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T cell immunity during co-infection with *Mycobacterium tuberculosis* and *Schistosoma mansoni*

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Abstract

T cell immunity during co-infection with *Mycobacterium tuberculosis* and *Schistosoma mansoni*

By Taryn A. McLaughlin

Tuberculosis (TB) and schistosomiasis are two of the greatest contributors to global health disparity. *Mycobacterium tuberculosis* (Mtb), the bacteria that causes TB, infects nearly one quarter of the world. While most infected people do not develop symptoms and are considered latently infected (LTBI), due to the high prevalence of infection, 10 million people developed TB disease in 2018 alone. In fact, TB results in 1.5 million deaths annually, making Mtb the deadliest pathogen in the world. Schistosomiasis affects 200 million people globally, over 90% of whom reside in Sub-Saharan Africa. While schistosomiasis is not often fatal, *Schistosoma mansoni* (SM), one of five species of schistosome helminths that infects humans, results in an estimated 130,000 deaths annually in Africa.

Classically, the immune response to Mtb and helminths such as SM are characterized by type 1 T cell responses and type 2 T cell responses, respectively. These immune responses have been shown to antagonize one another during mouse models of infection. Furthermore, co-infection with filarial or soil-transmitted helminths has been shown to result in impaired type 1 immunity to Mtb. It is currently unknown whether or not SM also impairs type 1 immunity in TB. Furthermore, the impact of Mtb on immunity to SM has not been evaluated. Due to the geographic overlap of TB and schistosomiasis, it is important to evaluate the immune response during coinfection with Mtb and SM and determine the effect of SM infection on TB disease. We hypothesized that SM infection would impair Mtb-specific type 1 immunity and lead to increased TB disease, but that Mtb would not impact SM immunity.

To address these hypotheses, we used a cohort of SM^- and SM^+ individuals across a spectrum of Mtb infection states from Kisumu Kenya, to conduct a thorough examination of T cell responses to both organisms. We found that while T cell responses to Mtb are preserved during co-infection with SM, T cell responses to SM are impaired in people with Mtb infection. Our findings also highlight a previously unrecognized response of peripheral γδ T cells to SM worms. In addition, we utilized a novel machine learning approach to measure the probability of TB disease due to co-infection with SM. We found that in HIV^+ but not HIV^- individuals, SM was associated with a higher probability of TB disease. Overall, our findings provide novel insights into T cell responses during co-infection and highlight the ability of the immune system to concurrently respond to diverse pathogens.

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"The single biggest threat to man's continued dominance on this planet is the virus"
-Dr. Joshua Lederberg (as seen in the opening of Outbreak)

This journey started 20 years ago with a movie about a virus. I guess it's appropriate it end with one too.

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Table of Contents

Figures and Tables

Chapter 1 Introduction

1.1 Tuberculosis

Tuberculosis (TB) is an ancient disease that has impacted humans for thousands of years. Paleo-microbiological studies have identified TB as the cause of death in the remains of ancient Egyptian mummies (1,2). The symptoms of TB have been described repeatedly throughout human history with references in the Old Testament and ancient Greek literature (3). It has gone by many names including: schachepheth, phthisis, consumption, and the white plague (4). These names are rooted in the symptomology of the disease, which includes wasting, loss of color, fatigue, fainting, and coughing blood (5). During the 18th century, these symptoms became inextricably entangled with the fashion choices and lifestyles of the elite in Europe. Writers such as Emily Bronte and Charles Dickens immortalized this glamorous image of TB in their work, all in spite of the incredible lethality of the disease (6). In fact, retrospective studies of patients prior to the implementation of short-term chemotherapy estimate that about 70% of people with microbiologically confirmed pulmonary TB died within 10 years of diagnosis (7). In the 19th century TB was responsible for one fourth of the deaths that occurred in Europe and North America, earning the title "Captain of all these men of death" (4). While the development of the Bacillus Calmette-Guérin (BCG) vaccine and the discovery of antibiotics have helped reduce the impact of TB in developed nations, many public health strategies have not been scalable (6). As such, today TB is one of the most common infections globally (8).

1.1.1 Cause

The causative agent of TB is *Mycobacterium tuberculosis* (Mtb), the bacteria famously discovered by Robert Koch in 1882 (9) and leading to the genesis of "Koch's Postulates" which

are still used in microbiology today (10). Koch was eventually awarded the Nobel prize for definitively proving that Mtb causes TB (11).

Mtb is an obligate intracellular pathogen that naturally infects humans, though other animals can be infected experimentally. It is an acid-fast (12), aerobic non-motile bacilli (5) that thrives in tissues with high oxygen levels such as the lung. It is incredibly slow growing, taking up to 20 hours to divide, 60 times as long as *Escherichia coli*. This combination of properties makes it difficult to study and to treat.

Mtb is thought to have originated from an environmental ancestor and co-evolved along with humans (13). It possesses a smaller genome than many other mycobacterial species and has gained a number of virulence factors including the ESX-1 secretion system which is essential for full virulence of Mtb (14). This system is responsible for secreting the Mtb immunodominant antigens[1] early secretory protein 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) which allow the bacteria to escape intracellular host defenses of the innate immune system (14). These virulence factors play a significant role in the transmission and pathogenesis of Mtb.

1.1.2 Global Burden of Disease

Nearly one quarter of the world is infected with Mtb (8). A majority of these individuals are latently infected and asymptomatic (15). Despite this, due to the high prevalence of infection, approximately 10 million people develop active TB disease and over 1 million people die every

[1] Antigen: a protein, lipid or carbohydrate structure that induces an immune response in the body

year (8) (Figure 1-1). Cumulatively TB is estimated to have killed 1 billion people over the last two centuries (16).

The US dramatically reduced the impact of TB by establishing a national surveillance program in 1953 and implementing streptomycin, isoniazid and rifamycin to treat TB. For three decades, the incidence of TB dropped 5.8% every year to 9.4 cases per 100,000 (17). In the 1980s the HIV epidemic and the rise of antibiotic resistant strains of Mtb lead to the reemergence of TB (18). In 1993 the World Health Organization (WHO) declared TB a public health emergency and in 2016 initiated the End TB Strategy (17,19). In response to both of these declarations countries like the US were once again able to successfully bring down the incidence of TB. In the 1990s the incidence of TB in the US dropped 7.3% every year (17).

Globally, however, we have not been as successful. In the 21st century TB incidence dropped by only 1.1% per year world-wide. This resulted in only an 11% reduction in the number of deaths over the last 3 years (8). This is true in many regions of the world including Africa. In 2018, 24% of new TB cases occurred in Africa (8). Nine of the ten countries with the highest TB incidence rates per capita were in Africa. Kenya, where the cohort for this research was enrolled, was ranked 15th in TB burden, with 150,000 incident cases and 33,000 deaths in 2018 (20). Because of the high burden in regions like Africa, TB is currently the leading cause of death due to a single infectious agent.

1.1.3 Pathogenesis

Although TB is typically thought of as a disease of the lung, Mtb can disseminate to nearly any part of the body. These extrapulmonary sites of infection commonly include lymph nodes, the

pleural cavity, bones, and joints, although any organ can be involved. Extrapulmonary TB accounts for approximately 15% of incident TB cases and primarily affects children and immunocompromised adults (21). The majority of TB cases though are pulmonary TB, the pathogenesis of which is determined by both the bacteria and the immune system (Figure 1-2). This is often referred to as the immunological life cycle of tuberculosis and is characterized by four stages: transmission, infection, equilibrium, and reactivation (22).

Mtb is transmitted through aerosol droplets containing as few as three bacteria. The small size of the droplet allows it to penetrate deep into the lungs where it is phagocytosed[2] by alveolar macrophages, a local innate immune cell (23). This results in localized inflammation which recruits additional phagocytic cells including monocytes. Due to the ability of Mtb to escape intracellular responses by phagocytes, this recruitment provides an unintended niche for Mtb to grow and replicate unabated (24). During this phase, individuals are often asymptomatic.

For reasons that remain unclear, the trafficking of live bacteria to the lymph node by the innate immune system is severely delayed. As such, by the time the adaptive immune system develops and migrates to the lung to fight the infection, the bacteria have already established a replicative niche. This is a contributing factor as to why infection with Mtb is rarely cleared without therapeutic intervention. The adaptive immune system is able to control and contain through the development of a granuloma, a walled structure of cells surrounding the bacteria. Granulomas are the hallmark pathological feature of TB disease and are meant to protect the host from damage by the bacteria (24). Historically, it was thought that the granuloma was a static structure that sequestered the bacteria throughout the course of an infection. In reality, granulomas

[2] Phagocytose: to engulf an extracellular object, usually for degradation

are heterogeneous structures that provide benefits to both the host and the bacteria (25). Infected cells within the granuloma are continuously dying and as a result new immune cells are continuously recruited to combat the bacteria and clear the dying cells. These cells occasionally traffic out of the granuloma and establish new granulomas within the lung tissue. Furthermore, it has been shown that, not only do immune cells traffic in and out of the granuloma, but bacteria can as well (26,27). As such, the granuloma is actually a highly dynamic structure within the lung environment and requires constant immune activity in order to maintain control of the bacteria. This immunological "equilibrium" does not result in active disease and is clinically classified as latent tuberculosis infection (LTBI) (22,28) (Figure 1-3).

In some individuals, this equilibrium is compromised leading to loss of control of the bacteria and development of active TB disease. When this occurs, cells in the granuloma die and the environment becomes hyper-inflammatory. This results in a loss of structural integrity in the surrounding tissue and leads to cavitation of the granuloma into neighboring airways resulting in the release of bacteria (24,25). Cavitation is associated with active TB disease symptoms including cough and hemoptysis and contributes to ongoing transmission of TB (29).

1.1.4 Diagnosis

Due to the complex clinical spectrum of Mtb infection, diagnosing and differentiating LTBI and active TB disease can be challenging. Diagnostic tools are divided into two groups: those that detect the presence of an immune response to Mtb and those that detect Mtb itself. The former is primarily used to detect LTBI, while the latter is used in the diagnosis of active TB disease. It is important to note that this is a practical strategy. The tests used to detect LTBI cannot differentiate LTBI from TB.

The two types of immunological tests are the tuberculin skin test (TST) and interferon-gamma (IFNγ) release assays (IGRA). TST, often referred to as the Mantoux test, involves detecting a delayed hypersensitivity reaction 48-72 hours following intradermal administration of purified protein derivative from sterilized Mtb culture filtrate proteins. This test is limited both by the requirement for follow-up evaluation as well as the potential for cross-reactivity in those that have been vaccinated with BCG. IGRAs are the modernized versions of this test (5). Currently, there are two U.S. Food and Drug Administration (FDA)-approved and commercially available IGRAs: QuantiFERON®-TB test (QFT) and T-SPOT®.TB test (T-Spot). IGRAs measure IFNγ[3] production in blood and generate a result within 24 hours. As such, they only require one patient visit. More importantly, IGRAs measure IFNγ production in response to CFP-10 and ESAT-6, which are immunodominant antigens that are present in Mtb but have been deleted from BCG. The test therefore is not cross-reactive with BCG, eliminating false positive results from individuals who are BCG-vaccinated but not infected with Mtb (30). QFT is an enzyme-linked immunosorbent assay (ELISA), whereas T-Spot is an enzyme-linked immunospot (ELISPOT)-based assay. While both tests are highly effective in detecting Mtb infection, neither test is able to differentiate between LTBI and active TB disease.

Microbiological confirmation of pulmonary TB requires a sample of sputum[4] from suspected patients. Liquid Mtb culture is the gold-standard diagnostic test because it is highly specific and able to detect live replicating bacteria (5). It does, however, have a long turnaround time. Smear and fluorescent microscopy, which visually detect Mtb in sputum, can be performed same day but have low sensitivity. Recently a number of commercially available molecular tests,

[3] A signaling protein produced in response to Mtb
[4] A combination of saliva and mucus expectorated from the lungs

such as the Xpert MTB/RIF and Hain MTBDRplus, have been implemented. These tests are relatively inexpensive, quick, and require little training to perform. In addition, they have the ability to detect drug resistance (31,32). Due to the higher accuracy of Xpert compared to sputum microscopy, the WHO now recommends Xpert MTB/RIF as the first-line diagnostic test in all adults (33). While the advent of molecular tests has made diagnosis of active TB disease easier and quicker, detection of subclinical and incipient TB continues to be challenging due to the low bacterial burden.

1.1.5 Treatment

The scientific field has made enormous strides in treating TB over the last 70 years. Up until the middle of the 20th century we had no definitive drugs to combat Mtb. The only treatments available to individuals with TB were life-style changes including bed rest, sunbathing and fresh air. Due to these recommendations and the contagious nature of TB, many individuals with TB were sent to sanatoria, which were essentially medical facilities in remote, often mountainous, locations. While sanatoria had moderate success, it is still contested whether they improved the chance of recovery over bed-rest at home (34). In 1944 streptomycin, the first antibiotic with demonstrable efficacy against Mtb, was discovered (35). This was quickly followed by the discovery of isoniazid (36) and the rifamycins (37) in treatment of TB. Over the following decades, combination therapy of these drugs was utilized to cure TB disease. The current standard course TB regimen consists of four drugs – isoniazid, rifampicin, pyrazinamide, and ethambutol – given for 2 months, followed by a continuation phase with isoniazid and rifampicin for an additional 4 months (38). This long treatment regimen, however, has been an obstacle in regions of the world with little health care infrastructure. This has led to a huge disparity in treatment outcomes across

the globe. In addition, the long treatment regimen often results in non-compliance and has led to the rise of antibiotic resistant strains of Mtb (39). In 2018, there were approximately 500,000 incident cases of rifampicin-resistant TB, of which 78% were resistant to additional front-line drugs (8). Fortunately, there are a number of new treatment options on the horizon to combat the threat of drug-resistant Mtb (40). Clinical trials are ongoing now in an effort to shorten the treatment regimens for TB.

1.1.6 Coinfections and Comorbidities

One of the perplexing features of tuberculosis is the spectrum of disease that results from Mtb infection. This can range from complete clearance of the bacteria to LTBI to active TB. The reasons underlying this spectrum are not well understood, but a number of risk factors have been identified (41). These predominantly include socioeconomic factors such as poverty and overcrowding, metabolic disorders such as diabetes, and conditions that affect the integrity of the lung such as smoking, chronic obstructive pulmonary disease, and silicosis. The greatest risk factor for developing active TB, however, is infection with human immunodeficiency virus (HIV), which increases the risk of developing TB disease by 20-40 fold (41). This risk is associated with the loss of CD4 T cells implicating a role for the immune system in control of Mtb infection (42). Despite it's obvious impact on TB outcomes, HIV only accounted for 8.6% of new TB cases and 17% of TB deaths in 2018 (8). There are therefore likely additional risk factors that have yet to be identified for TB.

In many regions of the world, tuberculosis is co-endemic with HIV, malaria and helminth infections (43,44), thus the probability of co-infection with some combination of these four diseases is high (Figure 1-4). The prevalence of a single helminth species, *Schistosoma mansoni*

(SM) in Kenya can reach well over 50% in endemic communities (44,45). Both Mtb and SM have co-evolved with humans for thousands of years. As such they have developed a unique balance with the immune system. Perturbation of this balance in either infection can result in severe disease. Furthermore, helminths, including SM have been shown to have robust immunomodulatory properties (46–48). It is therefore of great importance to understand the immune response during co-infection with Mtb and SM.

1.2 Schistosomiasis

Helminths are a diverse group of "worm-like" eukaryotic organisms that, despite similarities in body structure, are largely considered completely unrelated. There are a number of helminth species that cause disease in humans, and, similar to Mtb, helminths have had a long and memorable history with humans. Many passages in the Old Testament contain vague references to what are now assumed to be helminth infections. This includes allusions to guinea worm infections amongst the tribe of Moses and a ban on pork products due to trichinella (49,50). There are countless references to helminth infections in famous medical texts including those by Hippocrates and Aristotle (50). Indeed, roundworms and tapeworms are directly discussed in the famous medical text Ebers Papyrus, dating back to 1550 BC (50). While not directly addressed, schistosomiasis, also known as bilharziasis, is alluded to in this text as well. Furthermore, eggs from the helminth that causes schistosomiasis have been found in mummies dating back to 1100 BC (51).

1.2.1 Causes

Schistosomiasis is a specific helminthiasis caused by blood flukes of the genus *Schistosoma*. There are five species known to infect humans, but three of them account for a majority of the disease burden: *S. mansoni, S. japonicum, and S. haematobium* (52). In western Kenya where this research was conducted, *S. mansoni* (SM) is the dominant species that infects humans (45). As with most helminths, SM undergoes a complex life cycle involving both a snail host and a human host (Figure 1-5). Infected humans excrete SM eggs in feces that, upon contact with water, hatch releasing miracidia. The miracidia infect fresh-water snails and go through the next transition in the life cycle creating thousands of cercaria. Cercaria are released from snails in

response to sunlight at which point they attempt to locate a human host. When cercaria come into contact with humans, they release enzymes that allow them to penetrate the skin and transition to the next stage of the life cycle: the larval schistosomula. The schistosomula enters the circulatory system and migrates through the lung and then back into the circulatory system. If after re-entering circulation the schistosomula meets a member of the opposite sex, the two mature into adults and migrate to mesenteric veins together where they reside as a "mating pair" indefinitely. Here they mate and release eggs, which are passed into the intestine and excreted. A single worm pair can shed up to 300 eggs a day throughout the course of an infection. Occasionally these eggs are swept backwards into the circulatory system and become lodged in various tissues, predominantly the liver (53,54).

1.2.2 Global Burden of Disease

In 1947, Norman Stoll published a landmark paper entitled “This wormy world,” in which he estimated the number of people infected with helminths worldwide, with the estimated global prevalence of schistosome infections to be over 100 million (55). Today that estimate has increased to over 200 million infections (56). It is important to know that both these numbers are likely underestimates for the time. Historically, mapping schistosome prevalence has been based on microscopy, serology and clinical symptoms, although these tools may not be sufficiently sensitive to diagnose individuals with low intensity SM infections (57).

It is difficult to measure the impact of schistosomiasis on global health because it is rarely fatal. As such, indirect measures of morbidity have been developed to quantify the burden of schistosomiasis. The primary measure is the disability-adjusted life year (DALY) which is the sum of years of life lost (YLL) due to premature mortality and the years lived with disability (YLD)

(58). Schistosomiasis is responsible for 3.31 million DALYs, 2.99 YLLs, and 0.32 YLDs. It ranks third amongst all neglected tropical diseases (NTDs) for DALYS and has a disproportionately high YLL to DALY ratio (58,59). Another study reported that schistosomiasis was responsible for 2-15% Disability Weight (59). A Disability Weight reflects the severity of the disease on a scale from 0 to 100% where 0% is perfect health and 100% is certain death. While these measurements are somewhat abstract, they attempt to quantify the impact of schistosomiasis on the global burden of disease, especially in relation to other infections. To put it in perspective, schistosomiasis is amongst the top 5 NTDs in terms of its contribution to the global burden of disease (60).

Schistosomiasis disproportionately impacts people in Sub-Saharan Africa, which account for over 90% cases (61) (Figure 1-6). In this region, Schistosomiasis is responsible for 280,000 deaths per year (62). Approximately 25% of the population of Sub-Saharan Africa is infected with a schistosome species, of which the most common species are *S. haematobium* and *S. mansoni* (SM). As stated previously, the prevalence of SM infection alone reaches above 50% in communities along the shores of Lake Victoria in western Kenya (44,45). Indeed NTDs, including SM, rank in the top 10 cause of DALYs in Kenya. This is particularly striking because the observed DALYs for NTDs are 3.37 times the expected DALYs based on the Sociodemographic Index of Kenya (63). NTDs are therefore responsible for excess morbidity and mortality in this region.

1.2.3 Pathogenesis

As a part of the life cycle, SM eggs induce granuloma formation to help facilitate their passage into the gastrointestinal tract. This process involves recruitment of immune cells which support extravasation through the epithelial layer and digestion of the intestinal wall (64,65). In the natural course of infection, these eggs are released into the intestinal tract. At this point, the

damage to the intestine can be repaired. When eggs become lodged in other tissues, however, they still recruit immune cells, but are unable to exit the tissue. Instead, the granulomas become large and fibrous in an attempt to seal off the eggs.

Most of the pathology associated with schistosomiasis is caused by the immune response to eggs that are lodged in tissues (52,66). In non-immune individuals this often results in an overly robust immune response known as Katayama Fever, which is characterized by fever, malaise, headache, myalgia, and abdominal pain (67). In endemic settings, where individuals are continually being re-infected with SM, the immune response to SM eggs wanes over time. In this setting, individuals will develop more subtle chronic disease and non-specific symptoms such as abdominal pain and diarrhea (68). If left untreated, SM infection results in disabling morbidities such as anemia, malnutrition, and impaired childhood development (69).

1.2.4 Diagnosis

The most common method of diagnosing SM infection is done by detecting the presence of SM eggs in stool. While this test is highly specific, it has very low sensitivity. This is particularly true in individuals with HIV who shed fewer eggs (70). Serial sampling can improve the sensitivity, however SM infection cannot be definitively ruled out with a negative stool examination (71). Furthermore, stool examination cannot detect single sex infections which do not produce eggs. Serological assays which detect antibodies against schistosomal antigens have been useful in diagnosing acute schistosomiasis. In endemic settings, however, serology is unable to differentiate current infection from past exposure (72).

New molecular tests that detect circulating cathodic antigen (CCA) or circulating anodic antigen (CAA) have been able to overcome some of the limitations of previous tools. These assays are more sensitive than stool and more specific than serology. In particular, an inexpensive point of care test that is now commercially available (Rapid Medical Diagnostics, Pretoria, South Africa) can detect CCA in urine using a cassette. This permits rapid onsite diagnosis of SM infection (73,74). With the introduction of these tools, we have been better able to diagnose infections and estimate prevalence in areas with low intensity infections (57). Recent studies have found that the number of infected individuals detected by CCA/CAA is 6-10 times higher than the number detected by microscopic methods (57).

1.2.5 Treatment

The primary drug used to treat schistosomiasis for the last fifty years has been praziquantel. Praziquantel is effective against the adult worms of all five species of schistosome (52). Furthermore, it is inexpensive and effective at low doses in a wide range of populations (75). While the mechanism of action is not well understood, it requires an effective immune response for full efficacy. It is not active against the larval or egg stages of the schistosome life cycle, nor does it protect against reinfection (52).

Due to the long lifespan of a mating pair of schistosomes, a single infection can last for decades in the absence of treatment. Because an infection is not acutely severe, many individuals do not know they are infected and do not seek treatment. To combat this gap, the WHO recommends regular administration of praziquantel in endemic communities to control the prevalence of infection as well as reduce associated morbidities. In many areas, this involves regular mass drug administration (MDA) of praziquantel in schools and communities. Such MDA

efforts have been very effective. Community wide prevalence and intensity of SM infection can be reduced after just two rounds of treatment with praziquantel (76). Annual MDA has even been shown to reduce childhood malnutrition over time (77).

While naturally occurring praziquantel resistance has not been observed, it can be induced experimentally. This is particularly concerning given that treatment of schistosomiasis is a monotherapy. Drugs to treat other parasitic infections, such as artemisinin derivatives, which target alternative stages of the schistosome life cycle have been found to complement praziquantel treatment (52). There is still, however, an urgent need to develop additional tools and treatment strategies for schistosomiasis.

1.3 T cell immunity

1.3.1 The immune system and infection

The immune system is a complex organ system, encompassing a number of cell types and anatomical locations. It can be stratified, however, into two distinct arms: the innate and adaptive immune system. These two arms, while useful to discuss in theory, grossly oversimplify the dynamic and highly specialized cells of the immune system (78).

The innate immune system is primarily made of effector cell types, including monocytes, macrophages, granulocytes, natural killer (NK) cells, and dendritic cells. All of these cell types are able to respond early during infection but are only capable of non-specific responses. Monocytes and macrophages survey tissues phagocytosing foreign material and secreting effector molecules that combat extracellular pathogens. Granulocytes encompasses a group of immune cells that release granules full of effector molecules at the site of infection. Neutrophils are involved in the response against single cell extracellular pathogens such as bacteria. Basophils and eosinophils are involved in the response again multicellular organisms, such as helminths. NK cells respond to intracellular pathogens by identifying and killing infected cells. Dendritic cells are “professional” antigen presenting cells. They sample the site of infection in order to better prime the adaptive immune system. All of these cells work together during the initial stages of infection and are often capable of eliminating potential pathogens without the adaptive immune system (78).

The adaptive immune system consists of lymphocytes which respond in an antigen specific fashion and are usually slower to respond than the innate immune system. Lymphocytes are classified as either B cells or T cells depending on the type of receptor they express. Both cell

types go through a rigorous selection process during development such that each cell expresses a unique receptor that is highly specific to a single molecule, often from a single organism. These cells are able to coordinate memory responses against specific pathogens following either exposure or vaccination. B cells are responsible for antibody responses and are incredibly important. T cells, however, are the focus of this dissertation.

T cells are generally divided into three groups: CD4, CD8, and non-classical T cells. CD4 T cells are the master regulators of the immune system. They dictate the type of downstream responses generated in response to an insult through the secretion of cytokines. These cytokines specifically activate and polarize specific subsets of innate immune cells necessary to clear the pathogen. CD4 T cells are often referred to as "helper" T cells because of the support they provide to other arms of the immune system. CD8 T cells are referred to as "killer" T cells because they target and kill infected cells. CD4 and CD8 T cells also differ by their mechanism of antigen recognition. Peptide antigens are presented to both on major histocompatibility complex (MHC) molecules, however CD4 T cells recognize peptide antigen bound to MHC class II molecules, whereas CD8 T cells recognize antigen presented on MHC class I molecules and this difference relates to their function. MHC-II molecules are expressed only on immune cells and present peptide fragments from proteins sampled from the environment. Through this interaction, CD4 T cells determine whether an antigen is harmful or not and initiate downstream effector responses when necessary. MHC-I molecules are expressed ubiquitously on nucleated cells and present peptides from proteins expressed intracellularly, thus enabling CD8 T cells to recognize and kill infected cells throughout the body.

