This has been shown previously in studies of LTA4H mutations after found to be associated with clinical differences in TBM [159]. Due to the increased inflammatory burden in individuals with this mutation, administration of dexamethasone was found to play a significant role in patient survival that was dependent on LTA4H (rs17525495) genotype [177]. Further genetic research could identify similar candidate genes and pathways which have relevance across individuals and populations. Furthermore, by focusing on genetic characterization of *in vitro*, clinically important intermediate phenotypes, causal factors can be identified, validated, and mechanistically investigated. The hypothesis of this dissertation was that Mtb-induced cytokine expression in monocytes and macrophages is genetically regulated and can be discovered using a cellular GWAS approach. This project sought to define the Mtb-induced cytokine response in its relation to the overall Mtb-induced expression response and

define its genetic regulation (Chapter 2). We then defined the major genetic variants and pathways associated with inter-individual variation in Mtb-induced cytokine expression (Chapter 3). Finally, we identified variants which showed transferability in an independent cohort, validated results of their associated genes *in vitro*, and investigated their effect on other Mtb-response and clinical phenotypes (Chapter 4).

The first chapter of this dissertation identified multiple gene coexpression modules and the hub genes within these modules which were related to the Mtb-induced cytokine response. Mtb infection represents a substantial stimulation for a macrophage resulting in expression changes of over 20% of protein coding genes. Understanding how all of these gene expression changes function as a whole can help link the cytokine response to the total Mtb response. Using WGCNA, we identified two modules which were positively correlated with Mtb-induced IL1B expression and four modules which were negatively correlated. Go term enrichment analysis identified that modules associated with overall response to a stimulus were positively correlated whereas negatively correlated modules were enriched for DNA transcription factor activity and localization to the mitochondria. Although informative, the substantial module size led to these results being broad and limited ability to make definitive claims. As such we performed a thorough hub gene analysis with independent population validation, which identified 35 genes which were central to the response of these modules. Remarkably, 66% of the hub genes initially identified within our Ugandan cohort were shared in the South African cohort which supports that important elements of the Mtb-induced cytokine response may be shared between populations. These 35 hub genes were further centered around STAT3, and enriched for genes of the NFkB signaling pathway. Future genetic studies may benefit from this information since variants which perturb these hub genes and pathways will be known to effect central elements of

the overall response to Mtb. For example, SLIT3 was identified as a major regulator of Mtb-induced IL1B, and SLIT3 N-terminal ROBO1 interaction is known to lead to RHOA activation and subsequent STAT3 activation whereas SLIT3 C-terminal PLEXIN A interaction is known to lead to RAC1 activation which can effect NFkB [168,171,178]. As such, through the dual terminal effect of SLIT3, it may be able to simultaneously effect two of the major elements central to the Mtb-induced cytokine response. Further mechanistic research in this area may be warranted.

Additionally, we performed heritability estimates of the Mtb-induced cytokine response and identified that 32.2% of Mtb-induced IL1B expression and 9.2% of Mtb-induced IL6 expression was heritable. Although confidence intervals limit result confidence, it is interesting to note that the number of loci which surpass suggestive significance directly align with the projected heritability (IL1B = 51, IL6 = 24, TNF/IFNB1 = 6). Interestingly, our lead SLC1A1 variant, rs10974620, was found to explain a substantial amount of this heritability at 27.53% of variability in IL1B expression. This suggests that the use of a quantitative, *in vitro* phenotype that is directly measurable, may be an efficient method to detect genetic variation which explains a large portion of heritability.

The second chapter of this thesis sought to identify the major genetic regulators of Mtb-induced cytokine expression within the Ugandan population. In total, 77 loci and 2 pathways were identified to surpass thresholds of significance to warrant further investigation. Although no loci surpassed genome-wide significance, functional annotation revealed that many loci showed promise for potential to have causal effect. Of the 77 total loci, 44 mapped to protein coding genes within 20kb. Interestingly, 4 of these 44 genes (NEDD4, F13A1, RBFOX1, and SLIT3) have been mentioned in previous clinical TB GWAS studies [90,91,179]. Although

variants within these genes only reached suggestive significance, this points to the promise of the cellular GWAS for identifying genes with clinical importance. Obtaining genome wide significance has often proved difficult for clinical TB phenotypes. Potentially using this cellular GWAS approach to limit candidate genes could decrease multiple corrections burden and lead to more identifiable results. As such, we may be able to find previously unidentified genetic variation responsible for the missing heritability of these clinical phenotypes. Although we did not perform thorough *in vitro* validation of the genes associated with Uganda specific loci, many of these show promise for future efforts. In particular, the NEDD4 locus shows promise, containing many SNPs with high CADD scores, eQTL evidence, and NEDD4 has been shown to effect IL1B secretion in response to nigericin, and Mtb replication in THP1s [147,180]. Additionally, the IFNAR2 locus has previously been associated with effect on IFNAR2 protein levels, and IFNAR2 has also been shown to effect the cytokine response and intracellular replication of Mtb [70,137].