Non-classical T cells are less studied and therefore not as well understood. They encompass

a variety of T cells including mucosal associated invariant T (MAIT) cells, invariant natural killer T (iNKT) cells, and γδ T cells. While these cells also undergo a selection process during development, their receptors are less specific. This is largely due to the fact that they do not recognize peptide antigens. MAIT cells recognize metabolites produced by bacteria and fungus during vitamin B synthesis presented by the MHC-I related protein MR1 (79,80). iNKT cells recognize self and microbial derived lipids presented by the MHC-related glycoprotein CD1d (81). γδ T cells are divided based on whether they are tissue-resident or systemic. Tissue resident γδ T cells recognize several MHC-related molecules including lipids presented on CD1 molecules, as well as stress induced molecules. Peripheral γδ T cells largely recognize phosphoantigens which can be derived from either pathogens or self-antigens (82,83). Non-classical T cells can often respond to infection more quickly than CD8 and CD4 T cells. As such, they are often considered a "bridge" between the innate and adaptive immune system (78).

1.3.2 CD4 T Cell Subsets

In 1986 Mosmann et al. beautifully provided the first proof that CD4 T cells could be divided into two distinct subsets at a clonal level (84). These subsets were referred to as type 1 T helper cells (TH1 cells) and type 2 T helper cells (TH2 cells) based on the cytokines produced (then known as lymphokines) in response to the non-specific T cell stimulation Con A. Amazingly, this was done prior to the discovery and cloning of IL-4 which in the paper was referred to as B-cell Stimulating Factor-1 (BSF-1). In their paper TH1 cells and TH2 cells were predominantly separated based on the production of IFNγ and BSF-1 (IL-4) respectively (84). In 1989 another landmark paper followed wherein Richard Locksley and his colleagues demonstrated that upon Leishmania infection in mice either TH1 or TH2 cells would dominate and that the subset greatly

impacted disease outcome (85). Following these discoveries, a number of scientific groups contributed to what would become known as the TH1/TH2 paradigm (Figure 1-7). This paradigm characterizes TH1 and TH2 cells by the cytokines involved in polarizing the subsets, the lineage-specific transcription factors that controlled these programs, and the surface markers that could be used to identify subsets ex vivo. For example, TH1 cells produce TNFα and IFNγ, and express the transcription factor T-bet and the surface marker CXCR3. TH2 cells produce IL-4 and IL-13, and express the transcription factor GATA3 and the surface marker CCR4 (86–89). It was thought that these subsets represented terminally differentiated, lineage committed CD4 T cells.

In the last twenty years, a number of additional unique CD4 T cell subsets have been identified. As with TH1 and TH2 cells, some produced a hallmark cytokine for which they were named, such as TH9 (producing IL-9), TH17 (producing IL-17) and TH22 cells (producing IL-22) (90). Additional groups of CD4 T cells were identified based on their suppressive functions and broadly grouped into "regulatory" T cells (Tregs), characterized by expression of the transcription factor Foxp3 (91). Lastly, a subset of CD4 T cells were identified in B cell follicles and were shown to aid in class switching and B cell activation and were termed T follicular helper cells (Tfh cells). These CD4 T cell subsets have been useful in studying specific immune responses in controlled systems. However, because there is a great deal of overlap between the functions and expression patterns of these cells, extrapolating the findings of these studies to real life scenarios has been challenging.

1.3.3 Immunity to Tuberculosis

While many cell types are involved in the immune response to Mtb, CD4 T cells are absolutely critical. Indeed, the absence of CD4 T cells, either experimentally or due to HIV

infection, has been associated with poor control of Mtb and worse disease outcomes (42,92). Furthermore, it is well documented that a TH1 response, specifically, is essential for control of Mtb. TH1 cells are induced upon infection with Mtb and are responsible for orchestrating downstream components of the immune response through the release of the cytokines IFNγ and TNFα (93,94). These cytokines recruit and polarize type 1 effector cells such as classical macrophages (M1 cells), natural killer (NK) cells, and neutrophils. Activation by IFNγ increases the ability of macrophages to kill intracellular bacteria, the ability of NK cells to kill infected cells and the activity of neutrophils (78). Concordantly, TNFα inhibits bacterial growth and prevents macrophage death, thus blocking the spread of Mtb from dying cells (95,96). The necessity for IFNγ and TNFα in the control of Mtb has been demonstrated by studies with TNFα blockade or impaired IFNγ receptor signaling (97,98). In each case, individuals have an increased risk of developing active TB disease. Importantly, CD4 T cells are not the only source of TNFα and IFNγ. These cytokines can be produced by a variety of cells including γδ T cells, which have recently been shown to produce significant amounts of both cytokines during Mtb infection (99,100).

1.3.4 Immunity to Schistosomiasis

In contrast to Mtb, helminths are classic examples of TH2 immunity (101). TH2 cells orchestrate type 2 responses through secretion of the cytokines IL-4, IL-5, IL-13, IL-9 and IL-10 (103,104). IL-4 is the hallmark cytokine of type 2 responses and is responsible for polarizing macrophages to an M2 phenotype (104,105). IL-5 and IL-13 are responsible for the recruitment and activation of eosinophils, and even push the bone marrow to produce more eosinophils (104,106). IL-9 activates mast cells, which are present near epithelial barriers such as the lung and gastrointestinal tract (104,107). IL-10 is responsible for regulating the immune response and

limiting inflammation throughout the healing process (104,107).

Each effector cell involved in type 2 responses plays a role in both worm expulsion and wound repair. Eosinophils store pre-formed granules that contain cytokines, growth factors and molecules involved in tissue remodeling. The granules also contain molecules such as Major Basic Protein 1 and Eosinophil Cationic Protein which are directly toxic to helminths (106). Mast cells release pre-formed granules at epithelial barriers when antibody bound antigen crosslinks receptors on the surface of the cell. These granules are known for containing histamine, but they also contain molecules that act on epithelial barriers to increase mucus production and cell turnover. Increased mucus helps counteract the loss of barrier integrity and the turnover of cells disrupts the ability of many helminths to attach to epithelial surfaces (101,106). M2 macrophages produce chitinase like molecules that disrupt the protective tegument covering most helminths and arginase which starves helminths of nutrients. Their main role, however, is in wound repair and control of the immune response to prevent excessive damage due to inflammation. They secrete IL-10 as well as growth factors and molecules that promote angiogenesis (105). Together these cells support helminth expulsion and repair tissue damage causes by the helminth life cycle.

As a helminth, SM does induce a TH2 response. In fact, SM is one of the few helminths where a TH2 polarizing antigen has actually been identified (64,65,101). The eggs from SM are highly immunogenic and are responsible for the type 2 polarized immune responses seen during SM infections. Unique to SM, however, is the fact that the larval stage actually induces a strong TH1/TH17 immune responses (53,108). This only later gives way to a dominant TH2 response when adult worm pairs begin releasing eggs. The immune response to SM is therefore not only stage but antigen specific (Figure 1-8).

1.4 Dissertation Overview

During the initiation of the adaptive immune response, antigen-presenting cells secrete cytokines that polarize CD4 T cells to one of a number of subsets. Each subset is characterized by a set of transcription factors, surface markers and cytokines. This polarization determines the downstream effector functions that are utilized by the immune system. As mentioned previously, type 1 CD4 T cells (TH1 cells) are induced by infection with Mtb and are critical in the control of Mtb through production of IFNγ and TNFα. These cells are characterized by expression of the transcription factor T-bet and the chemokine receptor CXCR3 (86,109,110). In contrast, helminths, including SM eggs, elicit strong type 2 CD4 T cell (TH2 cell) responses characterized by the transcription factor GATA3, the chemokine receptor CCR4 and production of IL-4 (86,109,110). Considering these differential requirements for effective pathogen-specific immunity, determining the impact of co-infection on the immune response to both Mtb and SM is of critical importance.

The characterization of Mtb-specific CD4 T cell responses during coinfection with filarial worms and soil transmitted helminths is well documented (43,111). Both types of worms skew Mtb-specific immunity by decreasing TH1 cytokine production and increasing TH2 cytokine production in LTBI individuals, which is reversible upon treatment of helminth infections (112–115). However, there has been very little research examining the impact of SM on Mtb-specific CD4 T cells. In addition, there is no research examining the impact of Mtb infection on SM-specific T cell responses. The work described in this dissertation addresses these gaps in the scientific literature and evaluates the impact of co-infection with Mtb and SM on Mtb infection outcomes. This dissertation addresses the following hypotheses: (1) SM infection skews Mtb-specific TH1 immunity; (2) Mtb infection impairs SM-specific T cell function; and (3) SM

infection leads to increased probability of TB disease.

To test these hypotheses, we analyzed antigen-specific T cell responses using peripheral blood mononuclear (PBMC) samples obtained from individuals in a high TB and SM burden region of Kenya. We then characterized the cytokine profiles and proliferative capacity of these cells utilizing flow cytometry. We found that while SM infection does not inhibit Mtb-specific TH1 immunity, Mtb does impair SM-specific T cell responses. Furthermore, we used novel machine learning approaches to determine that there is no increased probability of active TB in HIV-uninfected SM^+ individuals; however, SM infection increases the probability of active TB in HIV-infected individuals.

1.5 Figures

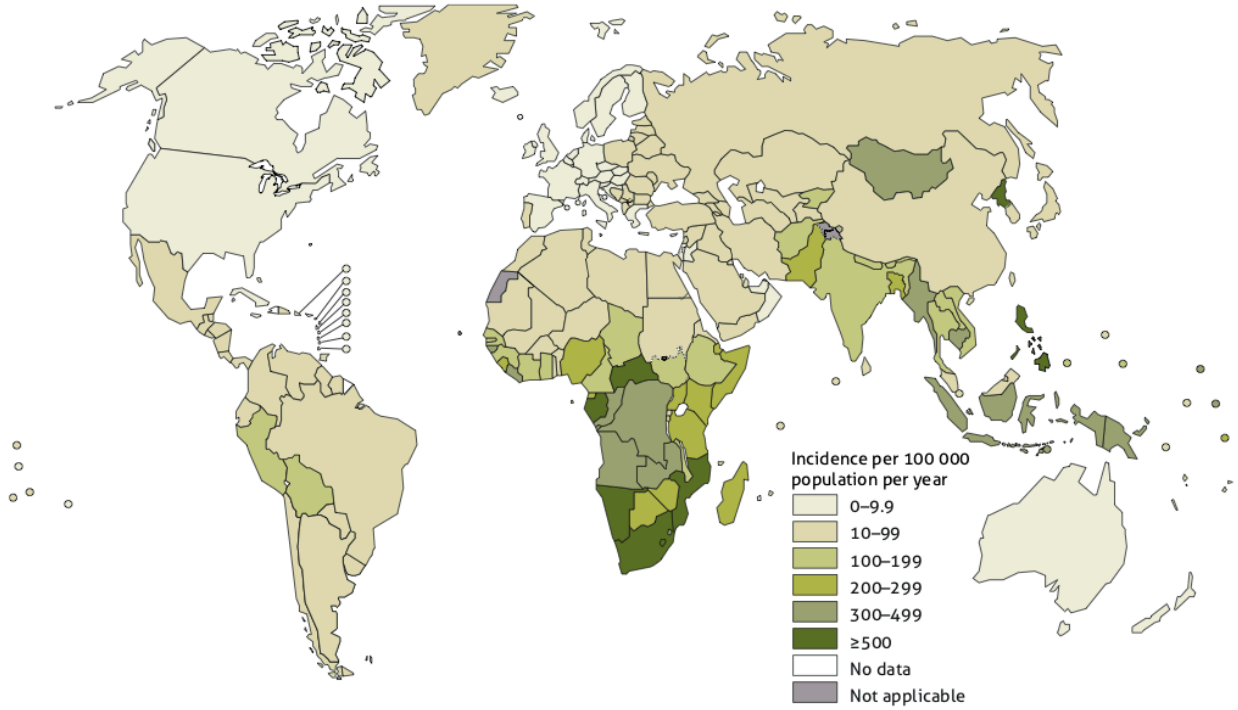


Figure 1-1: Estimated Incidence Rates of TB in 2018

Source: WHO Global Tuberculosis Report 2019 (8)

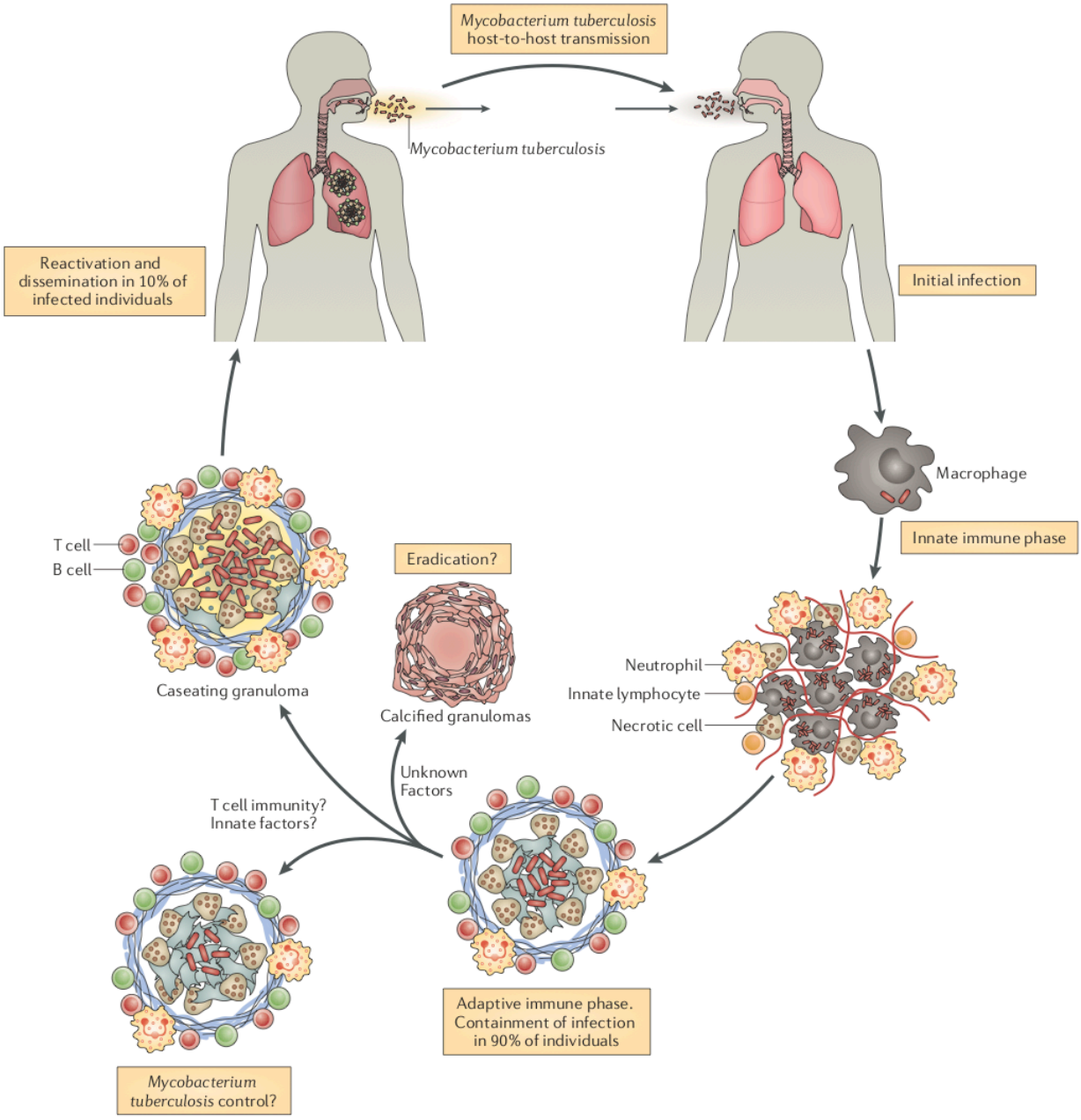
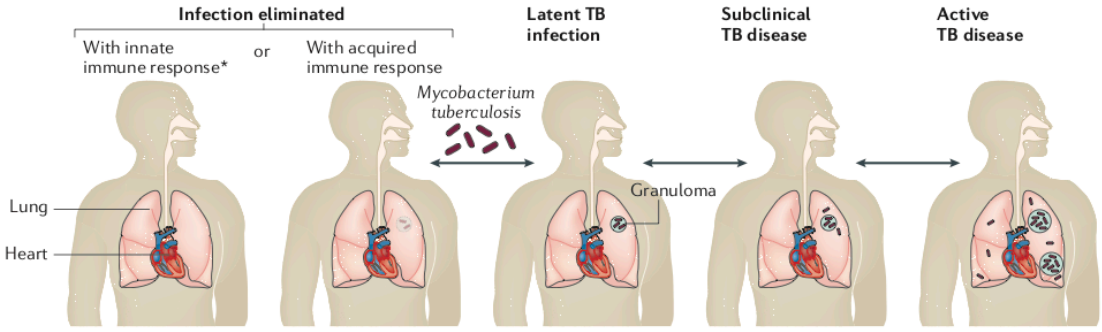


Figure 1-2: Transmission and pathogenesis of Mtb infection

Source: Nunes-Alves et al. (116)



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figure 1-3: Spectrum of Mtb Infection Outcomes

Source: Pai et al. (33)

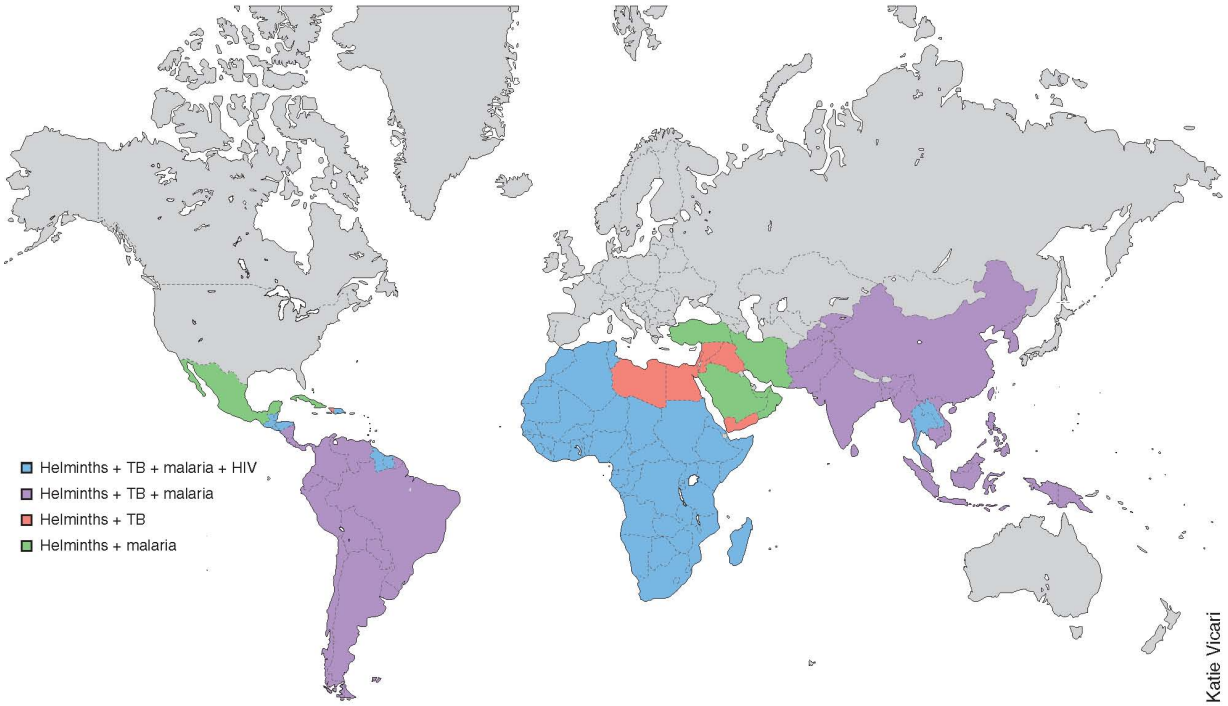


Figure 1-4: Geographic Overlap of TB and Helminths

Source: Salgame et al (43)

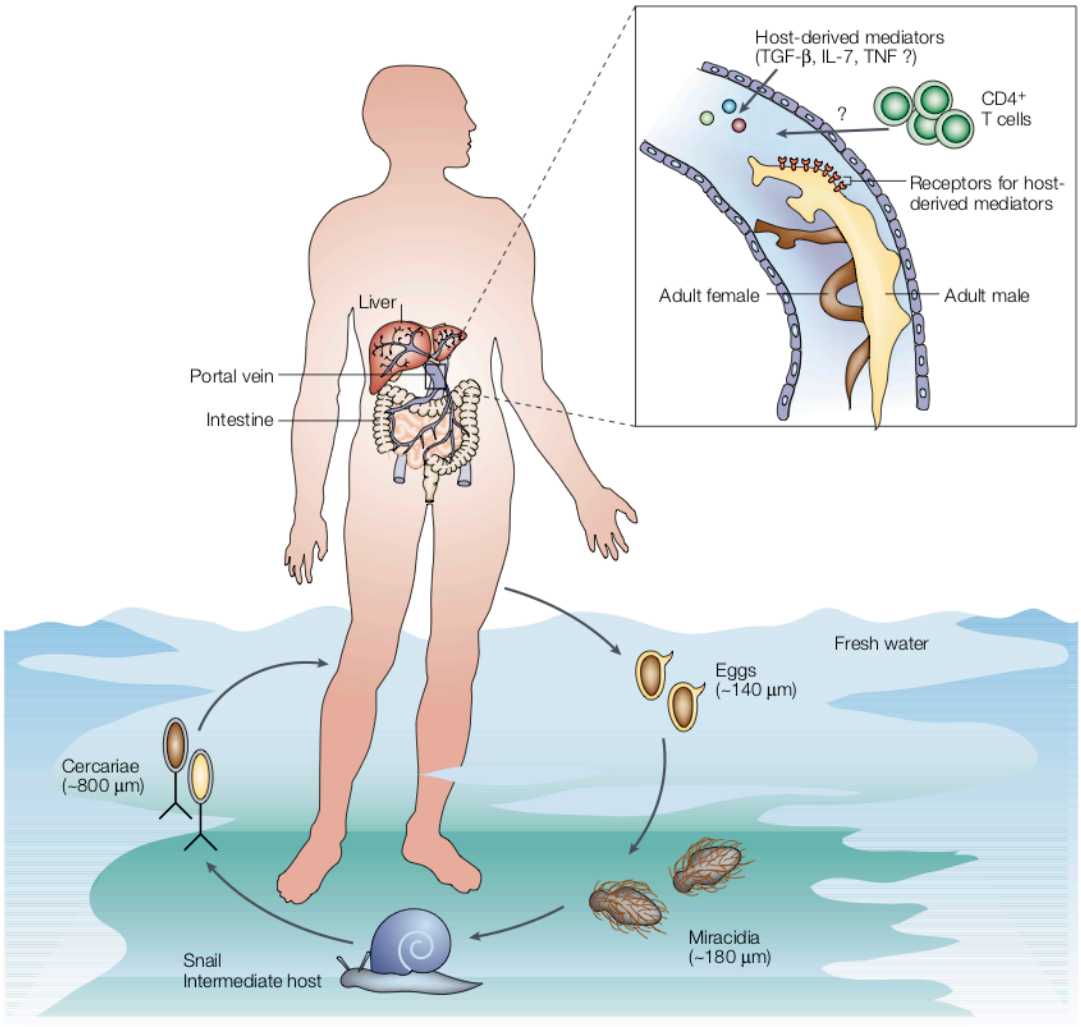


Figure 1-5: Life cycle of SM

Source: Dunne et al (117)

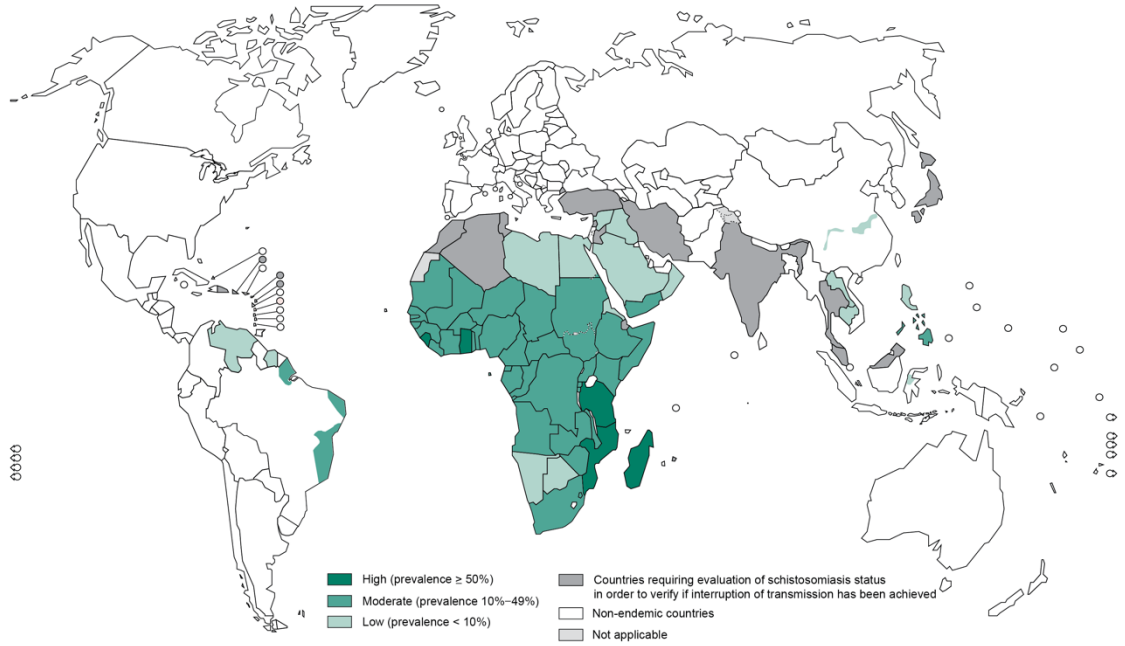


Figure 1-6: Global Prevalence of Schistosomiasis 2012

Source: WHO Schistosomiasis Progress Report and Strategic Plan (118)

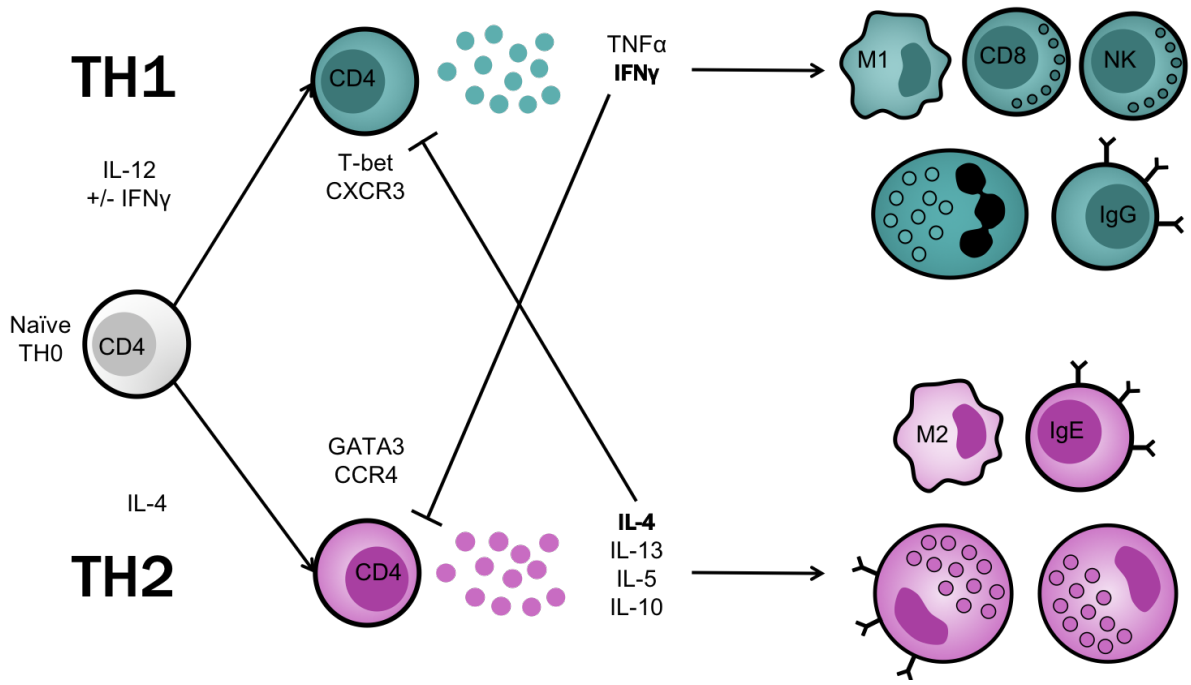


Figure 1-7: TH1/TH2 Paradigm

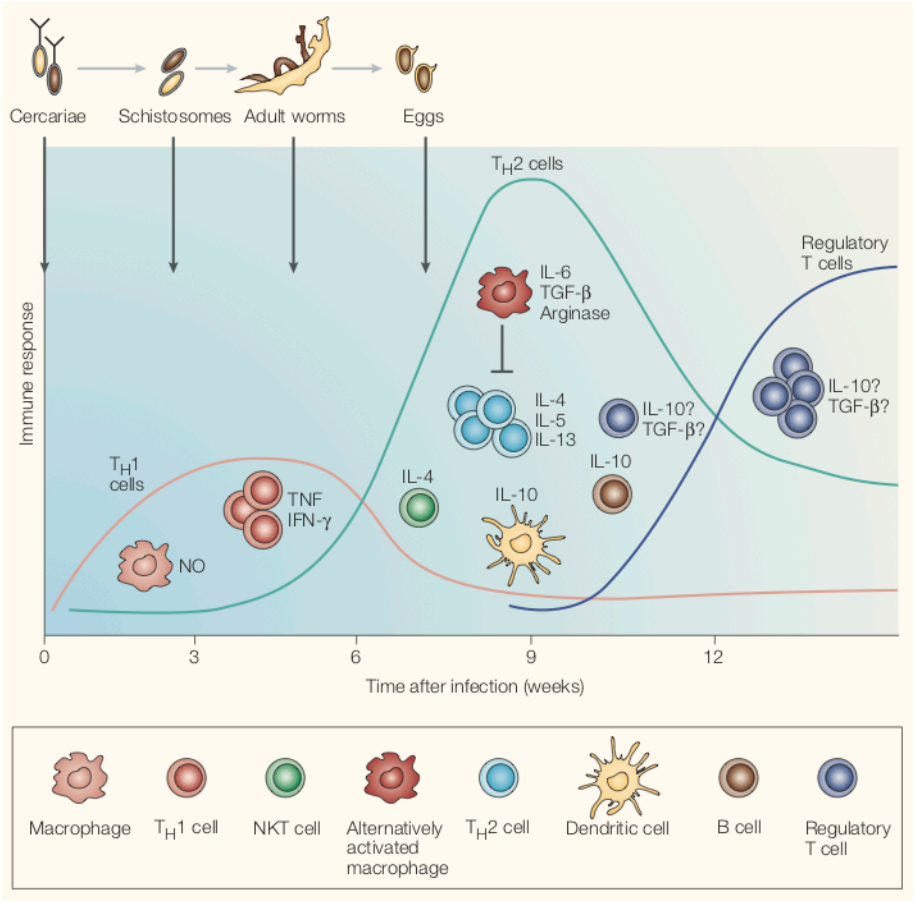


Figure 1-8: Stage Specific Immunity to SM

Source: Dunne et al (117)

Chapter 2 CD4 T cells in *Mycobacterium tuberculosis* and *Schistosoma mansoni* co-infected individuals maintain functional TH1 responses

This chapter is reproduced with minor edits from: McLaughlin TA, Khayumbi J, Ongalo J, et al. CD4 T Cells in Mycobacterium tuberculosis and Schistosoma mansoni Co-infected Individuals Maintain Functional TH1 Responses. Front Immunol **2020**; 11:127.

2. 1 Abstract

Mycobacterium tuberculosis (Mtb) is a serious public health concern, infecting a quarter of the world and leading to 10 million cases of tuberculosis (TB) disease and 1.5 million deaths annually. An effective type 1 CD4 T cell (TH1) immune response is necessary to control Mtb infection and defining factors that modulate Mtb-specific TH1 immunity is important to better define immune correlates of protection in Mtb infection. Helminths stimulate type 2 (TH2) immune responses, which antagonize TH1 cells. As such, we sought to evaluate whether co-infection with the parasitic helminth *Schistosoma mansoni* (SM) modifies CD4 T cell lineage profiles in a cohort of HIV-uninfected adults in Kisumu, Kenya. Individuals were categorized into six groups by Mtb and SM infection status: healthy controls (HC), latent Mtb infection (LTBI) and active tuberculosis (TB), with or without concomitant SM infection. We utilized flow cytometry to evaluate the TH1/TH2 functional and phenotypic lineage state of total CD4 T cells, as well as CD4 T cells specific for the Mtb antigens CFP-10 and ESAT-6. Total CD4 T cell lineage profiles were similar between SM^+ and SM^- individuals in all Mtb infection groups. Furthermore, in both LTBI and TB groups, SM infection did not impair Mtb-specific TH1 cytokine production. In fact, SM^+ LTBI individuals had higher frequencies of $IFN\gamma^+$ Mtb-specific CD4 T cells than SM^- LTBI individuals. Mtb-specific CD4 T cells were characterized by expression of both classical TH1 markers, CXCR3 and T-bet, and TH2 markers, CCR4 and GATA3. The expression of these markers was similar between SM^+ and SM^- individuals with LTBI. However, SM^+ individuals with active TB had significantly higher frequencies of $GATA3^+$ $CCR4^+$ TH1 $cytokine^+$ Mtb-specific CD4 T cells, compared with SM^- TB individuals. Together, these data indicate that Mtb-specific TH1 cytokine production capacity is maintained in SM-infected individuals, and that Mtb-specific TH1 $cytokine^+$ CD4 T cells can express both

TH1 and TH2 markers. In high pathogen burden settings where co-infection is common and reoccurring, plasticity of antigen-specific CD4 T cell responses may be important in preserving Mtb-specific TH1 responses.

2.2 Introduction

Despite advances in care in the past decades, tuberculosis (TB) disease is currently the leading cause of death due a single infectious agent. Nearly 25% of the world's population is infected with the bacteria that causes the disease, *Mycobacterium tuberculosis* (Mtb) (8). Infection with Mtb leads to a spectrum of clinical states ranging from complete clearance, to latent infection (LTBI), to active TB disease (28). The immunological states associated with these differences have not been completely defined, however it is clear that CD4 T cells are necessary to control Mtb infection (42,92). Furthermore, T cells must be capable of producing type 1 (TH1) cytokines, such as IFNγ and TNFα, which have been shown to be critical in the control of Mtb (22,93,94).

Co-infections, such as with HIV, and comorbidities, such as diabetes, are known to influence Mtb infection outcomes (8). In addition, infections with numerous helminth species are known to modulate the immune response in a variety of ways. Helminths can directly impair the immune system through the secretion of helminth-derived molecules that act on host immune cells and limit or alter their effector functions (48). Helminths also indirectly impact the immune system by inducing a strongly TH2 polarizing environment that primes immune responses to bystander antigens (46,47). Both these immune modulation strategies result in systemic immune dysregulation and have long term consequences for immune cell function and disease outcomes. Due to the overlapping geographic distributions of TB burden and helminth infections (43,44), determining the impact of helminths on Mtb immunity is important in determining correlates of protection against Mtb infection as well as against the development of TB disease. As such, many have investigated this phenomenon and reported differing conclusions. A number of studies in humans have demonstrated that both filarial worms and the soil transmitted helminths *Srongyloides stercoralis* and hookworm can globally dysregulate the immune response to Mtb

(119–123). Indeed, all three types of worm have been shown to skew Mtb-specific immune responses by limiting TH1 cytokine production and increasing TH2 cytokine production in response to Mtb antigens in individuals with LTBI (112–115); moreover, treatment of helminth infections in people with LTBI has been shown to result in increased the frequencies of Mtb-specific IFNγ^+ CD4 T cells (124). Others, however, have shown no demonstrable effect on either immunity to Mtb or disease outcomes during co-infection with helminths, including filarial worms and hookworm (125,126). One recent study even reported an increased ability to control Mtb growth in individuals infected with hookworm (127). This variation is particularly evident in a recent meta-analysis of epidemiological studies of individuals co-infected with Mtb and helminths. The report indicates that both the prevalence rate of co-infection as well as the measured associations between infections varies greatly between studies as well as between helminth species (128). The outcome of helminth co-infection on the immune response to Mtb is likely specific to both the helminth species, as well as the Mtb infection status of the individuals being studied.

Schistosomiasis, the disease caused by schistosome worms such as *Schistosoma mansoni* (SM), is estimated to affect 240 million people globally and is the second most common form of helminthiasis (56,118). Approximately 90% of affected individuals live in Sub-Saharan Africa and while not normally not a fatal infection, the mortality rates for schistosome infections in Sub-Saharan Africa are estimated to be 280,000 per year (62). Similar to both filarial worms and soil-transmitted helminths, SM has a complex life cycle. It enters a human host as a cercariae and then matures as it migrates throughout the body. Eventually adult worms take up residence in the portal vein and release eggs into the circulation, which are able to pass into the lumen of the gastrointestinal tract and are subsequently released in stool (54). Unlike other helminth species, however, the immune response to SM is stage dependent. Early stages of the worm life cycle

stimulate a mixed TH1/TH17 response, which only gives rise to a TH2 response upon egg secretion (53). As such, the impact of SM on Mtb-specific immune responses may be quite different from what has previously been reported in individuals co-infected with Mtb and either filarial or soil transmitted helminths. Despite this, the impact of co-infection with SM on Mtb-specific TH1 responses has not been thoroughly investigated in humans. One study in mice reported that SM infection impairs Mtb-specific TH1 CD4 T cell responses and increases arginase-1 expressing macrophages in type 2 granulomas (129). Furthermore, data regarding the impact of SM on Mtb-specific immune responses in general is conflicting. In mice, SM has no impact on Mtb-specific cytokine production or antibody responses generated by DNA vaccination (130,131); however, it impairs Mtb-specific cytokine production following BCG vaccination and results in higher CFU of both BCG and Mtb (132,133). In humans, SM infection does not impair the generation of Mtb-specific T and B cells by the TB candidate vaccine MVA85A (134). In vitro studies of human PBMCs exposed to SM antigens have demonstrated skewing of Mtb-specific CD4 T cells from a TH1 to a TH2 response; however, human monocyte-derived macrophages exposed to the same antigens have produced contradictory results with one study showing enhanced control and another showing impaired control of Mtb replication in vitro (135,136). It therefore remains unclear what impact, if any, co-infection with SM has on Mtb-specific immune responses, particularly in humans.

We sought to test the hypothesis that SM infection modifies the lineage profile of Mtb-specific CD4 T cells away from a dominant TH1 towards a TH2 phenotype. TH1 cells are characterized by their production of key TH1 cytokines such as IFNγ and their expression of specific lineage markers, namely the transcription factor T-bet and/or the chemokine receptor CXCR3 (86,109,110). TH2 cells can be similarly characterized by their production of TH2

cytokines such as IL-4 and their expression of the transcription factor GATA3 and/or the chemokine receptor CCR4 (86,109,110). While CD4 T cell subsets have been defined by canonical transcription factors and chemokine receptors, there is growing appreciation for the variability and plasticity of CD4 T cells (137–140). As such, we performed a comprehensive analysis of the lineage state of CD4 T cells in a well characterized cohort of Kenyan adults representing a spectrum of Mtb infection and disease. We enrolled individuals in groups defined by Mtb and SM infection status: healthy controls (HC), latent TB infection (LTBI), and active TB (TB), both with and without SM infection. In each group we examined CD4 T cells for the expression of cytokines, transcription factors, and chemokine receptors associated with TH1 and TH2 lineage commitment simultaneously. This allowed us to evaluate CD4 T cell lineage using both phenotypic and functional readouts; moreover, we measured co-expression of these markers to determine the variability and plasticity of the Mtb-specific CD4 T cell repertoire in the setting of human Mtb and SM co-infection.

2.3 Materials and Methods

Study Population and Sample Collection:

Participants 18-81 years old were recruited in Kisumu, Kenya as described previously (141). Healthy asymptomatic individuals with no previous history of TB disease or treatment were evaluated by QuantiFERON®-TB Gold In-Tube (QFT) assay: those with a negative QFT result (QFT^-; TB Ag-Nil <0.35 IU IFNγ/mL) were defined as healthy controls (HC); those with a positive QFT result (TB Ag-Nil >0.35 IU IFNγ/mL) were defined as having latent Mtb infection (LTBI). All HC and LTBI participants had normal chest x-rays. Patients with drug-sensitive active pulmonary tuberculosis disease (TB) were symptomatic individuals with a positive GeneXpert MTB/RIF result and a positive culture for Mtb growth. Blood was collected from individuals with active TB within the first 7 days of initiating the standard 6-month course of TB treatment, which was provided according to Kenyan national health guidelines. Chest x-rays were not performed on individuals with active TB. All participants are presumed to be BCG vaccinated due to the Kenyan policy of BCG vaccination at birth and high BCG coverage rates throughout Kenya (142,143). *Schistosoma mansoni* (SM) infection was determined using standard Kato Katz microscopy. Briefly, two thick Kato Katz smears were prepared from stool samples collected on two separate days. Slides were analyzed by experienced lab technicians who recorded the presence of SM eggs as well as the number of eggs counted. Participants were excluded if eggs belonging to other helminth species including *Ascaris lumbricoides*, *Trichuris trichuria* and hookworm were identified. Participants were not tested for lymphatic filariasis since it is not endemic in western Kenya (44). Other exclusion criteria included: pregnancy, hemoglobin value of <7.0 g/dl, HIV infection, and positive rapid malaria test. Blood was collected from patients in sodium heparin Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (BD Biosciences). PBMC were isolated

by density centrifugation, cryopreserved in freezing medium (50% RPMI 1640 + 40% heat-inactivated fetal calf serum [FCS] + 10% DMSO), and stored in LN_2 until use.

Ethics Statement:

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All participants gave written informed consent for the study, which was approved by the KEMRI/CDC Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

Antigens:

This study utilized peptide pools of the immunodominant Mtb antigens CFP-10 and ESAT-6 (1 μg/ml of each peptide). Pools of 15-mer overlapping peptides spanning the full-length sequences of CFP-10 and ESAT-6 were obtained through BEI Resources, NIAID, NIH (catalog numbers NR-50712 and NR-50711, respectively). For the overnight intracellular cytokine staining assay, phorbol 12-myristate 13-acetate, (PMA, 50 ng/mL, Adipogen) and ionomycin (1 μg/mL, Cayman Chemical) were used as a positive control. In the 5-day proliferation assay, Staphylococcal enterotoxin B (SEB; 1 μg/mL, Toxin Technology, Inc.) was used as a positive control for proliferation while PMA and ionomycin were used to induce cytokine production for the final 5 hours of the proliferation assay.

Antibodies:

The following human monoclonal fluorescently-conjugated antibodies were used in this study: anti-CD3 BV605 (clone OKT-3), anti-CD4 BV570 (clone RPA-T4), anti-CCR4 BV421

(clone L291H4), anti-T-bet PE-Cy7 (clone 4B10), anti-TNFα Alexa Flour 647 (clone Mab11), and anti-IL-4 PE-Dazzle594 (clone MP4-25D2), all from BioLegend; anti-CD4 BV786 (clone SK3), anti-CD8 PerCP-Cy5.5 (clone SK-1), anti-CXCR3 BV711 (clone 1C6), anti-GATA3 PE (clone L50-823), and anti-IFN-γ Alexa Fluor 700 (clone B27), all from BD Biosciences; and anti-IL-13 FITC (clone 85BRD) from eBiosciences.

PBMC overnight intracellular cytokine staining (ICS) assay:

Cryopreserved PBMCs were thawed in a 37°C water bath and immediately added to RPMI 1640 (Cellgro) containing deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich). Cells were washed twice in RPMI and then suspended in R10 media (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum [FCS], 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) and rested for a minimum of 3 h at 37°C and 5% CO_2 before the addition of antigens (described above). Cells incubated in R10 media alone served as a negative control. After 3 hrs, brefeldin A (10 μg/ml; Sigma-Aldrich) and monensin (1x, BioLegend) were added and the incubation continued for an additional 15 hrs.

PBMC proliferation assay:

Cryopreserved PBMCs were thawed, washed in PBS containing deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich). Cells were washed twice in PBS and then labeled with 0.5 μg/ml CellTrace™ Oregon Green® 488 carboxylic acid diacetate, succinimidyl ester (OG; Life Technologies). Cells were washed once more with PBS and resuspended in R10 media (RPMI 1640 supplemented with 10% heat-inactivated human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) containing recombinant human IL-2 (10 Units/mL, obtained

through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) (144). Cells were plated in 96-well plates and incubated for 5 days in a 37°C incubator with 5% CO_2. On day 5, 75 μl of cell culture supernatant per well were removed and stored for Luminex analysis (see below). Cells were then resuspended in 250 μL R10 media. With the exception of the negative control (wells containing cells in media alone) cells were re-stimulated with PMA and ionomycin (described above) and treated with brefeldin A (10 μg/ml; Sigma-Aldrich) and monensin (1x, Biolegend) for 5 hrs at 37°C to determine the cytokine capacity of proliferating CD4 T cells.

Antibody staining for flow cytometry:

Following stimulation, cells were washed with PBS and stained with the Fixable Viability Dye Zombie Near-IR (BioLegend) for 15 minutes at room temperature. Samples were then surface stained for 30 minutes at room temperature. For the ICS assay this included: anti-CD3 BV605, anti-CD4 BV570, anti-CD8 PerCP-Cy5.5, anti-CCR4 BV421, and anti-CXCR3 BV711. For the proliferation assay: anti-CD3 BV605, CD4 BV786, anti-CD8 PerCP-Cy5.5, anti-CCR4 BV421, and anti-CXCR3 BV711. Following the surface stain, cells were fixed and permeabilized on ice for 1 hr using the FoxP3 Transcription Staining Buffer Set (eBioscience). Cells were then stained for intracellular markers on ice for 40 min. For the ICS assay this included: anti-T-bet PE-Cy7, anti-GATA3 PE, anti-IFN-γ Alexa Fluor 700, anti-TNFα Alexa Flour 647, anti-IL-4 PE-Dazzle594, and anti-IL-13 FITC. For the proliferation assay this included: anti-T-bet PE-Cy7, anti-GATA3 PE, anti-IFN-γ Alexa Fluor 700, anti-TNFα Alexa Flour 647, and anti-IL-4 PE-Dazzle594. Finally, cells were washed in permeabilization buffer and resuspended in PBS. Samples were acquired using a BD LSR II flow cytometer.

Luminex:

Cell culture supernatants from each well were harvested on day 5 of the proliferation assay described above. Supernatants were frozen and stored at −80°C until use. The following cytokines were measured using a customized *R&D* Human Magnetic Luminex Assay kit (Biotechne) in batched analyses, following the manufacturer's instructions: IFNγ, TNFα, IL-21, IL-22, IL-17, IL-4, IL-5, IL-10, IL-13. The mean fluorescence intensity was read for each cytokine using a Luminex MAGPIX® system with xPONENT® software (Version 4.2) and analyzed using MILLIPLEX® Analyst 5.1 Software. Cytokine data from antigen-stimulated wells are reported after subtraction of corresponding cytokine levels in the negative control wells.

Data analysis:

Flow cytometry data were analyzed using FlowJo version 9.6.4 (BD). Compensation was calculated using single-stained anti-mouse Ig,κ CompBeads (BD Biosciences). Single cells were gated by plotting forward scatter-area versus forward scatter-height; lymphocytes were gated based on morphological characteristics. Viable cells were defined as Zombie Near-IR^{lo} cells. CD4 T cells were defined as CD3^{+}CD4^{+}CD8^{-} lymphocytes. Antigen-specific CD4 T cell populations were defined as cells producing cytokines (IFN-γ, TNF-α, IL-4 and/or IL-13) after stimulation with antigen. Proliferating cells were defined as those with low expression of the cytosolic dye Oregon Green (OG^{lo}). The flow cytometry gating strategy is indicated in Supplementary Figure 2-1. Responses were evaluated using the mixture models for single-cell assays (MIMOSA) method to determine positivity using a Markov Chain Monte Carlo algorithm with a prior of 0.01% in the ICS assay and a prior of 1% in the proliferation assay (145). Samples with a probability of response >70% and a false discovery rate (fdr/q-value) <3% were considered positive. Phenotypic

analysis of lineage marker expression on antigen-specific CD4 T cells was restricted to individuals who met the above criteria for a positive response.

Statistical analysis:

R programming software was used to perform all statistical analyses. Differences between SM^+ and SM^- individuals within each stratum of Mtb infection were evaluated using a non-parametric Mann-Whitney test. Differences between three or more groups were evaluated using a non-parametric Kruskal-Wallis test and corrected for multiple comparisons using the Bonferroni method. Correlations were evaluated using a non-parametric Spearman rank correlation. *P*-values < 0.05 were considered significant. Graphs were created using the R package ggplot2 and statistics were performed using the stats package.

2.4 Results

Study participants.

Participants were recruited and enrolled in Kisumu, Kenya and categorized into six groups based on their Mtb and SM infection status: HC, LTBI and TB, with or without concomitant SM infection (Table 2-1). Within each Mtb infection group, SM^+ and SM^- individuals had similar demographic profiles with two exceptions. First, there were more females in the LTBI SM^- group than the LTBI SM^+ group. In addition, amongst active TB individuals, SM^+ individuals were older than those that were SM^-.

Similar frequencies of CD4 T cells express TH1 and TH2 lineage markers in SM^+ and SM^- individuals independent of Mtb infection status.

Infection with helminths is associated with skewing of the immune system to a TH2 CD4 T cell response (112–115). To investigate the impact of SM infection on the CD4 T cell repertoire, we utilized flow cytometry to measure the expression of TH1 and TH2 lineage markers in total CD4 T cells (Figure 2-1A). This analysis was done within each stratum of Mtb infection to account for a possible differential impact of SM within diverse infection states. We first measured the frequency of CD4 T cells expressing the canonical TH1 and TH2 transcription factors, T-bet and GATA3, respectively (Figure 2-1B). Only HC exhibited significant differences in transcription factor expression, with SM^+ individuals having a lower frequency of T-bet^+ CD4 T cells than SM^- counterparts. This difference is not observed in LTBI or TB groups. Importantly, the frequency of transcription $factor^+$ CD4 T cells was not dominated by either transcription factor, demonstrating a balance of these responses in the total CD4 T cell repertoire. We then measured the expression of the chemokine receptors CXCR3 and CCR4, which are associated with TH1 and TH2 lineage

commitment, respectively (Figure 2-1B). CXCR3 expression was similar across groups whereas CCR4 expression was higher in SM^+ TB individuals compared to SM^- TB individuals. These data indicate that across Mtb infection states, SM does not substantially alter the baseline lineage phenotype of total CD4 T cells of individuals.

SM infection does not bias the capacity of CD4 T cells to produce TH1 and TH2 cytokines

In addition to phenotypic markers, CD4 T cell polarization can be evaluated by cytokine production. As such, we sought to investigate whether there were differences in the cytokine profiles of CD4 T cells in individuals infected with SM. To this end, we stimulated PBMC with PMA and ionomycin and then performed intracellular cytokine staining (ICS) for TH1 cytokines IFNγ and TNFα, as well as TH2 cytokines IL-4 and IL-13 (Figure 2-2A). We used flow cytometry to quantify the production of these cytokines individually as well as in combination. There were no differences in the total frequency of CD4 T cells producing any of the four cytokines between SM^+ and SM^- individuals across Mtb infection groups (Figure 2-2B). Furthermore, the median fluorescence intensity (MFI) of each cytokine measured did not differ between SM^+ and SM^- individuals (data not shown). Further analysis was done to evaluate combinations of cytokines being produced using a Boolean gating strategy. In all Mtb infection groups, the frequency of each combination of cytokines was similar between SM^+ and SM^- individuals (Supplementary Figure 2-2).