Lastly, within this chapter, we identified significant enrichment of two pathways associated with Mtb-induced TNF expression, including the alpha-linolenic acid metabolism pathway. This pathway and its downstream intermediates have many implications in the context of Mtb infection [47]. LTA4H mutations which can influence leukotriene availability have previously been shown to have major effects on the TNF response [159]. Our results pointed to PLA2 isoforms which act upstream of this in producing arachidonic acid. Validation of these results *in vitro* revealed that PLA2 activity can have dramatic effects on the cytokine response that are different based upon the stimulating Mtb being live and whole or dead and lysed. Although this adds complexity to interpretation, our data suggests that overall availability of

intermediates within this pathway can effect the Mtb-induced cytokine response, not just one particular downstream pathway.

Finally, we identified multiple variants with population spanning effect in an independent Seattle cohort and performed thorough *in vitro* validation, and phenotype investigation. The substantial genetic differences between these populations was underlined by the fact that less than half of our lead SNPs were able to be tested in the Seattle population. As such, variants which showed transferability in this population would have to overcome the burden of a very different genetic background. However, we identified four different loci which showed significant effect at a nominal $P < 0.05$ threshold. Due to the number of lead SNPs tested for each cytokine, 23 for IL1B and 4 for TNF, only the lead SNP (rs16947739) associated with Mtb-induced TNF was significant after multiple correction. Regardless, it is interesting to note that both of the candidate population spanning genes tested for validation *in vitro*, SLC1A1 and SLIT3, showed substantial effects on the Mtb-induced cytokine response. This suggests that the cellular GWAS may be a promising approach to identify real results with an impact on the phenotype used to discover them. Furthermore, SLIT3 regulated intracellular replication of Mtb, and variants of both the SLIT3 and SLC1A1 loci were found to be relevant in clinical TB phenotypes. As such, although only using an intermediate phenotype measuring Mtb-induced cytokine induction, the variants identified are relevant in the broader context of Mtb infection response. Interestingly, both variants of SLC1A1 and SLIT3 were associated with TBM phenotypes. SLC1A1 and SLIT3 have biased expression in the brain and substantial portion of the literature focuses on their role in this area [181]. Although SLC1A1 and SLIT3 expression was observed in monocytes and macrophages, expression levels were lower than found in neuronal tissue. Even at this lower level of expression, we still observed significant cytokine

effect after SLC1A1 and SLIT3 expression knockdown. Potentially, in areas of higher expression, this effect may be even more pronounced and lead to the associated differences in clinical progression identified.

In summary, this project sought to identify the major genetic regulators of the Mtb-induced cytokine response in monocytes and macrophages and investigate their effects. We hypothesized that Mtb-induced cytokine expression was genetically regulated and that the cellular GWAS approach was an effective way to determine causal variants. This study concluded that Mtb-induced cytokine expression was genetically regulated, especially for Mtb-induced IL1B and indicated heritability matched previous literature heritability estimates for these phenotypes. Using a cellular GWAS approach, we identified many candidate variants and pathways which may regulate the Mtb-induced cytokine response, and further *in vitro* efforts consistently validated their effect. We were able to identify variants relevant in multiple populations and these variants were found to be relevant in susceptibility to clinical TBM phenotypes. Although further effort is needed, these studies suggest that Mtb-induced cytokine expression is genetically regulated, and that using a cellular GWAS is an efficient mechanism to identify causal variants which may be important in the context of the host response to Mtb.

5.2 Limitations and Future directions

5.2.1 *Cohort sample size*

There are several limitations to the work performed in this dissertation which may be addressed in subsequent research efforts. Although addressed individually in the previous chapters, one major limitation in our studies was that they were often under powered, limiting result significance and our ability to make definitive conclusions from results. Even though there are numerous benefits to using the cellular GWAS approach that have been previously discussed,

this approach requires *in vitro* characterization which substantially increases the burden required to obtain data from each individual included. As such, our cohort sizes were limited, ranging from 40 to 100 individuals for our Uganda, Seattle, and South Africa cohorts. We were limited in our ability to increase our discovery samples sizes due to donor availability within the Ugandan cohort.