To evaluate the functional lineage state of CD4 T cells, we collapsed these Boolean gates into three categories of cells: TH1, TH2, and TH1/2 (Supplementary Figure 2-2). Briefly, TH1 cells were defined as cells producing IFNγ and/or TNFα but not IL-4 or IL-13, TH2 cells were defined as cells producing IL-4 and/or IL-13 but not IFNγ or TNFα, and TH1/2 cells were defined

as cells producing a combination of IFNγ or TNFα and IL-4 or IL-13. No significant differences in the frequencies of these cell subsets were observed between SM^+ and SM^- individuals in all three Mtb infection groups (Figure 2-2C). Furthermore, in all of the participant groups, the frequency of TH1 cytokine$^+$ CD4 T cells was significantly higher than that of either TH2 or TH1/2 cytokine$^+$ CD4 T cells. Together, these data provide further evidence that SM does not modify the TH1 and TH2 cytokine production capacity of CD4 T cells across Mtb infection groups.

TH1 cytokine$^+$ CD4 T cells express low to moderate levels of TH1 lineage markers while TH2 cytokine$^+$ CD4 T cells express high levels of TH2 lineage markers independent of SM and Mtb infection status

We next analyzed expression of lineage specific markers on cytokine$^+$ CD4 T cells following stimulation with PMA and ionomycin. We utilized our functional lineage gating definitions (Supplementary Figure 2-2) to define TH1 and TH2 cells and then used MIMOSA (see materials and methods) to evaluate samples with a positive cytokine$^+$ response for expression of T-bet, GATA3, CXCR3, and CCR4 on each cytokine subset of CD4 T cells (Figure 2-3A).

In HC, LTBI and TB groups, TH1 cytokine$^+$ CD4 T cells had intermediate expression of TH1 lineage markers (Figure 2-3B). As expected, TH1 cytokine$^+$ cells were almost exclusively GATA3$^-$, however only low to moderate frequencies were T-bet$^+$. In addition, TH1 cytokine$^+$ cells had low to moderate frequencies of CXCR3$^+$ and CCR4$^+$ cells. These expression profiles of TH1 cytokine$^+$ CD4 T cells did not differ by SM or Mtb infection status.

By contrast, TH2 cytokine$^+$ CD4 T cells have more distinct TH2-like lineage marker expression in HC, LTBI and TB groups (Figure 2-3B). Approximately half the TH2 cytokine$^+$ cells in each group were GATA3$^+$, with only a minority of cells expressing T-bet. TH2 cytokine$^+$ cells

were also predominantly CCR4$^+$ with low frequencies of CXCR3$^+$ cells. These expression profiles of TH2 cytokine$^+$ CD4 T cells did not differ by SM or Mtb infection status. These data demonstrate that CD4 T cells, as defined by TH1 and TH2 cytokine production, do not strictly adhere to canonical expression patterns of TH1 and TH2 phenotypic markers.

LTBI individuals co-infected with SM have higher frequencies of TH1 cytokine$^+$ Mtb-specific CD4 T cells, compared with SM$^-$ individuals with LTBI

Having established that SM infection does not impair the capacity of CD4 T cells to produce TH1 cytokines, nor does it skew CD4 T cells in general towards a TH2 phenotype, we next evaluated the impact of SM on Mtb-specific CD4 T cell responses in individuals with LTBI. We utilized our ICS assay to measure the frequency of cytokine$^+$ CD4 T cells after stimulation of PBMC with CFP-10 and ESAT-6 peptide pools (Figure 2-4A). The frequency of IFNγ^+ CD4 T cells was significantly higher in SM$^+$ than SM$^-$ individuals (Figure 2-4B). When cytokine production was analyzed across combinations of cytokines, this difference was found to be due to a population of cells co-producing IFNγ and TNFα (Supplementary Figure 2-3A). Importantly, there was no difference in Mtb-specific TH2 cytokine production between SM$^+$ and SM$^-$ individuals (Figure 2-4B) nor in the MFI of TH2 cytokines (data not shown). Furthermore, TH1 cytokines were the dominant response to CFP-10 and ESAT-6 peptide pools in both groups (Supplementary Figure 2-3B). These data indicate that unlike other helminth co-infections, SM infection is associated with increased frequencies of Mtb-specific TH1 cytokine$^+$ CD4 T cells amongst individuals with LTBI.

We next evaluated T-bet, GATA3, CXCR3, and CCR4 expression by TH1 cytokine$^+$ Mtb-specific CD4 T cells (Figure 2-4C). Similar to PMA/ionomycin-induced TH1 cytokine$^+$ CD4 T

cells, Mtb-specific TH1 cytokine$^+$ CD4 T cells expressed moderate levels of T-bet. Interestingly, there were also GATA3$^+$ TH1 cytokine$^+$ cells, though these frequencies were low (<10% of Mtb-specific TH1 cytokine$^+$ CD4 T cells) and did not differ by SM infection status (Figure 2-4C). Further analysis of these Mtb-specific TH1 cytokine$^+$ CD4 T cells indicated that they did not co-express GATA3 and T-bet (Supplementary Figure 2-3C). Moderate frequencies of Mtb-specific TH1 cytokine$^+$ cells expressed CXCR3$^+$ and/or CCR4$^+$, although this did not differ by SM infection status (Figures 2-4C and Supplementary 2-3C). These data indicate that Mtb-specific CD4 T cells in individuals with LTBI express predominately TH1 cytokines and are not skewed towards a TH2 phenotype in the presence of SM co-infection.

Individuals with active TB disease and SM co-infection have higher frequencies of GATA3$^+$CCR4$^+$ TH1 cytokine$^+$ Mtb-specific CD4 T cells

We next evaluated the impact of SM on Mtb-specific CD4 T cell responses in individuals with active TB disease (Figure 2-5A). The total frequency of each cytokine was similar between SM$^+$ and SM$^-$ individuals (Figure 2-5B). We confirmed these results by comparing the MFI of each cytokine, which did not differ between SM$^+$ and SM$^-$ groups (data not shown). Indeed, TH1 cytokines remained the dominant CD4 T cell response to Mtb in both SM$^+$ and SM$^-$ individuals with active TB disease, and the frequency of these cells did not differ between groups (Supplementary Figure 2-4A, 2-4B).

We next evaluated T-bet, GATA3, CXCR3, and CCR4 expression by TH1 cytokine$^+$ Mtb-specific CD4 T cells (Figure 2-5C). Consistent with the LTBI group, there were more T-bet$^+$ than GATA3$^+$ Mtb-specific TH1 cytokine$^+$ CD4 T cells; however, while T-bet$^+$ frequencies were similar between SM$^+$ and SM$^-$ individuals, GATA3$^+$ frequencies were significantly higher in SM$^+$

individuals compared to SM^- individuals. The total frequencies of $CCR4^+$ and $CXCR3^+$ TH1 $cytokine^+$ cells did not differ by SM infection (Figure 2-5C). However, there were significantly higher frequencies of Mtb-specific CD4 T cells co-expressing GATA3 and CCR4 in the SM^+ TB group, compared with SM^- TB group (Figure 2-5D). When co-expression of these markers was analyzed further, this population was strictly limited to $T\text{-}bet^-$ cells (Supplementary Figure 2-4C). Together these data indicate while Mtb-specific TH1 cytokine production is preserved in SM^+ individuals with active TB, these TH1 $cytokine^+$ cells express higher levels of the canonical TH2 markers GATA-3 and CCR4, compared with Mtb-specific CD4 T cells from SM^- TB individuals.

SM infection does not significantly impair CD4 T cell proliferative capacity

To further evaluate the functional capacity of CD4 T cells in the setting of SM infection, we next performed a proliferation assay. PBMC from each group were labeled with the cytosolic dye Oregon Green (OG) and incubated for 5 days either with media alone (negative control), SEB (positive control), or Mtb CFP-10 and ESAT-6 peptide pools. We then measured proliferation via flow cytometry (Figure 2-6A). No significant differences in CD4 T cell proliferative capacity were observed following stimulation with SEB between SM^+ and SM^- individuals in any of the Mtb infection groups (Figure 2-6B). We next measured the proliferative capacity of Mtb-specific CD4 T cells in individuals with either LTBI or active TB. There were no statistically significant differences in CD4 T cell proliferation following stimulation with Mtb peptides between SM^+ and SM^- individuals in either group; however, within the SM^+ groups, TB individuals had markedly lower proliferation in response to Mtb peptides than LTBI individuals (Figure 2-6C), consistent with previous reports of impaired Mtb-specific CD4 T cell proliferative capacity in individuals

with active TB disease (146). These data indicate that TB disease, but not SM infection status, impacts the ability of CD4 T cells to proliferate in response to Mtb peptides.

Proliferating CD4 T cells express TH1 and TH2 lineage markers in SM$^+$ and SM$^-$ LTBI individuals

We next assessed the lineage state of CD4 T cells that proliferate in response to SEB and Mtb peptides. On day 5 of the proliferation assay, PMA and ionomycin was added to OG-labeled PBMCs for 5 hrs to induce cytokine expression, and the cells evaluated by flow cytometry (Figure 2-7A, Supplementary Figure 2-5A). SEB stimulation induced proliferating CD4 T cells with robust cytokine production capacity, dominated by TH1 cytokines IFNγ and TNFα. The frequencies of these cells did not differ between SM$^+$ and SM$^-$ individuals in any Mtb infection group (Supplementary Figure 2-5B). Proliferating CD4 T cells were also evaluated for their expression of T-bet, GATA3, CXCR3, and CCR4 (Supplementary Figure 2-5C). After stimulation for 5 days with SEB, proliferating CD4 T cells expressed high levels of T-bet, GATA3, and CCR4. The frequency of T-bet$^+$ proliferating CD4 T cells was higher than the frequency of GATA3$^+$ CD4 T cells, whereas the frequency of proliferating CCR4$^+$ CD4 T cells was higher than the frequency of CXCR3$^+$ CD4 T cells (Supplementary Figure 2-5C). None of the markers differed in expression between SM$^+$ and SM$^-$ individuals. Together these data indicate that there are no intrinsic differences in the lineage phenotypes of proliferating of CD4 T cells attributable to SM infection.

We then evaluated the expression of lineage specific cytokines and phenotypic markers in proliferating Mtb-specific CD4 T cells (Figure 2-7A). Within the LTBI group, there were similar frequencies of cytokine$^+$ proliferating Mtb-specific CD4 T cells between SM$^-$ and SM$^+$ individuals (Figure 2-7B). Boolean analysis of cytokine$^+$ CD4 T cells indicated that proliferating Mtb-specific CD4 T cells were dominated by IFNγ$^+$TNFα$^+$ cells (Supplementary Figure 2-6A). Proliferating

Mtb-specific CD4 T cells expressed high levels of T-bet and moderate levels of GATA3 (Figure 2-7C). There were also moderate frequencies of $CXCR3^+$ and high frequencies of $CCR4^+$ proliferating cells. Expression of these four markers was also measured utilizing a Boolean strategy. Interestingly, in contrast to the overnight ICS assay where most Mtb-specific cells were $CXCR3^+$ and/or $CCR4^+$ but negative for transcription factors, a majority of proliferating Mtb-specific CD4 T cells expressed at least one transcription factor. There was also a substantial fraction of cells that expressed both T-bet and GATA3 (Supplementary Figure 2-6B). These data indicate that while Mtb-specific CD4 T cells predominantly produce TH1 cytokines, they are diverse with regards to their expression of TH1 and TH2 lineage markers.

IFNγ correlates more strongly with both TH1 and TH2 cytokines in SM^+ than SM^- LTBI individuals

We next used a Luminex assay to measure cytokine production in PBMC culture supernatants from the 5-day proliferation assay. This allowed us to evaluate a broader range of cytokines, representing multiple T helper subsets, than the ICS assay which was limited to 3-4 cytokines. We collected supernatants on day 5 following stimulation with Mtb CFP-10/ESAT-6 peptide pools, just prior to the addition of PMA and ionomycin. We then measured cytokines associated with TH1 (IFNγ, TNFα), TH2 (IL-4, IL-13, IL-5), TH17 (IL-17A, IL-22) and T-regulatory (IL-10) responses. All nine cytokines were produced at comparable levels between SM^+ and SM^- individuals to Mtb peptides (Figure 2-8A) as well as to SEB (data not shown).

We also performed correlation analysis to determine which cytokines were most strongly associated with one another. We separated this analysis by SM infection to further evaluate whether SM infection modulates the relationship between different cytokines produced in response

to Mtb CFP-10/ESAT-6 peptides. IL-5 did not correlate significantly with any other cytokine, and IL-17A correlated significantly with IL-22, but only in the SM^+ group. The remaining cytokines all displayed significant positive correlations with each other, many of which differed between SM groups (Figure 2-8B). In particular, the relationship of IL-10 and IFNγ to the remaining cytokines differentiated these two groups. In SM^- individuals, IL-10 correlated more strongly with IFNγ, IL-4, and IL-22, whereas in SM^+ individuals, IFNγ correlated more strongly with TNFα, IL-4, IL-13, IL-21, and IL-22 (Figure 2-8B). Together, these data suggest that while individual cytokine levels are similar between SM^+ and SM^- individuals, SM infection modifies the relationship between different cytokines produced in response to Mtb peptide stimulation, particularly with regards to the TH1 cytokine IFNγ.

2.5 Discussion

Infections with a variety of helminths have been shown to dysregulate Mtb-specific CD4 T cell immunity (113–115), however a thorough analysis of CD4 T cell lineage commitment during co-infection with SM and Mtb in humans has not been conducted. We hypothesized that SM infection would modulate the TH1 vs TH2 lineage profile of Mtb-specific CD4 T cell responses. While there are numerous CD4 T cell subsets, we focused on TH1 and TH2 cells because of their known associations with Mtb and helminth infections respectively. We found that TH1 cytokine responses are preserved in both the total and Mtb-specific CD4 T cell compartment of SM^+ and SM^- individuals. Moreover, SM^+ individuals with LTBI had significantly higher frequencies of Th1 cytokine$^+$ Mtb-specific CD4 T cells, compared with SM^- individuals with LTBI. We also provide evidence that TH1 cytokine$^+$ CD4 T cells are flexible in their expression of lineage markers irrespective of Mtb and SM infection status. Lastly, we found that SM modulates the lineage expression profile of TH1 cytokine$^+$ CD4 T cells in individuals with TB, but not LTBI.

Our data indicate that there are limited differences in the TH1 and TH2 profiles of total CD4 T cells attributable to SM infection. We observed fewer circulating TH1 cells, defined as T-bet$^+$ CD4 T cells, in SM^+ healthy controls than in SM^- healthy controls. However, this difference was not observed in the LTBI and TB groups, perhaps due to increased immune activation in Mtb infected individuals at baseline (28,33,147). We also observed higher frequencies of circulating CCR4$^+$ CD4 T cells in SM^+ TB individuals, consistent with a previous study indicating elevated CCR4 expression on CD4 T cells in individuals with active TB (148). Although CCR4 is predominately expressed on TH2 cells, it can be expressed on CD4 T cell subsets other than TH2 (149). Lastly, upon mitogen stimulation, the CD4 T cell cytokine profiles of SM^+ and SM^-

individuals did not differ in any of the three Mtb infection groups, thus providing further evidence that SM does not globally dysregulate TH1 and TH2 CD4 T cell subsets. Interestingly, there appeared to be a trend towards differences in lineage specific phenotype by Mtb infection status, with CD4 T cells from individuals with active TB having generally lower Th1 cytokine production capacity (Figure 2-2) and a trend towards higher expression of CCR4 and lower expression of CXCR3 on Mtb-specific CD4 T cells, compared with individuals with LTBI (Figures 2-4 & 2-5). This suggests that Mtb infection may have a dominant influence over SM infection on phenotypes of CD4 T cells in peripheral blood. Further studies would need to be conducted to directly evaluate TH lineage phenotype differences by Mtb infection and disease status.

Our findings also provide strong evidence that CD4 T cells are flexible in their expression of lineage specific phenotypic markers. We examined TH1 and TH2 cells, as defined by cytokine production, for the expression of canonical TH1 and TH2 transcription factors and chemokine receptors. T-bet and GATA3 were almost exclusively expressed in TH1 cytokine$^+$ and TH2 cytokine$^+$ CD4 T cells respectively and therefore did indeed stratify by functional CD4 T cell subset. By contrast, CXCR3 and CCR4 were expressed on both TH1 cytokine$^+$ and TH2 cytokine$^+$ CD4 T cells. In addition, while TH2 cytokine$^+$ CD4 T cells were predominantly GATA3$^+$ and/or CCR4$^+$, TH1 cytokine$^+$ CD4 T cells were often T-bet$^-$ and/or CXCR3$^-$. Taken together, this suggests that defining CD4 T cell subsets based on either function or phenotype is not sufficient and does not capture the full variability of these cells. Importantly, this variability is not explained by different immune states of the host since this phenomenon is not different between SM$^+$ and SM$^-$ individuals, nor between Mtb infection groups. There is growing appreciation that CD4 T cells do not strictly segregate into classical CD4 T cell subsets. Indeed, numerous studies in humans have observed co-production of cytokines as well as co-expression of transcription factors

from multiple CD4 T cell lineages at the single cell level (125,150–154). In many cases, these intermediate cells are associated with differential disease states. Our data supports the concept of CD4 T cell plasticity in humans, however the functional impact of this plasticity requires further study.

Importantly, lineage diversity was observed in Mtb-specific CD4 T cell responses in both LTBI and TB groups. Both groups had TH1 cytokine$^+$ Mtb-specific CD4 T cells that expressed not only TH1 but also TH2 lineage markers. Although there was a bias towards T-bet$^+$ cells in the overnight assay, GATA3$^+$ cells were also detected in the Mtb-specific CD4 T cell repertoire. Furthermore, in both SM$^+$ and SM$^-$ individuals in the LTBI group, proliferating Mtb-specific CD4 T cells expressed both TH1 and TH2 markers and expressed TH1 cytokines upon restimulation with PMA and ionomycin. This is consistent with previous studies which have reported of co-expression of lineage-specific transcription factors and transcriptional profiles in Mtb-specific CD4 T cells (125,153,155). Due to the low frequency of Mtb-specific proliferating CD4 T cells in TB group, consistent with previous reports (146), we were not able to evaluate TH1 and TH2 profiles of proliferating Mtb-specific CD4 T cells in individuals with active TB.

Our data also indicate that the impact of SM infection on Mtb-specific CD4 T cells is dependent on the Mtb infection status of the individual. Contrary to our initial hypothesis, we found that SM$^+$ LTBI individuals had higher frequencies of Mtb-specific TH1 cytokine producing CD4 T cells than SM$^-$ LTBI individuals. This difference was due to a subset of cells co-producing both TNFα and IFNγ, indicating that SM infection is associated with higher levels of polyfunctional TH1 cells. This differs from studies of co-infection with filarial worms and soil-transmitted helminths, which have reported lower Mtb-specific TH1 cytokine production and lower frequencies of Mtb-specific TH1 cytokine$^+$ CD4 T cells in LTBI individuals (112–115).

These differences may be due to differences in the life cycles of the worms, especially considering that the lung migrating stage of SM is TH1 stimulating (53). Amongst TB individuals, however, SM^+ and SM^- individuals did not differ in the frequency of Mtb-specific TH1 $cytokine^+$ cells. Again this is in contrast to most published studies which report lower TH1 responses in active TB individuals co-infected with either filarial worms or soil-transmitted helminths (115,121). Interestingly, SM^+ active TB individuals did have more $GATA3^+CCR4^+$ TH1 $cytokine^+$ Mtb-specific cells than SM^- active TB individuals. This is consistent with a recent study in Tanzania which reported mixed TH1/TH2 phenotypes in Mtb-specific CD4 T cells in individuals with active TB and helminth coinfection, although the participants in this study were not stratified by helminth species (125). It is difficult to determine whether this is due to TH1 $cytokine^+$ cells being skewed to a TH2 phenotype or whether phenotypically TH2 cells are being reprogrammed to produce TH1 cytokines. It has been shown that environmental cues can override programing inherent to lineage specific transcription factors thus providing immunity as needed (156).

The interpretation of our results is limited by cross-sectional enrollment with quota-based sampling. The order of SM and Mtb infection in these individuals may play a role in how CD4 T cells are polarized and respond to subsequent stimuli, although we are not able to confirm that order of SM and Mtb infection in our study cohorts. Infection with SM has been observed in children as young as 3 years old and reaches a prevalence rate of 60% in children 11-13 years old (157,158). Mass Drug Administration to treat SM infection in school-aged children in Kenya has been successful in reducing prevalence and intensity of SM infection, however reinfection still readily occurs (159). Furthermore, individuals defined as negative for SM infection may have had a previous SM infection are therefore not necessarily SM naïve. This may account for the lack of TH2 cytokine bias observed in the total CD4 T cell population. In addition, while we were able to

exclude participants infected with soil transmitted helminths, we did not test for current filarial infections in our study cohort. Although lymphatic filariasis is not endemic in western Kenya (44), other filarial infections have been shown to modulate host immunity (111,160) and could also influence T cell differentiation profiles of Mtb-specific CD4 T cell responses. Another limitation of our enrollment design is the inability to compare CD4 T cell profiles in SM^+ individuals before and after treatment for schistosomiasis. Studies in which individuals were treated for helminth infections have reported increased levels of Mtb-specific chemokines and cytokines following treatment (112,120,122,124). Our analysis was also confined to CD4 T cells specific to the Mtb antigens CFP-10 and ESAT-6, which are immunodominant Mtb antigens known to elicit robust IFNγ responses (161). CD4 T cell responses to other antigens in Mtb may have different cytokine profiles and may be more or less malleable to a TH2 stimulus such as a helminth co-infection. Furthermore, our analysis was limited to evaluation of CD4 T cells circulating in peripheral blood. It is possible that there is more pronounced TH2 skewing of CD4 T cells at the site of Mtb infection in the lung, which has been observed in mice co-infected with Mtb and SM (129). Lastly, by using flow cytometry to evaluate CD4 T cell lineage profiles, we were limited to specific markers for TH1 and TH2 lineage commitment. Future studies, using RNA-sequencing of Mtb-specific CD4 T cells can provide more comprehensive analysis of the Mtb-specific CD4 T cell repertoire including additional CD4 T cell subsets and functions.

The environment in which immune cells function is vast, diverse and constantly changing. It is of critical importance that CD4 T cells, which orchestrate the immune response to pathogens, commensals, and self, be able to interpret mixed signals and initiate the responses required for survival of the host. Furthermore, they must be able to flexibly respond to changes in the environment, whether they be competing stimuli, changes in antigen load, or changes in tissue

structure. We provide evidence that while infection with SM can skew the phenotype of CD4 T cells under certain conditions, it does not compromise the ability of CD4 T cells to mount a functional TH1 response to Mtb. Furthermore, the impact of SM on functional TH1 responses to Mtb depends on the clinical status of the Mtb infection, with co-infected LTBI individuals actually having higher Mtb-specific TH1 cytokine responses than those with LTBI alone. When considering that pathogens such as Mtb and SM are co-endemic in many areas of the world, this flexibility in the immune system is highly advantageous to the host in being able to mount immune responses to multiple different pathogens simultaneously.

2.6 Tables and Figures

Table 2-1: Characteristics of study participants

	Healthy Controls (HC)		Latent Mtb Infection (LTBI)		Active TB Disease (TB)	
	SM⁻ ***N=24***	**SM⁺** ***N=13***	**SM⁻** ***N=26***	**SM⁺** ***N=24***	**SM⁻** ***N=26***	**SM⁺** ***N=16***
Age (years) [a] (IQR)	24 (21-28)	25 (21-32)	36 (23-55)	34 (25-38)	28 (21-35)	40 [b] (26-45)
sex: (%M)	37.5	23.1	23.1	58.3 [c]	69.2	75.0
(%F)	62.5	76.9	76.9	41.7	30.8	25.0
Hemoglobin g/dl [a] (IQR)	13.5 (12.6 – 14.7)	13.6 (12.6 - 14.4)	12.9 (11.3 – 14.1)	14.0 (12.4 – 15.0)	12.1 (10.7 – 14.1)	11.7 (10.8 – 12.2)
SM eggs/gram [a] (IQR)	0	36 (12-120)	0	150 (36-333)	0	48 (21 - 87)
QFT IU/mL [a] (IQR)	0.00 (0.00 - 0.05)	0.00 (0.00 - 0.10)	7.94 (2.91 - 9.25)	9.11 (5.36 - 9.54)	ND [d]	ND [d]

[a] Value denotes median

[b] $p < 0.05$, compared with TB/SM⁻

[c] $p < 0.05$, compared with LTBI/SM⁻

[d] Not done

IQR, interquartile range

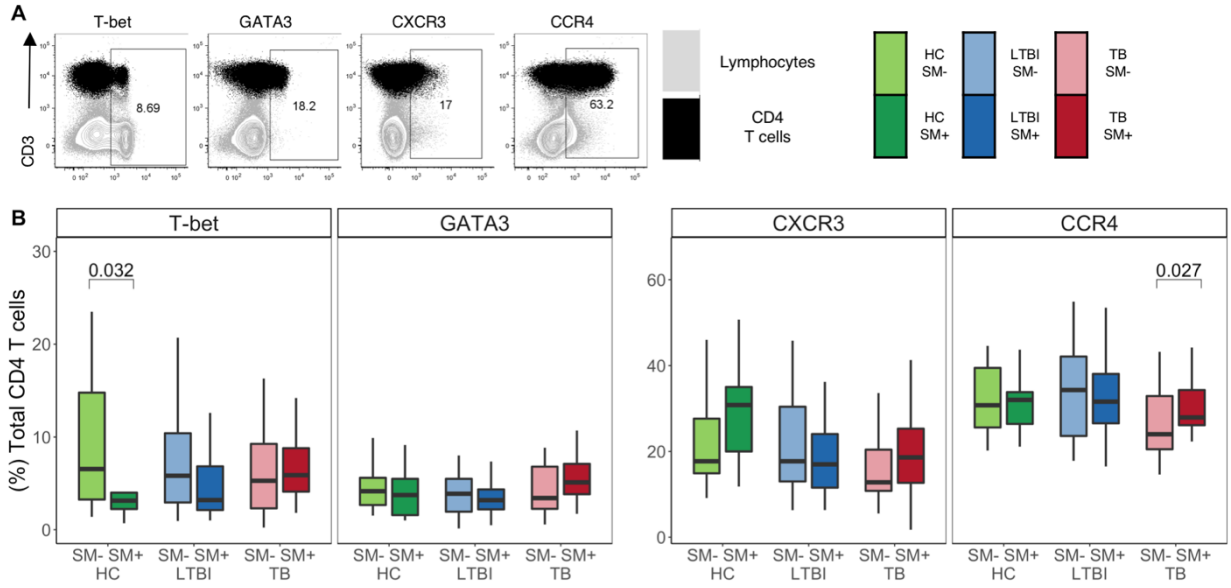


Figure 2-1: Similar frequencies of CD4 T cells express TH1 and TH2 lineage markers in SM$^+$ and SM$^-$ individuals, independent of Mtb infection status.

Expression of transcription factors and chemokine receptors was measured by flow cytometry using PBMC samples obtained from individuals in each of six groups defined by Mtb and SM infection status: HC, LTBI and TB, with or without concomitant SM infection (HC SM$^-$, n=24; HC SM$^+$, n=13; LTBI SM$^-$, n=25; LTBI SM$^+$, n=25; TB SM$^-$, n=25; TB SM$^+$, n=15). (A) Representative flow cytometry data from an SM$^+$ individual with active TB. Plots show cells gated on live CD3$^+$CD4$^+$CD8$^-$ lymphocytes in black dots overlaying live lymphocytes in gray. (B) Frequency of indicated transcription factor$^+$ and chemokine receptor$^+$ amongst total CD4 T cells. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each lineage marker$^+$ CD4 T cell population between SM$^+$ and SM$^-$ individuals were assessed using a Mann Whitney U test.

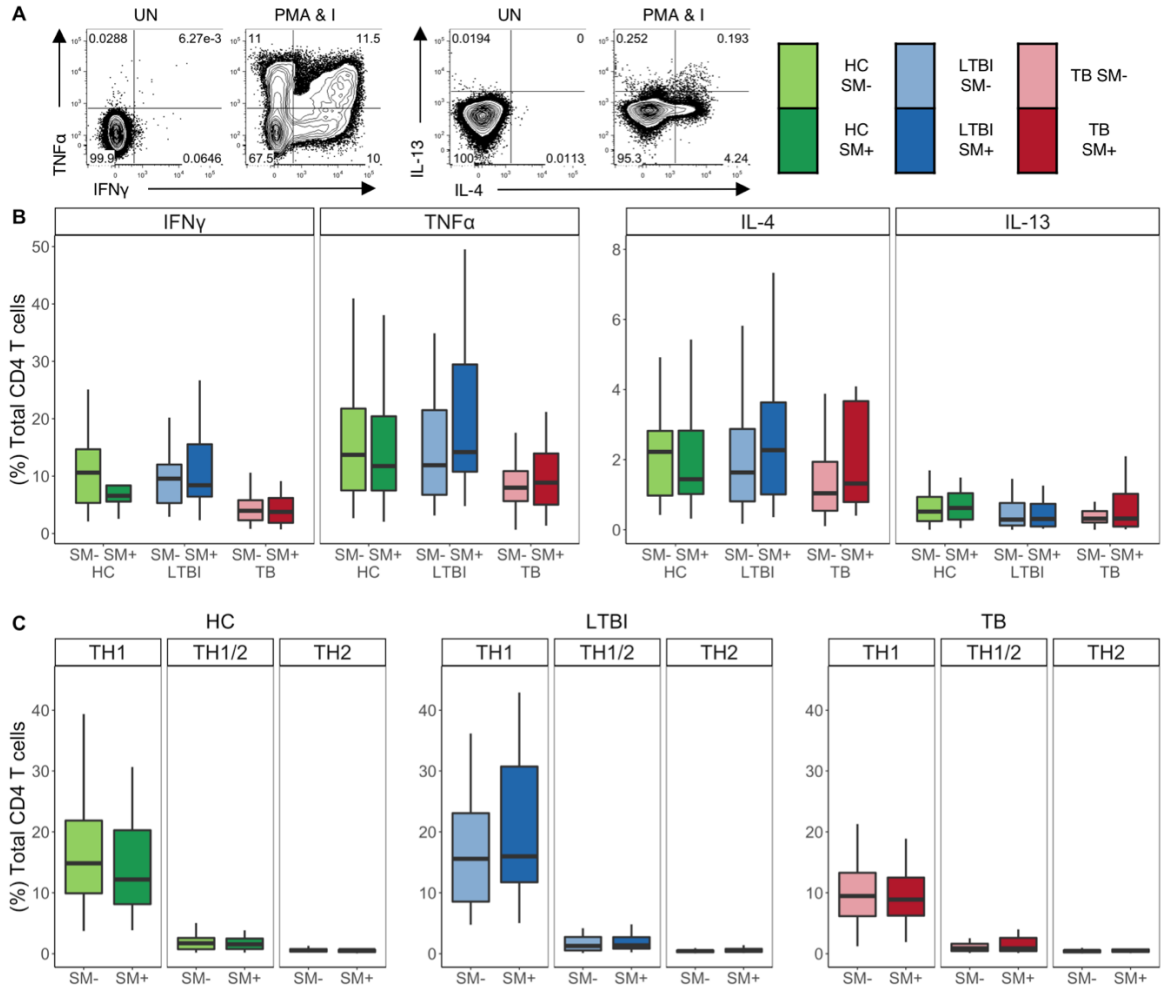


Figure 2-2: SM⁺ and SM⁻ individuals have similar frequencies of TH1 and TH2 cytokine⁺ CD4 T cells, with TH1 cytokines being the dominant response independent of SM and Mtb infection status.

PBMC samples obtained from individuals in each of six groups defined by TB and SM infection status were incubated for 18 h in media alone (negative control) or stimulated with PMA and ionomycin. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry (HC SM⁻, n=24; HC SM⁺, n=13; LTBI SM⁻, n=25; LTBI SM⁺, n=25; TB SM⁻, n=25; TB SM⁺, n=15). **(A)** Representative flow cytometry data from an SM⁺ HC. Plots are shown gated on live CD3⁺CD4⁺CD8⁻ lymphocytes from the unstimulated (UN) and stimulated condition. **(B)** Frequencies of total CD4 T cells expressing each indicated cytokine. **(C)** Cytokine⁺ cells were aggregated by T cell lineage (Figure S2) and the frequency of each group was reported. Data are shown after subtraction of background cytokine production in the unstimulated negative control condition. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each cytokine⁺ CD4 T cell population between SM⁺ and SM⁻ individuals were assessed using a Mann Whitney U test. Differences in the frequencies of TH1, TH1/2, and TH2 CD4 T cells within each group were evaluated using a Kruskal Wallis test. TH1 cytokine frequencies were statistically higher than the both TH1/2 and TH2 frequencies after applying the Bonferroni correction for multiple comparisons.

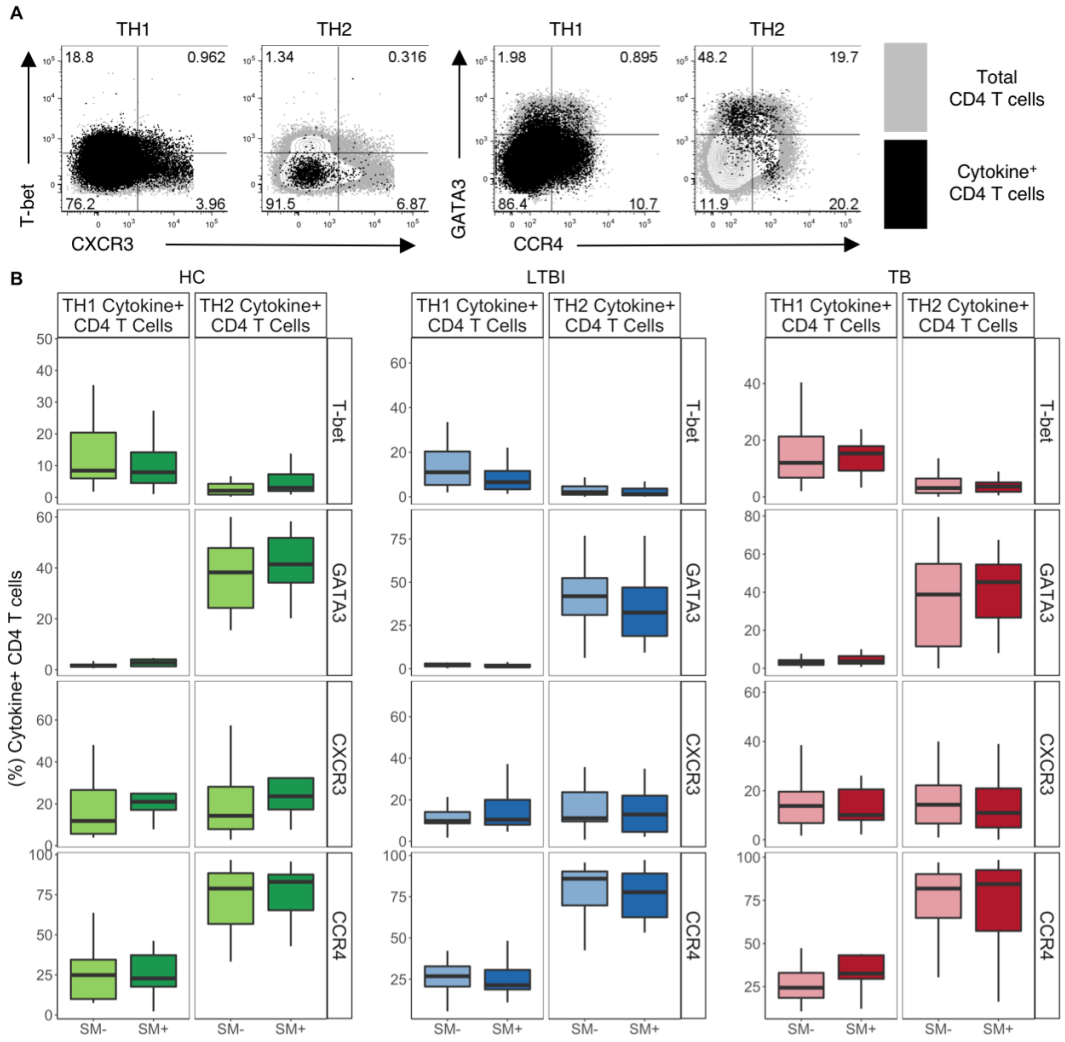


Figure 2-3: TH1 cytokine⁺ cells express low levels of TH1 lineage markers while TH2 cytokine⁺ CD4 T cells express high levels of TH2 lineage markers independent of SM and TB infection status.

PBMC samples obtained from individuals in each of six groups defined by Mtb and SM infection were incubated for 18 h in media alone (negative control) or stimulated with PMA and ionomycin. Intracellular expression of IFN-γ, TNF-α, IL-4 and IL-13 was evaluated by flow cytometry and cytokine⁺ cells were aggregated by functional T cell lineage (Figure S2). Samples meeting the criteria for a positive response (see Materials and Methods) were evaluated for expression of lineage specific transcription factors and chemokine receptors by flow cytometry. **(A)** Representative flow cytometry data from an SM⁻ HC. Plots show either TH1 cytokine⁺ or TH2 cytokine⁺ live $CD3^+CD4^+CD8^-$ lymphocytes in black dots overlaying total live $CD3^+CD4^+$ lymphocytes in gray. **(B)** Frequency of indicated transcription factor⁺ and chemokine receptor⁺ amongst TH1 cytokine⁺ or TH2 cytokine⁺ CD4 T cells in HC (SM^- , n=24 ; SM^+, n=13), LTBI (SM^-, n=25; SM^+, n=25), and TB (SM^-, n=25; SM^+, n=14). Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each lineage marker⁺ CD4 T cell population between SM^+ and SM^- individuals were assessed using a Mann Whitney U test.

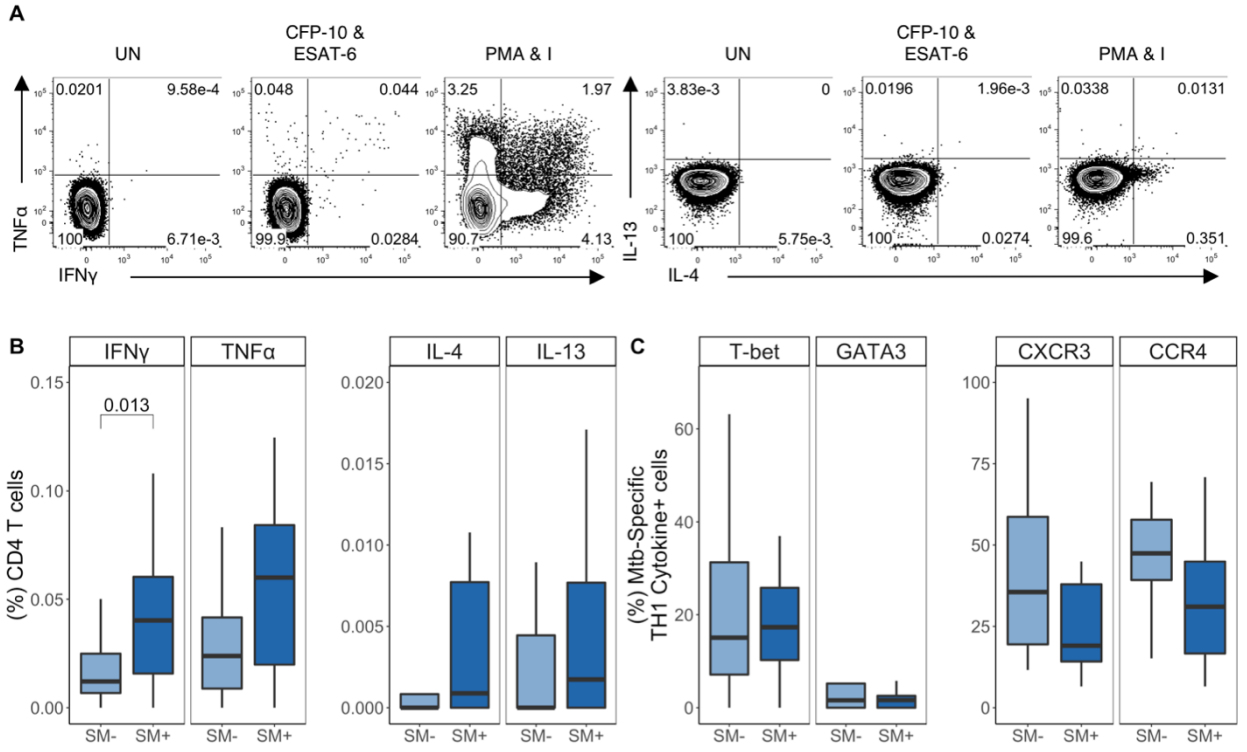


Figure 2-4: SM⁺ LTBI individuals have higher frequencies of TH1 cytokine⁺ Mtb-specific CD4 T cells, which express both TH1 and TH2 lineage markers.

PBMC samples obtained from SM⁺ and SM⁻ LTBI individuals were incubated for 18 h in media alone (negative control), PMA and ionomycin (positive control), or CFP-10 and ESAT-6 peptide pools. Intracellular expression of IFN-γ, TNF-α, IL-4 and IL-13 was measured by flow cytometry. **(A)** Representative flow cytometry data from an SM⁺ individual. Plots are shown gated on live $CD3^+CD4^+CD8^-$ lymphocytes from the unstimulated and stimulated conditions. **(B)** Frequency of each subset of TH1 cytokine⁺ and TH2 cytokine⁺ CD4 T cells (SM⁻, n=24; SM⁺, n=22). Data are shown after subtraction of background cytokine production in the unstimulated negative control condition. **(C)** Samples meeting the criteria for a positive TH1 cytokine response (see Materials and Methods) were evaluated for expression of lineage specific transcription factors and chemokine receptors by flow cytometry. The frequency of indicated transcription factor⁺ and chemokine receptor⁺ TH1 cytokine⁺ CD4 T cells are reported (SM⁻, n=16; SM⁺, n=18). Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each CD4 T cell population between SM⁺ and SM⁻ individuals were assessed using a Mann Whitney U test.

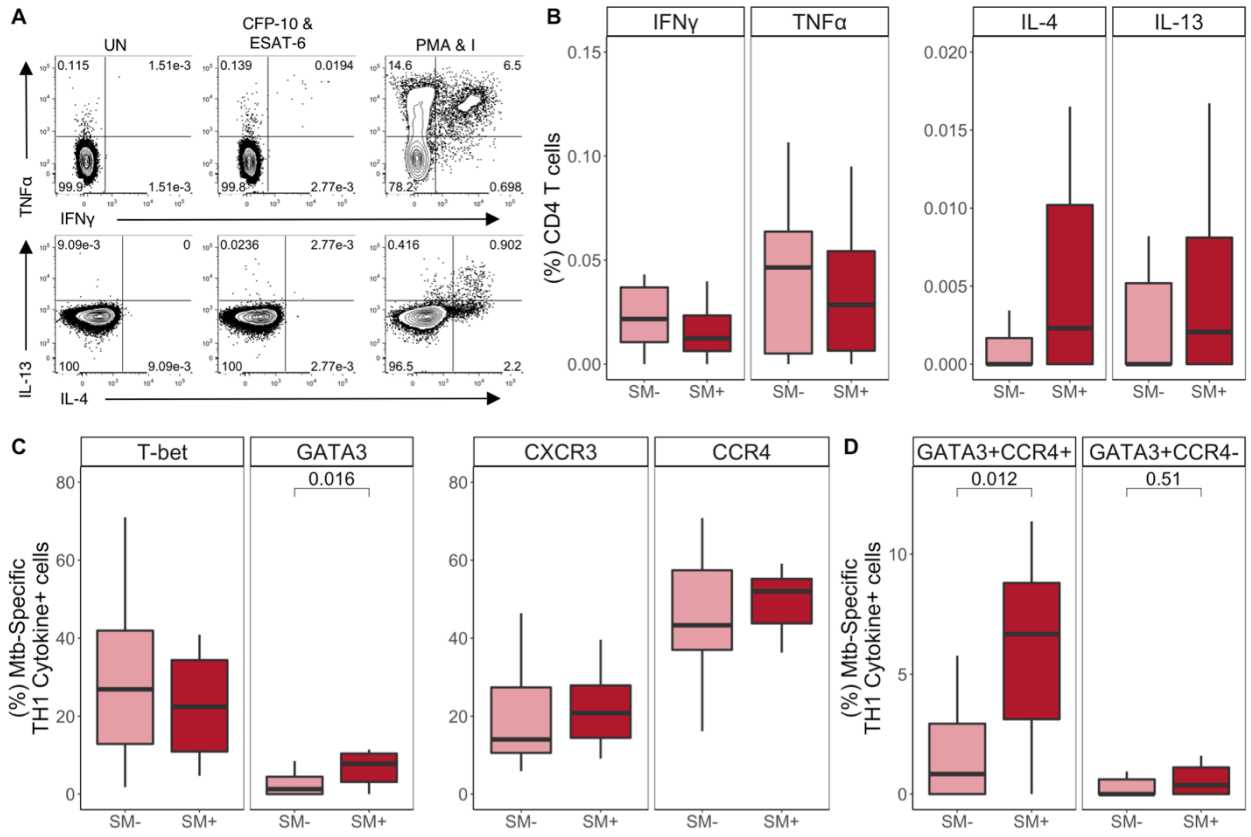


Figure 2-5: SM⁺ individuals with active TB disease have similar frequencies of TH1 cytokine⁺ Mtb-specific CD4 T cells, but higher expression of GATA3 and CCR4, compared with SM⁻ TB patients.

PBMC samples obtained from SM⁺ and SM⁻ TB individuals were incubated for 18 h in media alone (negative control), PMA and ionomycin (positive control), or CFP-10 and ESAT-6 peptide pools. Intracellular expression of IFN-γ, TNF-α, IL-4 and IL-13 was measured by flow cytometry. **(A)** Representative flow cytometry data from an SM⁺ patient with active TB disease. Plots are shown gated on live $CD3^+CD4^+CD8^-$ lymphocytes from the unstimulated and stimulated conditions. **(B)** Frequency of Mtb-specific CD4 T cells producing each indicated cytokine (SM⁻, n=25; SM⁺, n=15). Data are shown after subtraction of background cytokine production in the unstimulated negative control condition. **(C, D)** Samples meeting the criteria for a positive Mtb-specific TH1 cytokine response (see Materials and Methods) were evaluated for expression of lineage specific transcription factors and chemokine receptors by flow cytometry. The frequency of Mtb-specific TH1 cytokine⁺ CD4 T cells expressing the indicated transcription factor and chemokine receptor **(C)** as well as co-expressing cells **(D)** are reported (SM⁻, n=15; SM⁺, n=9). Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each CD4 T cell population between SM⁺ and SM⁻ individuals were assessed using a Mann Whitney U test.

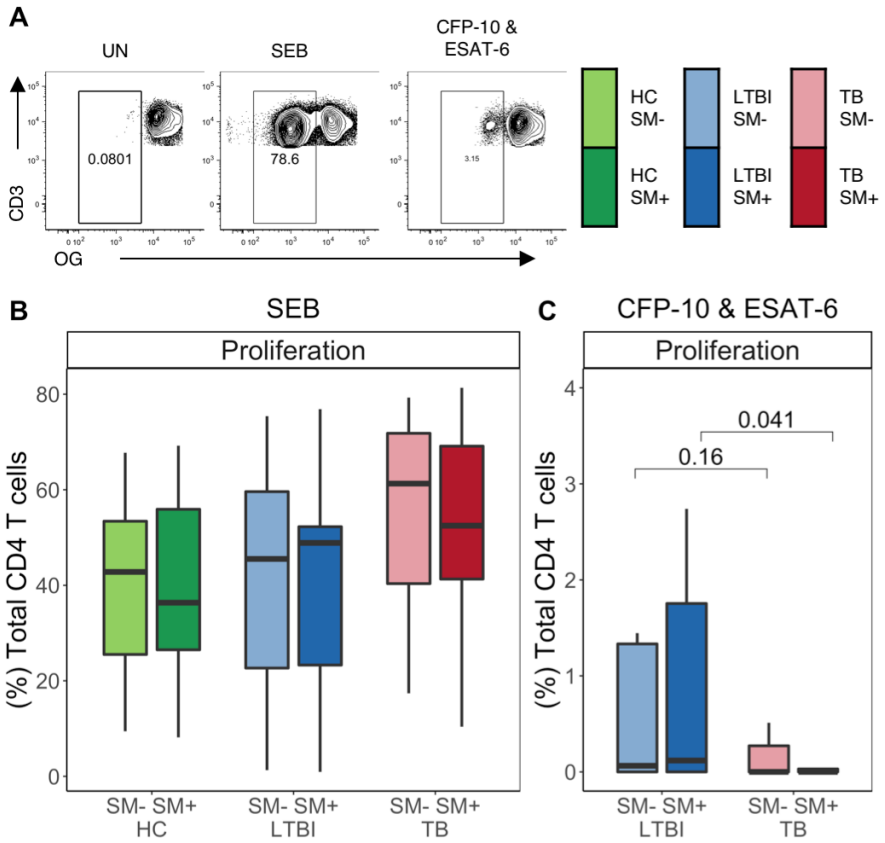


Figure 2-6: Similar frequencies of CD4 T cells proliferate in SM$^+$ and SM$^-$ individuals independent of Mtb infection status.

Proliferation assays were performed using PBMC samples obtained from individuals in each of six groups defined by Mtb and SM infection status (HC SM$^-$, n=24; HC SM$^+$, n=13; LTBI SM$^-$, n=25; LTBI SM$^+$, n=25; TB SM$^-$, n=25; TB SM$^+$, n=15). Cells were labeled with Oregon Green (OG) and incubated for 5 days under the following conditions: media alone (negative control), SEB (positive control) or CFP-10 and ESAT-6 peptide pools. **(A)** Representative flow cytometry data from an SM$^+$ individual with LTBI. Plots show cells gated on live CD3$^+$CD4$^+$CD8$^-$ lymphocytes. **(B, C)** Frequency of OG^{lo} (proliferating) CD4 T cells to SEB and CFP-10 and ESAT-6 peptide pools. Data are shown after subtraction of background proliferation in the unstimulated negative control condition. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each CD4 T cell population between SM$^+$ and SM$^-$ individuals were assessed using a Mann Whitney U test.