For our heritability calculations, this resulted in wide confidence intervals with standard deviations spanning more than 50%. As such, we could not make definitive heritability estimates. This is not something unique to our study, and multiple heritability estimates of similar phenotypes have shown similar issues in their work even with sample sizes of hundreds of individuals [98,114]. These studies have overcome these difficulties by showing concordance of heritability estimates using multiple methods. Although not performed in this study, future efforts could follow this approach, and use self-indicated family structure within the Ugandan cohort to calculate this effect. Additionally, heritability estimates could be compared with those in the Seattle cohort to see if they correlate and heritability is consistent across populations.

Our limited power additionally resulted in minimal ability to determine significance with our Uganda discovery GWAS approach. Our SLC1A1 lead variant, rs10974620, explained 27.53% of the variation in Mtb-induced IL1B expression, even though it only had an indicated heritability of 32.2% total. Even with this level of effect, we did not surpass genome-wide significance, suggesting our power was too small to detect genetic signal even with large effect sizes. Although not investigated in this dissertation, we have profiled an additional 55 donors within the Seattle population and are currently working on RNAseq characterization, and genotyping of the entire cohort. Future efforts with these samples may allow for subsequent investigation with greater power. Furthermore, we will investigate the possibility of utilizing a

meta-analysis approach in which significance is determined based on the combined contribution from both cohorts. As such, we are focusing on variants that show population spanning effect, and increasing our power to detect variants within the Uganda population without adding additional Uganda samples.

5.2.2 *In vitro investigation*

Our study was also limited by the methods used during *in vitro* investigation. Validation of identified genetic associations is seldom performed and greatly lacking in the field. As such, we wanted to confirm that genes associated with our candidate loci were having causal effects when recreated *in vitro*. In order to assess multiple genes and assess this effect in a relevant cell type, we optimized and employed a siRNA knockdown approach in human macrophages. After differentiated, human macrophages no longer reproduce which limits ability to perform meaningful, lasting, genetic manipulation. However, using an siRNA approach limited us to investigation of genes with sufficient expression to knockdown by siRNA and to short term phenotypes that could be measured during the transient period of expression perturbation introduced by siRNA. As such, we could not assess the gene KSR2, which was associated with our only SNP that passed multiple correction in the population spanning analysis (rs16947739). KSR2 was found to have nearly undetectable expression as assessed by RNAseq in our Uganda cohort, eliminating siRNA knockdown as a viable validation option. Future research efforts could focus on transient overexpression of KSR2 in human macrophages and would be necessary to validate if this gene is a possible effector mechanism of the genetic variants identified.

Additionally, as previously mentioned, siRNA limited the experiments which we could investigate mechanistically. Using our siRNA approach, we identified consistent knockdown of gene expression at 48 hours after nucleofection exceeding 70%. However, siRNA knockdown

efficiency can often fall off at later time points, or be overcome by compensatory mechanisms in response to a strong stimulus such as Mtb infection. As such, we were unable to use siRNA knockdown to measure effect on Mtb replication. We overcame this limitation for SLIT3 by using exogenous application of purified N- and C-SLIT3 protein fragments. However, for SLC1A1, we were unable to test for any phenotypic effect. Subsequent research efforts could focus on overexpression of SLC1A1 and SLIT3 which may result in expression perturbation for a longer period, or genetic knockout using CRISPR approaches in a replicating cell line.

5.2.3 *Genetic mechanism identification*

Finally, this dissertation did not focus on the mechanism by which candidate causal variants were affecting Mtb-induced cytokine expression. After candidate variants were identified in our initial cellular GWAS screen, we performed extensive functional annotation to try and characterize possible mechanisms by which genetic effect could be occurring. For some loci, this was highly informative, as in the case of the chr 16p13.3 locus which contains a SNP predicted to have detrimental effects on the protein structure of CORO7. However, for the genes SLIT3 and SLC1A1, the mechanism of effect was not clarified. No substantial eQTL effect was identified for either gene and CADD scores only reached a maximum of 10.8. Further investigation of the SLC1A1 and SLIT3 loci identified that the sentinel SNPs of the Uganda and Seattle populations, respectively (rs10974620, rs62378623) were significant methylation quantitative trait loci (mQTL) in monocytes within the iMETHYL database [182]. The SLC1A1 SNP (rs10974620) was located at a potential methylation site, and the C to G mutation could result in loss of methylation at this location. Although the mechanism of mQTL effect for the SLIT3 SNP (rs62378623) was less clear, multiple SNPs in linkage also demonstrated mQTL status and may be the causal SNP. Although these mechanisms may be promising for future

research, the methylation sites for both SLIT3 and SLC1A1 are intronic, and without being able to link this to differences in expression, we are unable to state how this methylation difference would act functionally. However, loss of methylation of SLIT ligands has previously been implicated to have major effects in certain forms of cancer supporting the importance that methylation has in regulating this gene [183]. Future efforts to define the causal mechanisms of these SNPs is an important future direction. If methylation differences are responsible, this could be clarified using targeted demethylation approaches in which a demethylating enzyme attached to a dead-Cas9 (dCas9) and guided to the target region in the genome.