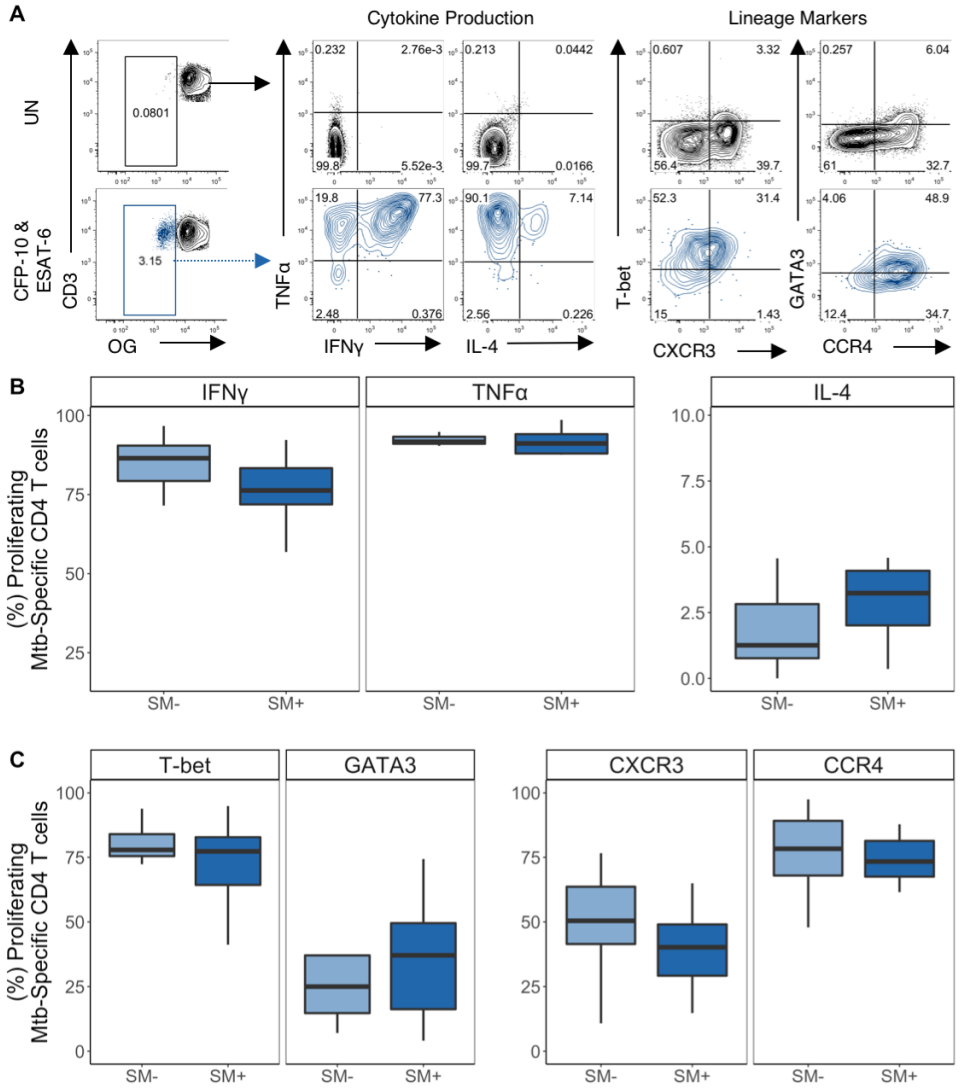


Figure 2-7: Proliferating Mtb-specific CD4 T cells have equivalent expression of TH1 and TH2 cytokines and lineage markers in SM$^+$ and SM$^-$ LTBI individuals.

PBMCs from the CFP-10 and ESAT-6 stimulated condition were restimulated on day 5 with PMA and ionomycin for 5 hours to induce cytokine production. Samples meeting the criteria for a positive proliferative response (see Materials and Methods) were evaluated for cytokine production and expression of lineage specific transcription factors and chemokine receptors by flow cytometry (SM$^-$, n=10; SM$^+$, n=11). **(A)** Representative flow plots from an SM$^+$ LTBI individual. Unstimulated samples (upper) show cytokine production and phenotypes on cells gated on live CD3$^+$CD4$^+$CD8$^-$ lymphocytes. CFP-10/ESAT-6-stimulated samples (lower) show cytokine production and phenotypes on cells gated on live OG^{lo}CD3$^+$CD4$^+$CD8$^-$ lymphocytes. **(B)** Frequency of TH1 cytokine$^+$ and TH2 cytokine$^+$ cells amongst proliferating CD4 T cells. **(C)** Frequency of transcription factor$^+$ and chemokine receptor$^+$ cells amongst proliferating CD4 T cells. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each CD4 T cell population between SM$^+$ and SM$^-$ individuals were assessed using a Mann Whitney U test.

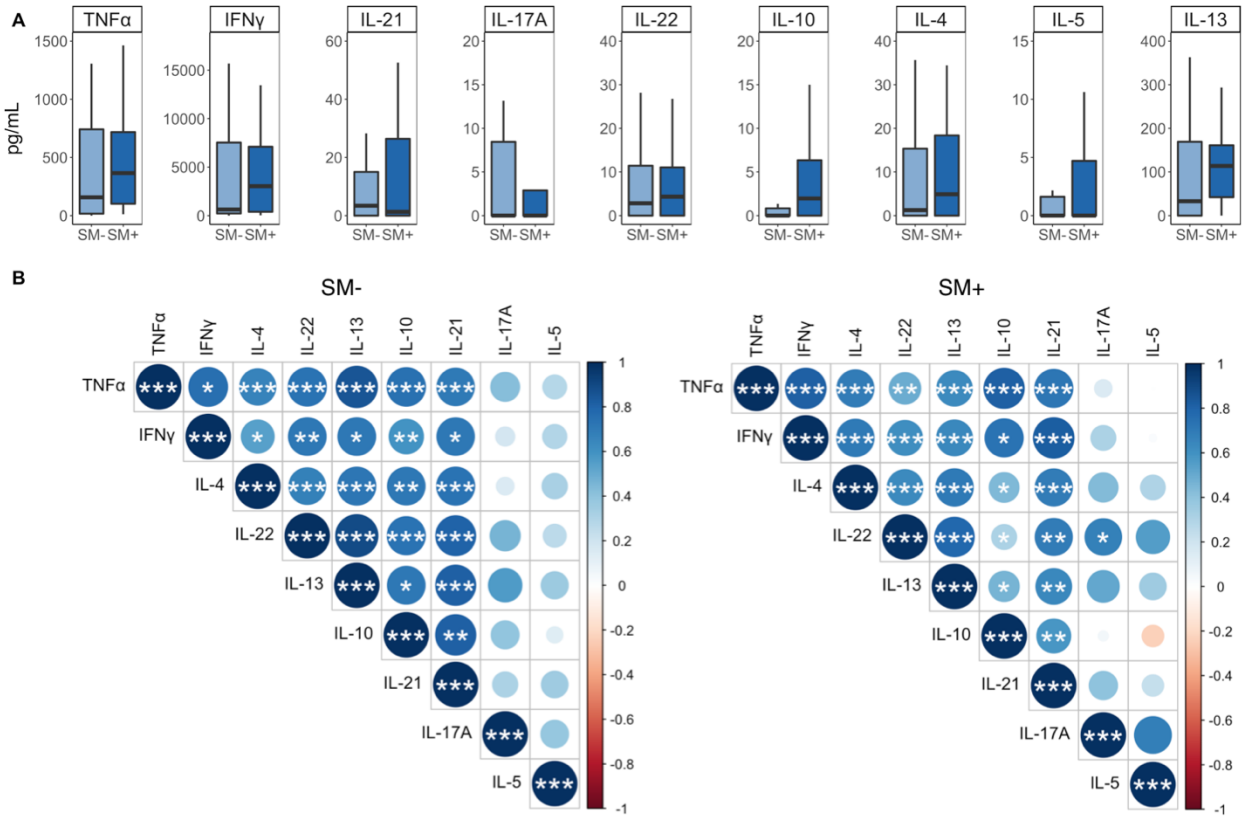
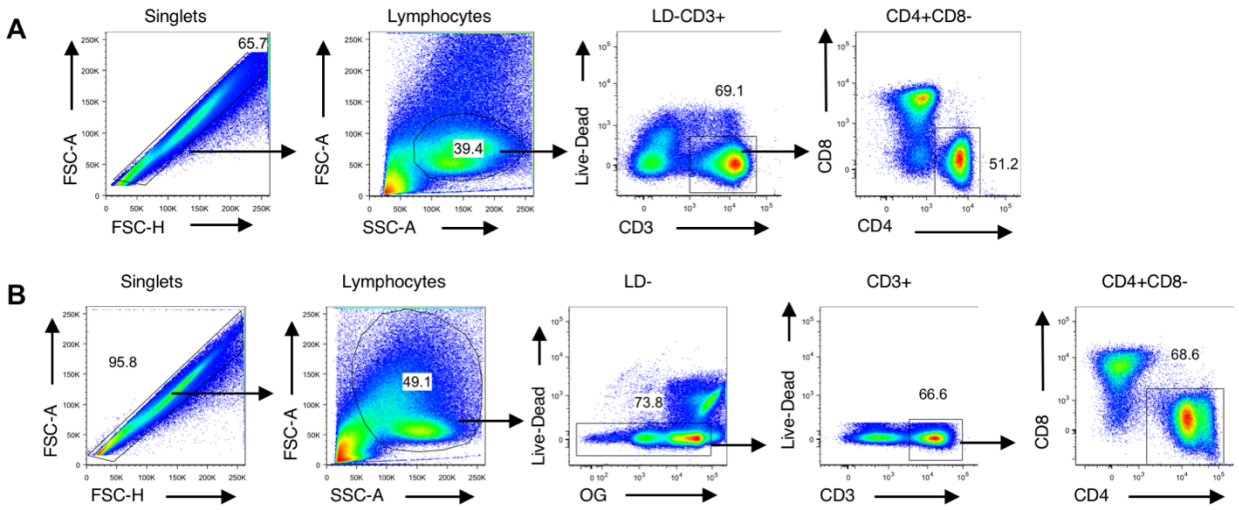


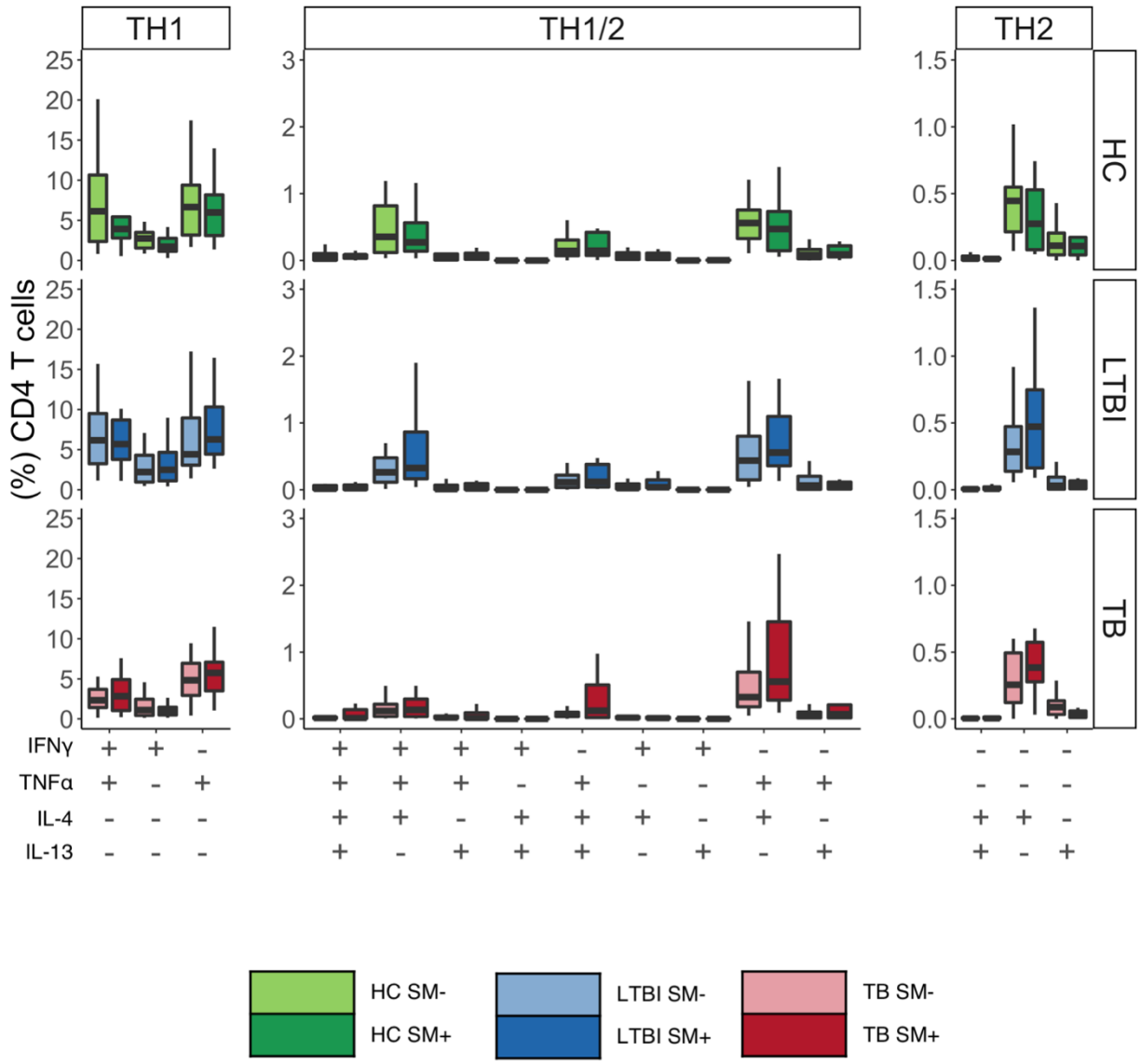
Figure 2-8: IFNγ is more strongly correlated with the production of additional cytokines in SM⁺ than in SM⁻ LTBI individuals.

Supernatants were collected from PBMCs incubated for 5 days under the following conditions: media alone (negative control) or CFP-10 and ESAT-6 peptide pools. **(A)** Levels of each cytokine were quantified by Luminex (SM^-, n=22; SM^+, n=22). Data are shown after background subtraction of the respective cytokine in the negative control condition. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the amount of each cytokine between SM^+ and SM^- individuals were assessed using a Mann Whitney U test. **(B)** Correlogram plots of each cytokine, separated by SM infection status. Correlations were evaluated using a non-parametric Spearman rank correlation. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Cytokines within each plot were ordered using the centroid method of hierarchical clustering within the SM^- data. This order was then applied to the SM^+ data. *** $p<0.001$; ** $p< 0.01$; * $p< 0.05$



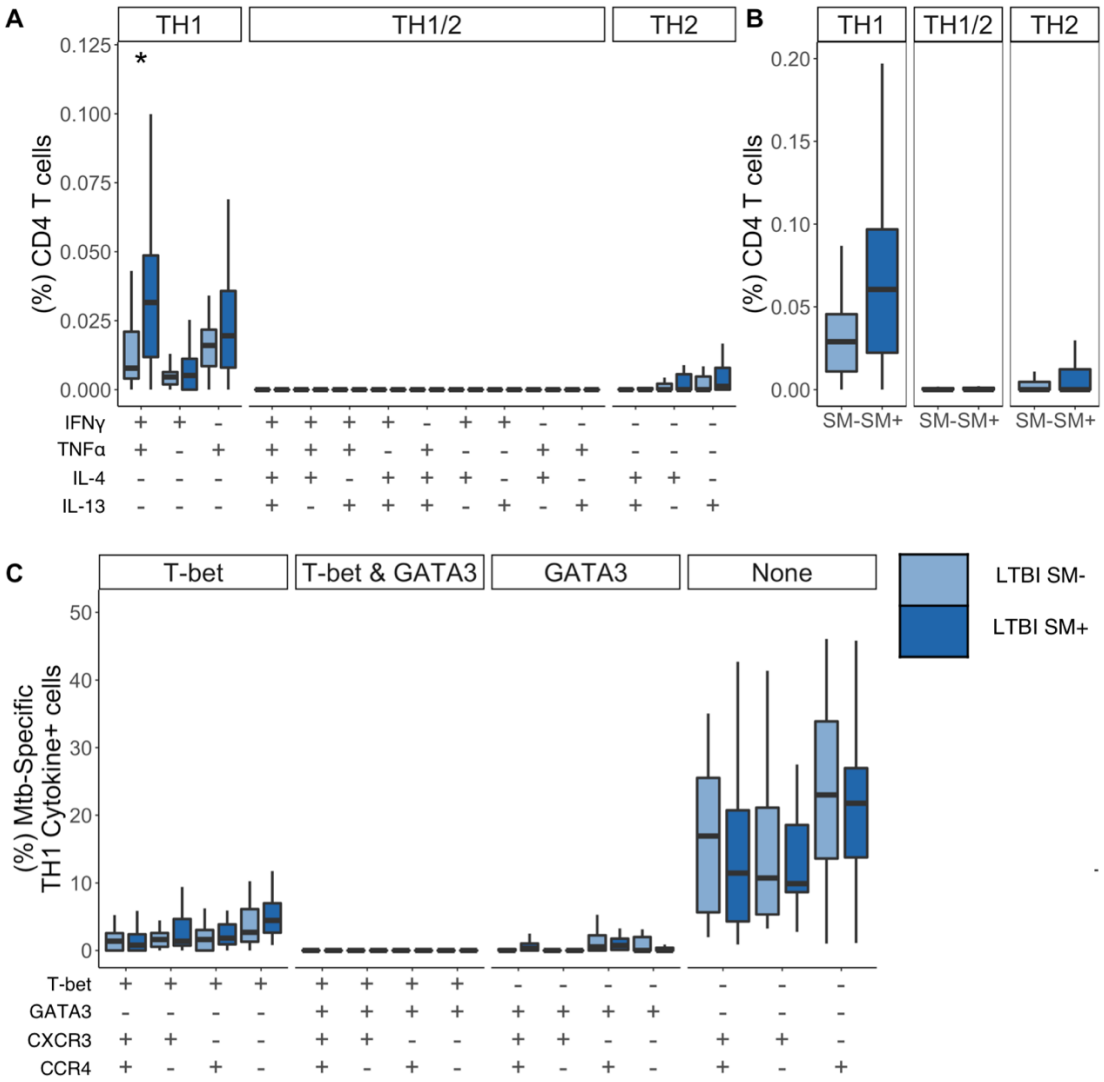
Supplementary Figure 2-1: Gating strategy for flow cytometry analysis

(A) In this sample gating for the overnight ICS assay, cells were first gated for singlets (FSC-H vs. FSC- A) and lymphocytes (SSC-A vs. FSC- A). The lymphocyte gate is further analyzed for their uptake of the Zombie IR Live/Dead stain to determine live versus dead cells and their expression of CD3 (Zombie Near-IR^{lo}, CD3^{+}). CD4 and CD8 surface expression is then determined from this gated population. (B) In this sample gating for the Proliferation ICS assay, cells were first gated for singlets (FSC-H vs. FSC- A) and lymphocytes (SSC-A vs. FSC-A). The lymphocyte gate is further analyzed for their uptake of the Zombie IR Live/Dead stain to determine live versus dead cells (Zombie Near-IR^{lo}). Live cells are then gated for their expression of CD3 (CD3^{+}) and CD4 and CD8 surface expression is then determined from this gated population.



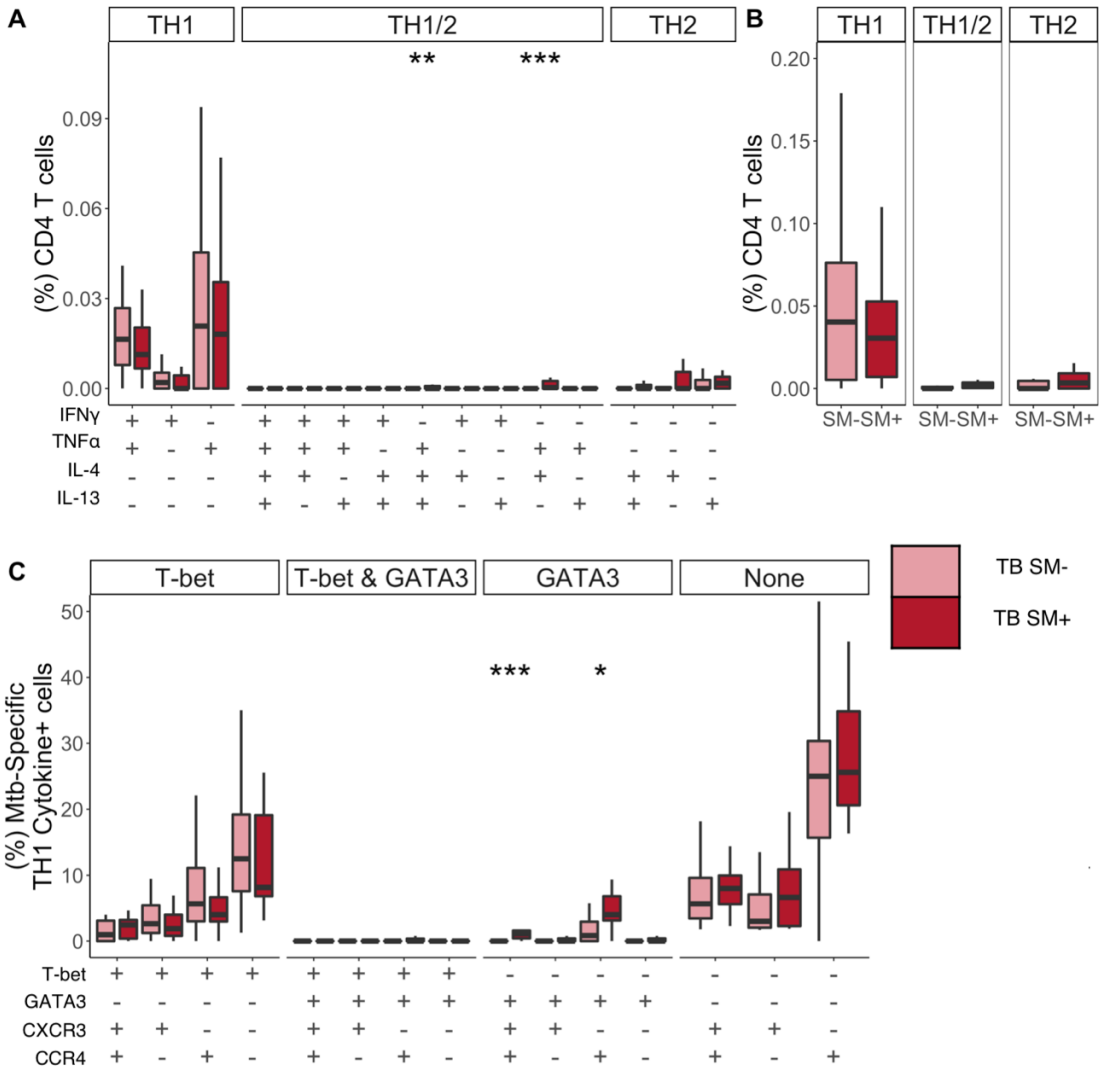
Supplementary Figure 2-2: SM⁺ and SM⁻ individuals have similar frequencies of cytokine+ CD4 T cells across all combination of TH1 and TH2 cytokines.

PBMC samples obtained from individuals in each of six groups defined by TB and S. mansoni infection status were incubated for 18 h in media alone (negative control) or stimulated with PMA and Ionomycin. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry (HC SM⁻, n=24; HC SM⁺, n=13; LTBI SM⁻, n=25; LTBI SM⁺, n=25; TB SM⁻, n=25; TB SM⁺, n=15). Frequency of each combination of cytokines using a Boolean gating strategy grouped by TH lineage. Data are shown after subtraction of background cytokine production in the unstimulated negative control condition. Boxes represent the median and interquartile ranges; whiskers represent 1.5*IQR. Differences in the frequency of each cytokine⁺ CD4 T cell population between SM⁺ and SM⁻ individuals were assessed using a Mann Whitney U test. P-values < 0.05 were considered significant.



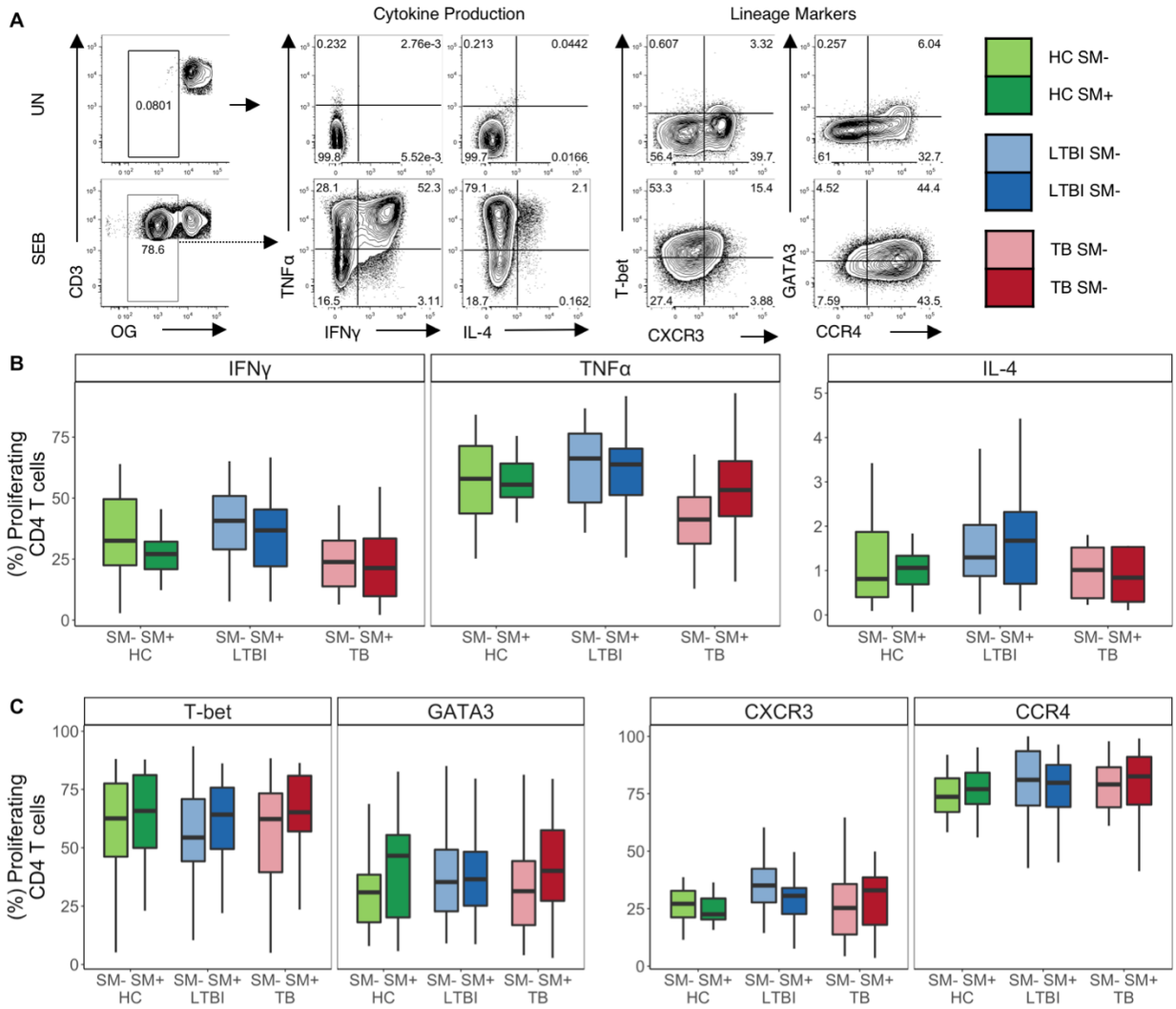
Supplementary Figure 2-3: SM⁺ LTBI individuals have higher frequencies of IFNγ⁺TNFα⁺ Mtb-specific CD4 T cells, which express both TH1 and TH2 lineage markers.

PBMC samples obtained from SM⁺ and SM⁻ LTBI individuals were stimulated for 18 h with Mtb peptides CFP-10 and ESAT-6. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry (SM⁻, n=24; SM⁺, n=22). (A) Frequency of each combination of cytokines using a Boolean gating strategy. (B) Frequency of each aggregated group of TH cytokine⁺ CD4 T cells as defined in S4A. Samples meeting the criteria for a positive response (see Materials and Methods) were evaluated for expression of lineage specific phenotypic markers using a Boolean gating strategy. (C) Frequency of each combination of transcription factors and chemokine receptors amongst TH1 cytokine⁺ CD4 T cells (SM⁻, n=16; SM⁺, n=18). Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequencies of TH1, TH1/2, and TH2 CD4 T cells within each group were evaluated using a Kruskal Wallis test. TH1 cytokine frequencies were statistically higher than the both TH1/2 and TH2 frequencies after applying the Bonferroni correction for multiple comparisons. Differences in the frequency of each CD4 T cell population between SM⁺ and SM⁻ individuals were assessed using a Mann Whitney U test. **: $p < 0.01$; *:$p < 0.05$



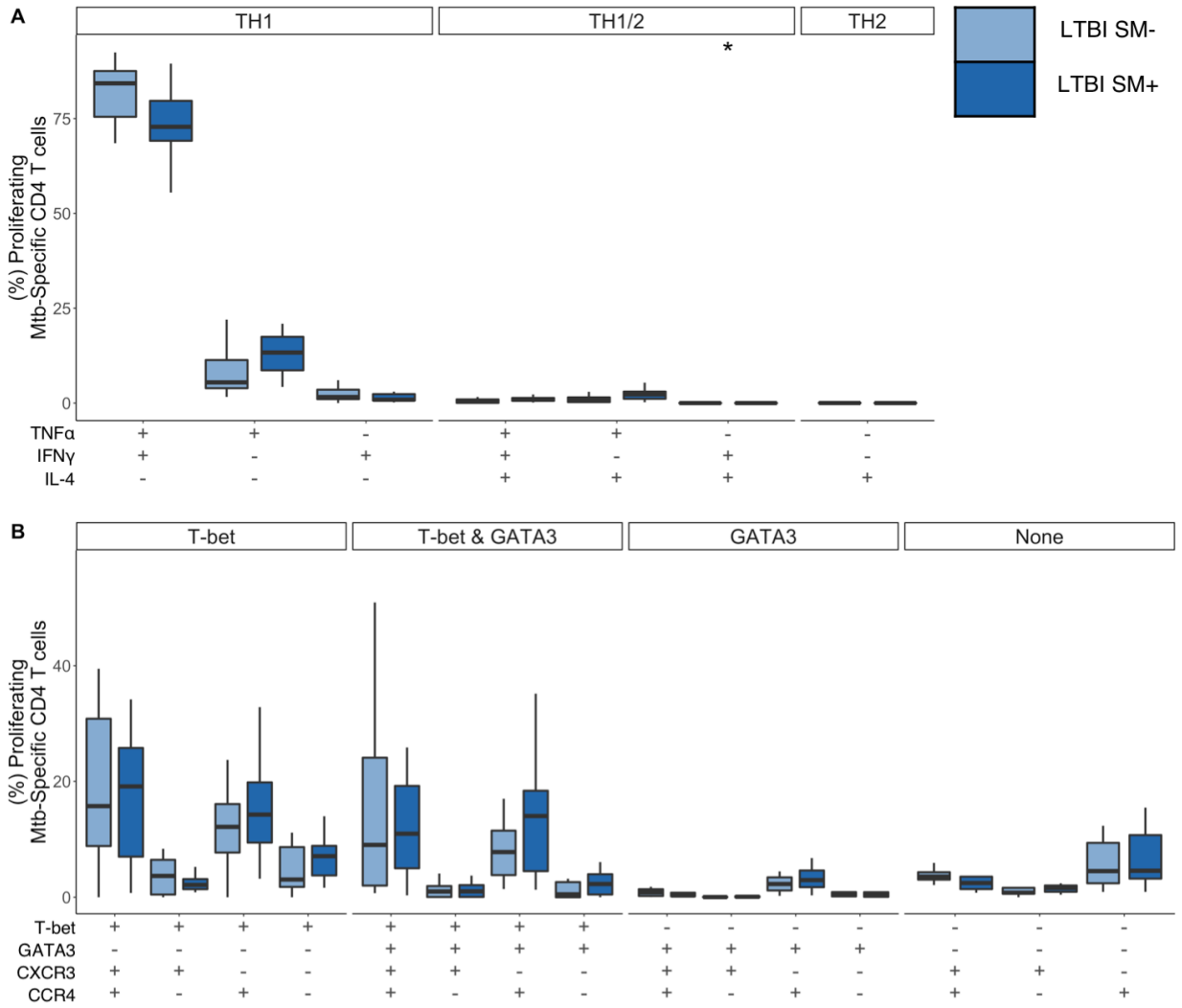
Supplementary Figure 2-4: SM⁺ TB individuals have higher frequencies of IL-4⁺TNFα⁺ and GATA3⁺CCR4⁺ Mtb-specific CD4 T cells.

PBMC samples obtained from SM⁺ and SM⁻ TB individuals were stimulated for 18 h with Mtb peptides CFP-10 and ESAT-6. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry (SM⁻, n=25; SM⁺, n=15). (A) Frequency of each combination of cytokines using a Boolean gating strategy. (B) Frequency of each aggregated group of TH cytokine⁺ CD4 T cells as defined in S5A. Samples meeting the criteria for a positive response (see Materials and Methods) were evaluated for expression of lineage specific phenotypic markers using a Boolean gating strategy. (C) Frequency of each combination of transcription factors and chemokine receptors amongst TH1 cytokine⁺ CD4 T cells (SM⁻, n=15; SM⁺, n=9). Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequencies of TH1, TH1/2, and TH2 CD4 T cells within each group were evaluated using a Kruskal Wallis test. TH1 cytokine frequencies were statistically higher than the both TH1/2 and TH2 frequencies after applying the Bonferroni correction for multiple comparisons. Differences in the frequency of each CD4 T cell population between SM⁺ and SM⁻ individuals were assessed using a Mann Whitney U test. **: $p < 0.01$; *: $p < 0.05$



Supplementary Figure 2-5: Proliferating CD4 T cells have equivalent expression of TH1 and TH2 cytokines and lineage markers in SM$^+$ and SM$^-$individuals across Mtb infection groups.

PBMC from SEB stimulated condition were restimulated on day 5 with PMA and Ionomycin for 5 hours to induce cytokine production. Samples meeting the criteria for a positive proliferative response (see Materials and Methods) were evaluated for cytokine production and expression of lineage specific transcription factors and chemokine receptors by flow cytometry. (A) Representative flow plots from an SM$^+$ LTBI individual. Unstimulated samples (upper) show cytokine production and phenotypes on cells gated on live CD3$^+$CD4$^+$CD8$^-$ lymphocytes. SEB samples (lower) show cytokine production and phenotypes on cells gated on live OG^{lo}CD3$^+$CD4$^+$CD8$^-$ lymphocytes. (B) Frequency of TH1 cytokine$^+$ and TH2 cytokine$^+$ cells amongst proliferating CD4 T cells. (C) Frequency of transcription factor$^+$ and chemokine$^+$ cells amongst proliferating CD4 T cells. Boxes represent the median and interquartile ranges; whiskers represent the minimum and maximum 1.5*IQR. Differences in the frequency of each CD4 T cell population between SM$^+$ and SM$^-$ individuals were assessed using a Mann Whitney U test. P-values < 0.05 were considered significant.



Supplementary Figure 2-6:Proliferating Mtb-specific CD4 T cells produce TH1 cytokines and express both TH1 and TH2 lineage markers in SM$^+$ and SM$^-$ LTBI individuals.

PBMC from the CFP-10 and ESAT- 6 stimulated condition were restimulated on day 5 with PMA and Ionomycin for 5 hours to induce cytokine production. Samples meeting the criteria for a positive proliferative response (see Materials and Methods) were evaluated for cytokine production and expression of lineage specific transcription factors and chemokine receptors by flow cytometry (SM$^-$, n=10; SM$^+$, n=11). (A) Frequency of each combination of TH1 and TH2 cytokine$^+$ cells amongst proliferating CD4 T cells. (B) Frequency of each combination of transcription factor$^+$ and chemokine receptor$^+$ cells amongst proliferating CD4 T cells. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each CD4 T cell population between SM$^+$ and SM$^-$ individuals were assessed using a Mann Whitney U test. *:p< 0.05

Chapter 3 Adults from Kisumu, Kenya have robust γδ T cell responses to *Schistosoma mansoni,* which are modulated by tuberculosis

This chapter is reproduced with minor edits from: McLaughlin TA, Khayumbi J, Ongalo J, et al. Adults from Kisumu, Kenya have robust γδ T cell responses to *Schistosoma mansoni,* which are modulated by tuberculosis. PLoS Negl Trop Dis (In preparation)

3.1 Abstract

Schistosoma mansoni (SM) is a parasitic helminth that infects over 200 million people and causes severe morbidity. It undergoes a multi-stage life cycle in human hosts and as such stimulates a stage-specific immune response. Very little is known about the human T cell response to SM, particularly with regard to the adult worm stage of the life cycle. In addition, while a great deal of research has uncovered mechanisms by which co-infection with helminths modulates immunity to other pathogens, there is a paucity of data on the effect of pathogens on immunity to helminths. As such, we sought to characterize the breadth of the T cell response to SM and determine whether co-infection with *Mycobacterium tuberculosis* (Mtb) modifies SM-specific T cell responses in a cohort of HIV-uninfected adults in Kisumu, Kenya. SM-infected individuals were categorized into three groups by Mtb infection status: healthy controls (HC), latent Mtb infection (LTBI) and active tuberculosis (TB). US adults that were seronegative for SM antibodies served as naïve controls. We utilized flow cytometry to characterize the T cell repertoire to SM egg and worm antigens. We found that T cells had significantly higher proliferation and cytokine production in response to worm antigen than to egg antigen. The T cell response to SM was dominated by γδ T cells that produced TNFα and IFNγ. Furthermore, we found that in indiviudals infected with Mtb, γδ T cells proliferated less in response to SM worm antigens and had higher IL-4 production compared to naïve controls. Together these data demonstrate that γδ T cells respond robustly to SM worm antigens and that Mtb infection modifies the γδ T cell response to SM.

3.2 Introduction

Though considered a neglected tropical disease (NTD), helminthiasis is a serious global health burden. Over 1 billion people worldwide are estimated to be infected with one or more helminth species (56). The most common helminth infections are schistosomes, lymphatic filarial worms and the soil transmitted helminths ascaris, trichuris and hookworm. Each of these individually infects hundreds of millions of people globally (56). While not normally fatal, infections with helminths cause a great deal of morbidity and are collectively responsible for 26 million Disability Adjusted Life Years (DALYs) (56).

People in Sub-Saharan Africa are disproportionately impacted by helminth infections (61). This is particularly true for schistosomiasis, which affects over 200 million people globally, 90% of whom reside in Sub-Saharan Africa (61). Indeed, it is estimated that one in four people in Sub-Saharan Africa is infected with a schistosome species. The mortality rate for *Schistosoma mansoni* (SM), which is only one of five schistosome species known to infect humans, is estimated at 130,000 per year in Sub-Saharan Africa alone (62).

As with most helminths, SM has a complex life cycle which involves both a human and intermediate host (54). Within the human host, SM larvae migrate through the body, passing through a variety of tissues including the lung, as they develop into adult worms. When adult worms reach sexual maturity, they take up residence in the portal vein. If left untreated, adult mating pairs can reside in the bloodstream for years releasing hundreds of eggs into circulation every day (54). These eggs are passed through the endothelium into the gastrointestinal tract and are shed in stool, which begins the life cycle over again.

In contrast to other helminths, which are strictly type 2, the immune response to SM is stage specific. The migrating larval stage of the worm elicits a type 1 response which gives rise to

a type 2 response upon egg laying and transitions to a regulatory phenotype when the infection becomes chronic (53,162). Much more is known about the immune response to SM eggs than either the larvae or adult worms. This is largely due to the role the eggs play in immunopathology and their ability to modulate antigen presentation and CD4 T cell phenotypes (64,65). Indeed, previous studies on SM T cell immunity have focused exclusively on CD4 T cells (108). Very little is therefore known about CD8 T cell responses and even less about non-classical T cell responses, such as γδ T cells, which can have diverse roles during helminth infections.

γδ T cells in humans are divided based on their V chain expression and anatomical location, which in turn dictates their function. Intraepithelial lymphocytes (IELs) are largely Vδ1 cells and are involved in surveillance and maintenance of barrier tissues (163,164). IELs have been shown to be involved in the expulsion of the helminths *Nippostrongylus brasiliensis* and *Trichinella spiralis* from the guts of infected mice (165–167). Hepatic γδ T cells are often Vγ4 cells and secrete the cytokine IL-17 and/or IL-10 (168). Studies in mice indicate a role for IL-17 producing γδ T cells in liver fibrosis and immune pathology during infection with *Schistosoma japonicum* (169–172) and SM (173). They can either be pathogenic as in the case of *S. japonicum*, or protective in the case of *Listeria monocytogenes* (168). In the blood, γδ T cells are predominantly Vγ2Vδ2 (alternatively called Vγ9Vδ2) (174–176). They canonically proliferate and produce IFNγ in response to phosphoantigens produced by pathogens. Vγ2Vδ2 T cells have recently been shown to be expanded in the peripheral blood of people infected with a variety of infections (177,178) including SM (179). While it is clear that γδ T cells respond to infection with helminths, whether they respond to helminth antigen directly or are merely bystander cells is still unknown.

Peripheral γδ T cells respond to phosphoantigens, which are produced by a variety of microbes (83,178). For this reason, the role of γδ T cells during SM infection is of particular

interest because SM is co-endemic with a variety of pathogens known to elicit γδ T cell responses including *Mycobacterium tuberculosis* (Mtb) (43,44). Co-infection with SM and Mtb is common throughout the world, particularly in sub-Saharan Africa, and may impact the immune response to both pathogens. A great deal of research has been conducted evaluating the impact of helminth infections on immunity to Mtb (43,111); however, the effect of Mtb on the immune response to helminths is less clear. There are no published studies that have evaluated the impact of Mtb infection on SM-specific T cell responses.

In this study, we characterized the T cell repertoire to two SM antigens from distinct stages of the SM life cycle. We enrolled individuals in groups defined by Mtb and SM infection status: SM-naïve controls (N), active TB (TB), latent TB infection (LTBI), and Mtb-uninfected healthy controls (HC). In each group we examined the ability of T cells to produce cytokines and proliferate in response to adult worm and egg antigens. In addition, we measured the impact of Mtb infection status on T cell functions. We hypothesized that Mtb infection would dampen the immune response to SM.

3.3 Materials and Methods

Study Population:

Participants ≥18 years old were recruited in either Kisumu, Kenya as described previously (180) or Atlanta, GA, United States. Individuals in Kenya were enrolled based on Mtb infection status into three groups: active TB disease (TB), latent TB infection (LTBI) and Mtb-uninfected healthy controls (HC). Patients with drug-sensitive active pulmonary TB disease were symptomatic individuals with a positive GeneXpert MTB/RIF result and a positive culture for Mtb growth. Healthy asymptomatic individuals with no previous history of TB disease or treatment were evaluated by QuantiFERON®-TB Gold In-Tube (QFT) assay: those with a positive QFT result (TB Ag-Nil >0.35 IU IFNγ/mL) were defined as having LTBI; those with a negative QFT result (TB Ag-Nil <0.35 IU IFNγ/mL) were defined as HC. All LTBI and HC participants had normal chest x-rays. Blood was collected from individuals with active TB within the first 7 days of TB treatment, which was provided according to Kenyan national health guidelines. SM infection was determined using standard Kato Katz microscopy. Briefly, two thick Kato Katz smears were prepared from stool samples collected on two separate days. Slides were analyzed by experienced lab technicians who recorded the presence of SM eggs as well as the number of eggs counted. Participants were excluded if eggs belonging to other helminth species including *Ascaris lumbricoides*, *Trichuris trichuria* and hookworm were identified. Other exclusion criteria included: pregnancy, hemoglobin value of <7.0 g/dl, HIV infection, and positive rapid malaria test.

Individuals in Atlanta were enrolled as a naïve control group for both SM and Mtb infection. All individuals were US based and had not been BCG vaccinated. Serologic testing was

performed for antibodies against SM eggs as previously described (181). All US participants in the naïve control group had SM Ab levels <40 units/ml.

Sample Collection:

Blood was collected in sodium heparin Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (BD Biosciences). PBMC were isolated by density centrifugation, cryopreserved in freezing medium (50% RPMI 1640 + 40% heat-inactivated fetal calf serum [FCS] + 10% DMSO), and stored in LN_2 until use.

Ethics Statement:

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All participants gave written informed consent for the study, which was approved by the KEMRI/CDC Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

Antigens:

This study utilized crude antigen extracts from two distinct stages of the SM life cycle: Soluble Egg Antigen (SEA, 20 μg/ml) and Soluble Worm Antigen Prep (SWAP, 2.5 μg/ml). SEA and SWAP were produced at the CDC. The positive control for the overnight intracellular cytokine staining assay was phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Adipogen) and ionomycin (1 μg/ml, Cayman Chemical). Staphylococcal enterotoxin B (SEB; 1 μg/ml, Toxin Technology, Inc.) was used as a positive control for proliferation in the 5-day proliferation assay. During the

last 5 hours of the proliferation assay, PMA and ionomycin were used to induce cytokine production.

Antibodies:

The following human monoclonal fluorescently-conjugated antibodies were used in this study: anti-CD3 BV605 (clone OKT-3), anti-CD4 BV570 (clone RPA-T4), anti-TNFα Alexa Flour 647 (clone Mab11), and anti-IL-4 PE-Dazzle594 (clone MP4-25D2), all from BioLegend; anti-CD4 BV786 (clone SK3), anti-CD8 PerCP-Cy5.5 (clone SK-1), anti-TCR γδ BV480 (clone 11f2), and anti-IFN-γ Alexa Fluor 700 (clone B27), all from BD Biosciences; and anti-IL-13 FITC (clone 85BRD) from eBiosciences.

PBMC overnight intracellular cytokine staining (ICS) assay:

Cryopreserved PBMCs were thawed in a 37°C water bath and immediately added to RPMI 1640 (Cellgro) containing deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich). Cells were washed in RPMI twice and then suspended in R10 media (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum [FCS], 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine). Cells were rested for a minimum of 3 h at 37°C and 5% CO_2 before the addition of antigens (described above). Cells incubated in R10 media alone served as a negative control. Brefeldin A (10 μg/ml; Sigma-Aldrich) and monensin (1x, BioLegend) were added for the last 15 hrs of an 18 hr incubation.

PBMC proliferation assay:

Cryopreserved PBMCs were thawed, washed in PBS containing deoxyribonuclease I (DNase, 10 μg/ml, Sigma-Aldrich). Cells were washed in PBS twice and then labeled with 0.5 μg/ml CellTrace™ Oregon Green® 488 carboxylic acid diacetate, succinimidyl ester (OG; Life Technologies). Cells were washed once more with PBS and resuspended in R10 media containing recombinant human IL-2 (10 units/ml, obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) (144). Cells were plated in 96-well plates and incubated for 5 days in a 37°C incubator with 5% CO_2. On day 5, with the exception of the negative control (wells containing cells in media alone), cells were re-stimulated with PMA and ionomycin (described above) and treated with brefeldin A (10 μg/ml; Sigma-Aldrich) and monensin (1x, BioLegend) for 5 hrs at 37°C to determine the cytokine capacity of proliferating T cells.

Antibody staining and Flow cytometry:

Following stimulation, cells were washed with PBS and stained with the Fixable Viability Dye Zombie Near-IR (BioLegend) for 15 minutes at room temperature. Samples were then surface stained for 30 minutes at room temperature. For the ICS assay this included: anti-CD3 BV605, anti-CD4 BV570, and anti-CD8 PerCP-Cy5.5. For the proliferation assay: anti-CD3 BV605, anti-CD4 BV786, anti-CD8 PerCP-Cy5.5, and anti-TCR γδ BV480. Following the surface stain, cells were fixed and permeabilized on ice for 1 hr using the FoxP3 Transcription Staining Buffer Set (eBioscience). Cells were then stained for intracellular markers on ice for 40 min. For the ICS assay this included: anti-IFN-γ Alexa Fluor 700, anti-TNFα Alexa Flour 647, anti-IL-4 PE-Dazzle594, and anti-IL-13 FITC. For the proliferation assay this included: anti-IFN-γ Alexa Fluor 700, anti-TNFα Alexa Flour 647, and anti-IL-4 PE-Dazzle594. Finally, cells were washed in permeabilization buffer and resuspended in PBS. Samples were acquired using a BD LSR II flow

cytometer. 6 peak Rainbow Calibration Particles (BioLegend) were used to standardize instrument settings.

Data analysis:

Flow cytometry data were analyzed using FlowJo version 9.6.4 (BD). Compensation was calculated using single-stained anti-mouse Ig,κ CompBeads (BD Biosciences). Single cells were gated by plotting forward scatter-area versus forward scatter-height; lymphocytes were gated based on morphological characteristics. Viable cells were defined as Zombie Near-IR^{lo} cells. In the overnight assay, CD4 T cells were defined as CD3^{+}CD4^{+}CD8^{-} lymphocytes, CD8 T cells were defined as CD3^{+}CD4^{-}CD8^{+} lymphocytes, and a third population of T cells, referred to as CD4^{-}CD8^{-} T cells, were defined as CD3^{+}CD4^{-}CD8^{-} lymphocytes. In the proliferation assay, CD4 T cells were defined as CD3^{+}CD4^{+}CD8^{-}γδ^{-} lymphocytes, CD8 T cells were defined as CD3^{+}CD4^{-}CD8^{+}γδ^{-} lymphocytes, and γδ T cells were defined as CD3^{+}CD4^{-}CD8^{-}γδ^{+} lymphocytes. Antigen-specific T cell populations were defined as cells producing cytokines (IFN-γ, TNF-α, IL-4, or IL-13) after stimulation with antigen. Proliferating cells were defined as those with low expression of the cytosolic dye Oregon Green (OG^{lo}). The flow cytometry gating strategies are indicated in Supplementary Figure 3-1. Responses in the proliferation assay were evaluated using the mixture models for single-cell assays (MIMOSA) method to determine positivity using a Markov Chain Monte Carlo algorithm with a prior of 1% (145). Samples with a probability of response >70% and a false discovery rate (fdr/q-value) <3% were considered positive. Cytokine production from proliferating T cells was restricted to individuals who met the above criteria for a positive response.

COMPASS analysis of flow cytometry data:

Cell counts were analysed using the COMbinatorial Polyfunctionality Analysis of Antigen-Specific T cell Subsets (COMPASS) algorithm as described previously (182). COMPASS uses a Bayesian computational framework to identify antigen-specific changes across all observable functional T cell subsets without the need to limit the analysis to specific cytokine combinations. Each analysis was therefore unbiased and considered all 16 cytokine combinations, across each of three T cell subsets (CD4, CD8, and $CD4^{-}CD8^{-}$) to both SM antigens. For a given participant, COMPASS was also used to compute a functionality score which summarizes the magnitude of the cytokine response and a polyfunctionality score which summarizes the cytokine repertoire.

Statistical analysis:

R programming software was used to perform all statistical analyses. Differences between SEA and SWAP responses within each infection group or cell type were evaluated using a non-parametric Mann-Whitney test. Differences between three or more groups were evaluated using a non-parametric Kruskal-Wallis test and corrected for multiple pairwise comparisons using the Nemenyi method. P-values < 0.05 were considered significant. Graphs were created using the R package ggplot2 and statistics were performed using the stats and PMCMRplus package.

3.4 Results

Study participants.

Participants were recruited and enrolled in Atlanta, GA and Kisumu, Kenya. All participants from Atlanta were seronegative for SEA-specific antibodies and served as naïve controls (N) for SM infection. Participants from Kenya were SM^+ and categorized into three groups based on their Mtb infection status: HC, LTBI and TB (Table 3-1). Participants in the HC group were younger than the other participant groups. In addition, there were more females in the N and HC groups than LTBI and TB groups. The median egg burden in the LTBI group is classified as a moderate intensity infection, whereas the HC and TB groups both had light intensity infections as defined by the WHO (118).

T cell cytokine production is higher to SWAP than to SEA, irrespective of Mtb and SM infection status

While the CD4 T cell response to SEA is well characterized (108,183), less is known about the CD4 T cell response to SWAP. In addition, SM-specific CD8 T cell responses and non-classical ($CD4^-CD8^-$) T cell responses have not been thoroughly investigated. To characterize the T cell repertoire to SM antigens from distinct stages of the SM life cycle, we stimulated PBMC overnight with media alone (negative control), PMA and ionomycin (positive control), Soluble Egg Antigen (SEA) and Soluble Worm Antigen Prep (SWAP). We then performed intracellular cytokine staining (ICS) for the type 1 cytokines IFNγ and TNFα, as well as the type 2 cytokines IL-4 and IL-13 for analysis by flow cytometry (Figure 3-1A). Total cytokine frequencies were evaluated in CD4, CD8 and $CD4^-CD8^-$ T cells and were higher following stimulation with SWAP than SEA (Supplementary Figure 3-2). To analyze this data in an unbiased manner, we utilized the

statistical package COMPASS which allowed us to evaluate all possible cytokine combinations across all three T cell subsets to both SEA and SWAP (Figure 3-1B). The dominant cytokine producing subset in both the SEA and SWAP stimulation was TNFα single positive T cells. More individuals had IL-4^{+} T cell responses in the SEA condition than the SWAP condition. More individuals had TNFα^{+}IFNγ^{+} T cells in the SWAP condition than in the SEA condition, predominantly amongst CD4^{-}CD8^{-} T cells. To confirm that these observations were specific to the SWAP stimulation, the same analysis was performed on T cells following stimulation with PMA and ionomycin. Overall, the cytokine response to PMA and ionomycin was very robust, with CD4 T cells having an overall higher FS than CD8 and CD4-CD8- cells (Supplementary Figure 3-3).