5.3 Final Thoughts

The number of lives lost due to TB disease every year remain at unacceptable levels. Despite significant efforts, we have been unable to reduce the disease burden at a rate close to WHO projected milestones for its end TB strategy [2]. As such, new avenues of research are desperately needed which can lead to new response strategies. This is even more crucial in an era of antibiotic resistance and when current prevention efforts can be disrupted by global pandemics. Utilization of the host response to clear infection is greatly underutilized, and has resulted in only a few clinical trials targeting select pathways [184]. Humans have coevolved with Mtb over 70,000 years of infection, and have likely developed numerous genetic adaptations which can modulate infection outcomes [5]. This is supported by the high genetic effect indicated in familial and SNP based heritability methods [29,85,86,130]. However, we have not identified the causal variants responsible and a lack of mechanistic evaluation has led to little clinical impact.

This dissertation has thoroughly investigated the use of a cellular GWAS approach to uncover novel and previously underappreciated TB response elements. We hypothesized that the Mtb-induced cytokine response was genetically regulated and could serve as an important intermediate phenotype for investigation. This hypothesis was supported with the identification of multiple genetically regulated genes and pathways which were validated with *in vitro* macrophage assays. Furthermore, they were found to be associated with clinical TB response phenotypes. Although the clinical signal was not at a level of genome-wide significance, we were able to limit our multiple correction penalty to only 2 tests, increasing the strength of this claim. By identifying these important variants, and further validating and mechanistically defining them, we can begin to fill in the gaps of missing heritability explaining heterogeneity in clinical TB response. As we uncover the underlying biology modulated by these causal variants, we can then discover essential elements of host response which should be investigated for further research.

This dissertation identified 3 such areas for further research. Regulation of the downstream intermediates of alpha-linolenic acid metabolism has already shown substantial impact on the Mtb response and has led to clinically impactful dexamethasone treatment options in individuals with mutations effecting these pathways [159,177]. This study identified that major effects on the Mtb-induced cytokine response may be seen with differences in the upstream metabolite, arachidonic acid, not just specific downstream intermediates. How arachidonic acid metabolism modulates the host response to Mtb is complex and has been found to display many inconsistencies between studies [184]. However, this data suggests that further definition of this pathway and its effects on Mtb infection are worth investigating.

Additionally, the genes SLC1A1 and SLIT3 were identified as major regulators of the Mtb-induced cytokine response which had significant effects in clinical TBM phenotypes. With further confirmation and mechanistic definition of these results, it could lead to treatment decisions for individuals with these mutations. Furthermore, SLC1A1 and SLIT3 pathways should be further investigated. Metabolic intermediate transport has been identified multiple times to effect the macrophage response [173,174]. Greater understanding of the interplay between changes in metabolite transport and macrophage function are necessary. Lastly, SLIT biology should continue to be further investigated for its effect on the cytokine response and effects on Mtb infection. A recent paper indicated that Mtb acted to increase levels of SLIT2 in order to modulate infection response [172]. Whereas our research indicated that SLIT3 was steeply downregulated in response to Mtb infection. If control of SLIT ligand abundance is a major factor in Mtb infection outcome, therapies targeting these ligands could be a new avenue for host directed therapeutic research.

The research outlined in this dissertation focuses on only one Mtb response element, at one time point, in one cell type. Although we believe the cytokines responses, time point, and cell type we chose were well justified in importance in the context of Mtb infection, many other avenues for future research exist. Further investigation of the numerous other Mtb response phenotypes can only broaden our understanding of the host Mtb response and the heterogeneity of outcomes seen after pathogen exposure. As such, further research in this area is crucial and may hopefully one day lead to an increased ability to respond to the devastating global impact of TB.

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VITA

Joshua Ivie was born in Aurora, Colorado in 1992 and attended and graduated from Evergreen High school in 2011. In 2015, he graduated from the University of Colorado, Boulder with a degree in Molecular, Cellular, and Developmental Biology. During undergraduate studies, he researched endosomal maturation systems in yeast working with Dr. Greg Odorizzi. After graduation, he joined the lab of Dr. Carol Sartorius at the University of Colorado, Anschutz and progressed to researching hormonal regulation of breast cancer. Joshua joined the Pathobiology program at the University of Washington, Seattle in 2019 to pursue a PhD. During his doctoral studies, he completed his dissertation research in the laboratory of Dr. Tom Hawn where he studied genetic regulation of innate immune responses to Mtb infection.