We also utilized COMPASS to generate summary scores for both the overall functionality (FS), as well as the polyfunctionality (PFS) of each sample (Figure 3-1C). In CD8 and CD4^{-}CD8^{-} T cells, SWAP responses were higher than SEA responses with regard to both FS and PFS. Upon stratification by Mtb and SM status, this held true in the HC and LTBI groups, but not the N or TB groups (Supplementary Figure 3-4). Together these data indicate that T cell responses to SWAP are more robust than T cell responses to SEA.

T cell responses to SWAP are dominated by non-classical T cells

We next utilized COMPASS to determine which T cell subsets had the strongest response to SEA and SWAP (Figure 3-2A). SEA-specific T cell FS were equivalent between CD4, CD8, and CD4^{-}CD8^{-} subsets in all four groups (Figure 3-2B). In contrast, SWAP-reactive FS were higher in CD4^{-}CD8^{-} T cells, compared to both CD4 and CD8 T cells in the LTBI and TB groups

(Figure 3-2B). This was also true for SWAP-reactive PFS (Supplementary Figure 3-4). These data indicate that while there is no dominant SEA-specific T cell type, $CD4^{-}CD8^{-}$ T cells are the dominant T cell population responding to SWAP.

Since these differences were only observed in some groups, we next evaluated whether Mtb or SM infection affected T cell functionality scores. Importantly, the only differences in FS and PFS scores between Mtb groups were in CD8 T cells (Supplementary Figure 3-5). SEA-specific CD8 T cell scores were higher in N compared to HC. SWAP-reactive CD8 T cell scores were higher in N compared to TB individuals. These data indicate that SM but not Mtb infection modestly alters SM-specific CD8 T cell responses.

SWAP induces more robust T cell proliferation than SEA

To further characterize the SM T cell repertoire, we next performed a proliferation assay. PBMC from each group were labeled with the cytosolic dye Oregon Green (OG) and incubated for 5 days with media alone (negative control), SEB (positive control), SEA or SWAP. We then measured proliferation of total T cells via flow cytometry (Figure 3-3A). Similar to what was observed in the overnight ICS assay, the $CD3^{+}$ T cell proliferative response to SWAP was higher than to SEA in all groups (Figure 3-3B). These data indicate that T cells proliferate more robustly to SWAP than to SEA.

γδ T cells are the dominant population of T cells responding to SWAP

Peripheral γδ T cells have been found highly expanded in the blood of people infected with a variety of pathogens (177,178). Since we observed a robust response of $CD4^{-}CD8^{-}$ T cells to SWAP during the overnight ICS assay, we therefore included an antibody to detect γδ T cell

receptor expression in the proliferation assay. We used MIMOSA (see Materials and Methods) to determine which samples had a positive proliferative response. These samples were evaluated for the distribution of CD4, CD8 and γδ T cells using flow cytometry (Figure 3-4A). Amongst SWAP-reactive proliferating T cells, γδ T cells were the dominant subset in the N, HC, and LTBI groups (Figure 3-4B). Due to the low frequency of SEA-specific proliferating T cells across all groups, we were not able to evaluate which T cell subsets proliferated in response to SEA.

TB modifies the SWAP-reactive γδ T cell response

We next determined whether Mtb infection status modified the γδ T cell response to SWAP. We first measured the frequency of γδ T cells at day 5 in the unstimulated condition, which did not differ between the four groups (Figure 3-5A). We next evaluated the frequency of proliferating γδ T cells in the SEB, SEA and SWAP conditions. The frequency of proliferating cells did not differ across the groups in the SEB or SEA condition (Figure 3-5B, 3-5C). However, SWAP-reactive γδ T cell proliferation was significantly lower in the LTBI and TB groups compared to the N group. Together these data indicate that there is not an inherent defect in γδ T cell numbers or proliferative capacity due to Mtb infection. We next evaluated whether the difference in proliferative capacity was specific to γδ T cells. Importantly, the proliferative capacity of total $CD3^+$ T cells in response to SEB, SEA and SWAP did not differ between the groups (Supplementary Figure 3-7). These data indicate that Mtb infection status specifically impacts the proliferative capacity of γδ T cells to SWAP and not to other antigenic stimuli.

We next evaluated the cytokine profiles of proliferating SWAP-reactive γδ T cells. On day 5 of the proliferation assay, we restimulated cells using PMA and ionomycin for 5 hours and performed intracellular cytokine staining for analysis by flow cytometry (Figure 3-6A). Similar to

the overnight assay, high frequencies of SWAP-reactive γδ T cells produce TNFα and IFNγ, while very low frequencies produce IL-4. While TNFα and IFNγ production capacity did not vary between groups, there were higher frequencies of IL-4 producing SWAP-reactive γδ T cells in the active TB group compared to the N group. Taken together, these data demonstrate that not only are γδ T cells impaired in Mtb-infected individuals with regard to proliferative capacity, but they also have skewed cytokine profiles in individuals with active TB.

3.5 Discussion

SM has a complex life cycle which involves multiple distinct morphological stages and immunological phases in the host (53). This is largely characterized by a type 1 CD4 T cell response to the larval stage which shifts to a type 2 CD4 T cell response upon egg laying (108). However, little is known about the T cell response to the adult stage of the worm and which T cell subsets contribute to the overall immune response to SM. In this study, we sought to characterize the T cell repertoire to different stages of the SM life cycle, as well as determine the impact of Mtb infection on SM-specific T cell responses. We determined that the T cell response to SWAP was significantly more robust than the T cell response to SEA. We also found that the dominant T cell subset to respond to SWAP consisted of γδ T cells. Lastly, we determined that Mtb infection impaired the ability of γδ T cells to proliferate in response to SWAP and altered the cytokine profile of these T cells in patients with active TB disease.

Our data indicate that T cell responses are higher to SWAP than to SEA with regard to both cytokine production and proliferative capacity. This phenomenon is particularly evident in the CD8 and non-classical T cell compartments of SM infected individuals without active TB. While SEA is strongly immunogenic during acute infection and experimental models, it is generally accepted that in endemic settings, the immune response to egg antigens wanes over time in order to limit immunopathology (162,184). It is also generally accepted that SWAP responses are maintained during chronic infection. This is largely based on the fact that antibody responses to worm stage antigens have been shown to rise during infection and stay high in chronically infected individuals (184,185). The data supporting the preservation of SWAP responses has not, however, directly addressed the role of T cells. In studies measuring PBMC proliferation after stimulation with SEA or SWAP, the proportion of acutely infected individuals who respond to SEA is much

higher than the proportion of chronically infected individuals. In contrast, the proportion of individuals who respond to SWAP is consistent between groups of acute and chronically infected patients (186). In addition, PBMCs from chronically infected patients show higher cytokine production and phosphorylation of T cell signaling molecules following stimulation with SWAP than SEA (187,188). These studies and our data suggest that in chronically infected individuals, the T cell response to SWAP is maintained while the T cell response to SEA is not.

One strength of our study is that we did not limit our analysis to CD4 T cells which allowed us to detect a strong γδ T cell response to SWAP antigen. γδ T cells have been found elevated in the blood of individuals with acute schistosomiasis, however this analysis was not done in an antigen-specific fashion (179). Here we provide evidence that γδ T cells respond to SM in an antigen specific manner in that they are activated by SWAP but not by SEA. Furthermore, SWAP-reactive responses were characterized by high production of TNFα and IFNγ. In other infections, type 1 cytokine producing γδ T cells have been shown to link the innate and adaptive arms of the immune system (189). Thus, our study has uncovered a potential novel role for SM-specific γδ T cells during infection which should be investigated further.

Our findings also provide strong evidence that Mtb infection alters the response of γδ T cells to SWAP. Indeed, we observe decreased proliferative capacity of SWAP-reactive γδ T cells in individuals infected with Mtb. Furthermore, in individuals with active TB disease, we observed an increase in IL-4 producing γδ T cells. Previous work on Mtb-specific γδ T cells has shown a decrease in blood γδ T cells during active TB disease and has suggested that Mtb infection induces chemokines in the lung which recruit blood dwelling γδ T cells into the lung (190). It is possible that SM-specific γδ T cells respond to similar signals and are therefore depleted from the blood, leading to the diminished responsiveness observed in this study.

The interpretation of our study is limited by the cross-sectional enrollment of our study participants. As such, we are not able to determine the order of infection of SM and Mtb, nor we were able to determine the duration of current SM infection or the number of previous infections. All of these factors may play a role, not only in how T cells respond to SM antigens from different stages of the SM life cycle, but also in the potential for Mtb to impair these responses. This is particularly relevant since in western Kenya children as young as 3 years old have been shown to be infected with SM (158) and SM infections reach a prevalence of 60% by the time children are 11-13 years old (157). As such, there is a high probability that our study participants have had previous exposure to SM. We have tried to account for previous SM infection by including a naïve control group of US adults, who are seronegative for SM antibodies indicating no previous exposure. These individuals, however, are US residents and therefore likely differ in other unmeasured ways from the Kenyan participants.

It should be noted that we were not able to characterize the full T cell repertoire in this study. Notably we did not include markers to identify mucosal associated invariant T cells (MAIT cells) or invariant natural killer T cells (iNKT cells). Because both of these non-classical T cell subsets are $CD4^{-}CD8^{-}$, we cannot conclude that all of the $CD4^{-}CD8^{-}$ T cells observed in our overnight assay were γδ T cells. However, the robust responses of γδ T cells in the proliferation assay suggest that the cytokine responses observed in the overnight assay were due to γδ T cells. Indeed, correlation analysis found a significant positive correlation between the proliferation of γδ T cells and the frequency of cytokine producing $CD4^{-}CD8^{-}$ (data not shown). Furthermore iNKT cells constitute a very small portion of T cells in the blood (~0.5%) (81) and there are no currently published studies that suggest that MAIT cells are involved in the immune response to helminth infections. Indeed, it is believed that MAIT cells respond specifically to metabolites derived from

bacterial and fungal vitamin B synthesis pathways (79,80,191). Similarly, we were unable to confirm that cells defined as CD8 T cells in the overnight assay did not include γδ T cells. A subset of γδ T cells, termed intraepithelial lymphocytes, have been shown to express CD8, however these are tissue-resident and therefore not likely to be present in our PBMC samples (192,193). Lastly, we were not able to determine the v-chain usage of the SWAP responding γδ T cells. In humans γδ T cells expressing the Vδ2 and the Vγ2/Vγ9 (used interchangeably in the literature) chain are the dominant subset in the blood (82,174–176,194). As such, we assume these are Vγ2Vδ2 cells. This is of particular interest since Vγ2Vδ2 T cells have been shown to possess memory-like properties in murine models of Listeria and staphylococcal infection and primate models of BCG vaccination and Mtb infection (189,195). Furthermore, in primate models of Mtb infection, priming γδ T cells using the γδ specific ligand (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) protected animals against Mtb challenge (196).

Lastly, because SWAP is a complex mixture of antigens, we were not able to determine which specific antigen is recognized by γδ T cells. Previous literature has demonstrated that low molecular weight fractions of SWAP are responsible for inducing IFNγ production, however the specific antigens present in this fraction are still unknown (197). Peripheral blood γδ T cells respond to phosphoantigens from a variety of microbes (82). Two known γδ phosphoantigens, HMBPP and isopentenyl pyrophosphate (IPP), are both intermediates of isoprenoid synthesis pathways (83). Genetic analysis has found that SM possesses genes for the enzymes involved in the mevalonate pathway of isoprenoid synthesis and therefore may produce IPP during the adult stage of the life cycle (198,199). Furthermore, the drug mevinolin, which targets isoprenoid synthesis has been shown to impair egg production from schistosome worms, suggesting it is actively produced by adult worms (200). IPP is therefore a strong candidate ligand for γδ T cells.

In this study, we provide evidence that SM-specific γδ T cells have the capacity to respond to SM worms by producing type 1 cytokines. The immune response to SM is complex and dynamic but has mostly been characterized by type 2 responses. Indeed, the focus of most helminth immunology has been on type 2 and regulatory responses for their potential applications in other fields. While the contribution of type 1 cytokine producing γδ T cells during SM infection remains unclear, there are many potential roles for these γδ T cells in infection with SM. Studies in mice have shown that early IFNγ production can provide protection against SM infection, however this was directed at cercarial antigen and not adult worms (201). Furthermore, high IFNγ responses to SEA antigen were associated with protection in a cohort of persistently uninfected individuals from an endemic area in Brazil (202). Whether type 1 cytokines produced in response to the adult worm can provide similar benefits is unknown, particularly because adult worms are heavily shielded from the immune system by their external tegument (203). As such, the antigens in SWAP that induce γδ T cell responses may be hidden until the worm dies. It has been hypothesized, however, that these antigens may become exposed following treatment with praziquantel and that SWAP-specific immune responses may synergize with praziquantel to achieve worm death and expulsion (204). Robust SWAP-specific responses in PBMCs have also been associated with resistance to reinfection (205). Future studies in mice or primates would benefit from expanding γδ T cells at different stages of infection in order to determine their function in vivo and their potential in mediating protection from SM.

3.6 Tables and Figures

Table 3-1: Characteristics of study participants

	Naïve (N) n=12	Healthy Controls (HC) n=13	Latent TB Infection (LTBI) n=24	Active TB Disease (TB) n=16	p-value
Age (years)[a] [IQR]	42 [33 - 43]	25 [21 - 32]	34 [25 - 38]	40 [26 - 45]	0.005
sex: (%F)	75%	77%	42%	25%	0.01
(%M)	25%	23%	58%	75%	
SM eggs/gram[a] [IQR]	ND[b]	36 [12 - 120]	150 [36 - 333]	48 [21 - 87]	0.077
QFT IU/mL[a] [IQR]	ND[b]	0.00 [0.00 - 0.14]	9.11 [5.36 - 9.54]	ND[b]	

[a] Value denotes median
[b] ND, not done
IQR, interquartile range

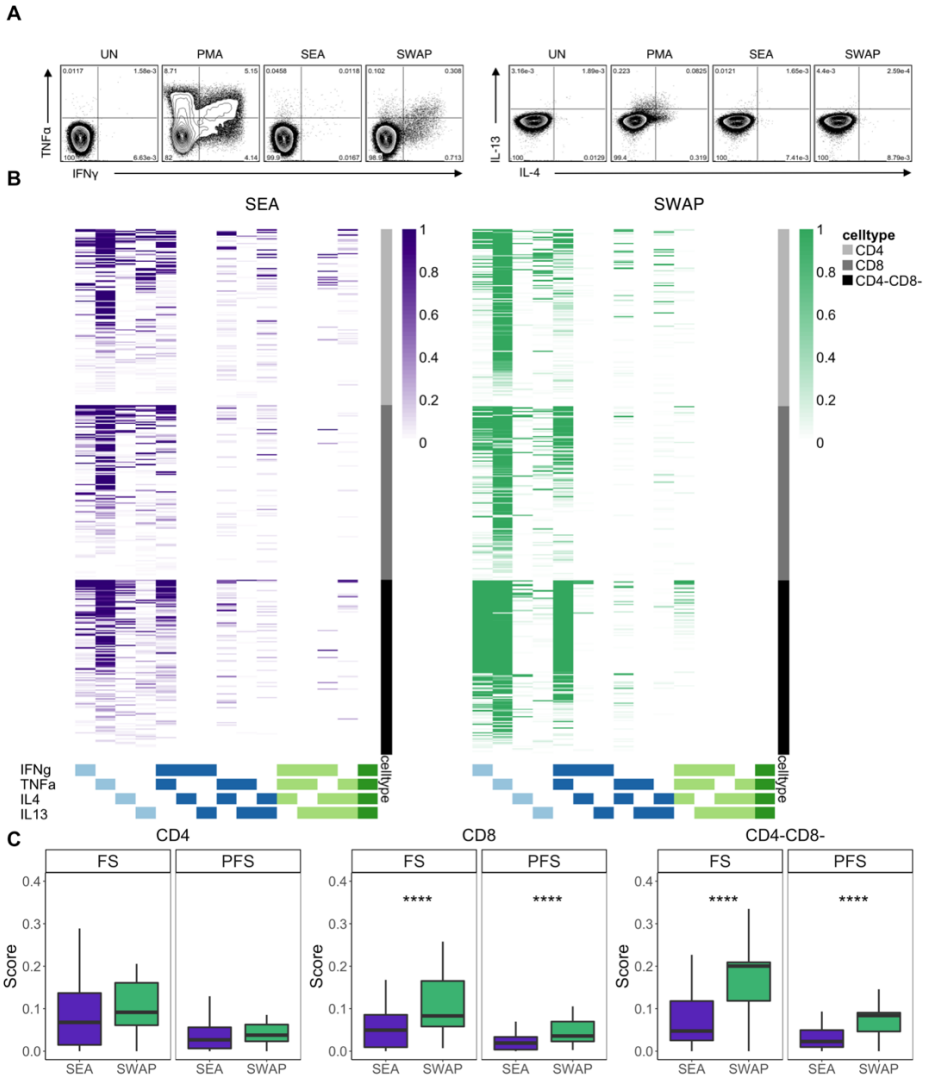


Figure 3-1: SWAP cytokine responses are higher than SEA cytokine responses amongst CD8 and CD4⁻CD8⁻ T cell subsets.

PBMC samples obtained from individuals in each of four groups defined by TB and SM infection status (N, n=12; HC, n=12; LTBI, n=23; TB, n=15) were incubated for 18 h in media alone (negative control) or stimulated with SEA, SWAP or PMA and ionomycin (positive control). Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry. (**A**) Representative flow cytometry data from a Naïve individual. Plots show cells gated on live CD3⁺ lymphocytes from the unstimulated (UN), PMA, SEA and SWAP stimulated conditions. (**B**) ICS data were analyzed using COMPASS and the results from each cytokine subset are displayed as a heatmap. Rows represent study subjects and columns represent cytokine combinations. The intensity of shading represents the probability of detecting a response above background. (**C**) Subject-specific COMPASS results were summarized for 63 individuals using the functionality and polyfunctionality scores. Scores from CD4, CD8 and CD4⁻CD8⁻ T cell subsets were aggregated across all groups. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the scores of each T cell population between SEA and SWAP were assessed using a Mann Whitney U test. **** p<0.0001

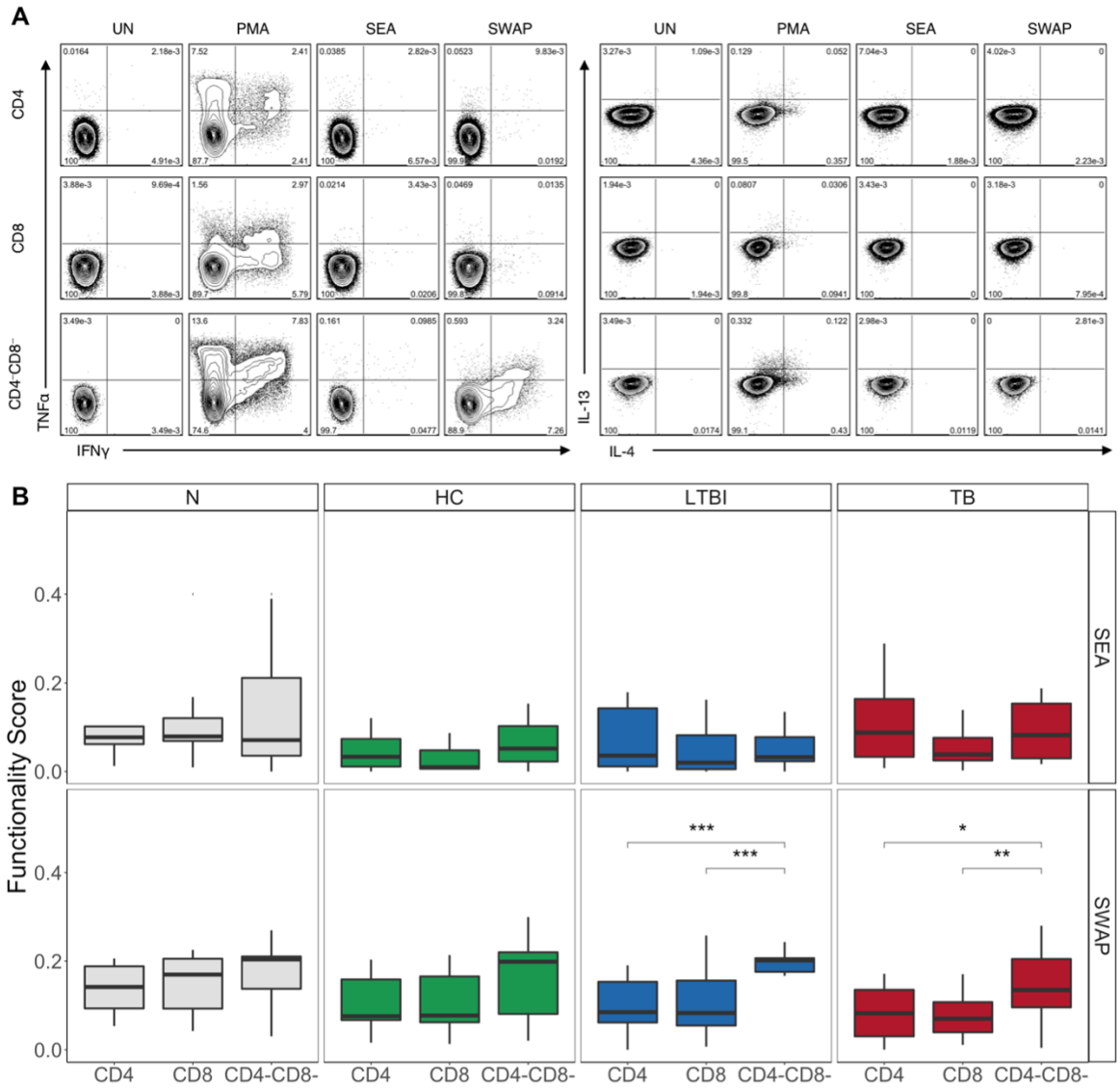


Figure 3-2: CD4⁻CD8⁻ T cells have a greater functional response to SWAP than CD4 and CD8 T cells in LTBI and TB individuals.

PBMCs from individuals in each group were stimulated and analyzed by flow cytometry as described in Figure 1. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry. **(A)** Representative flow cytometry data from a Naïve individual. Plots are shown gated on live $CD3^{+}CD4^{+}CD8^{-}$, $CD3^{+}CD4^{-}CD8^{+}$, and $CD3^{+}CD4^{-}CD8^{-}$ lymphocytes from the unstimulated (UN), PMA, SEA and SWAP stimulated condition. **(B)** COMPASS functionality scores among each T cell subset. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the scores of each T cell subset were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. *** $p<0.001$; ** $p< 0.01$; * $p< 0.05$

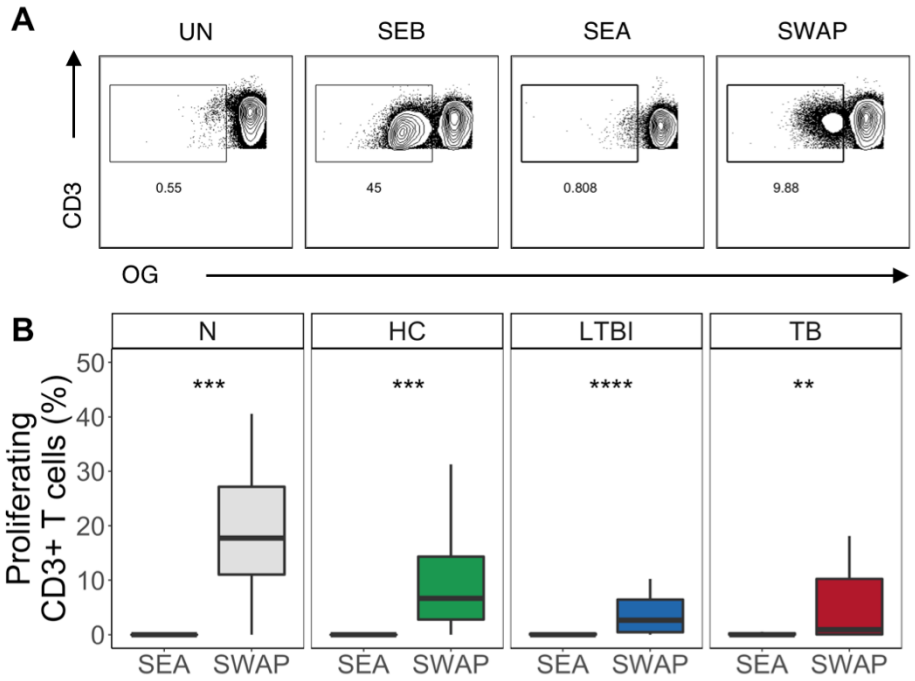


Figure 3-3: Higher frequencies of T cells proliferate in response to SWAP than in response to SEA, independent of Mtb and SM infection status.

Proliferation assays were performed using PBMC samples obtained from individuals in each of four groups defined by Mtb and SM infection status (N, n=10; HC, n=13; LTBI, n=24; TB, n=16). Cells were labeled with Oregon Green (OG) and incubated for 5 days under the following conditions: media alone (negative control), SEB (positive control), SEA or SWAP. **(A)** Representative flow cytometry data from an LTBI individual. Plots show cells gated on live $CD3^+$ lymphocytes. **(B)** Frequency of OG^{lo} (proliferating) T cells to SEA and SWAP. Data are shown after subtraction of background proliferation in the unstimulated negative control condition. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of proliferating T cells between SEA and SWAP were assessed using a Mann Whitney U test. **** $p<0.0001$; *** $p<0.001$; ** $p< 0.01$

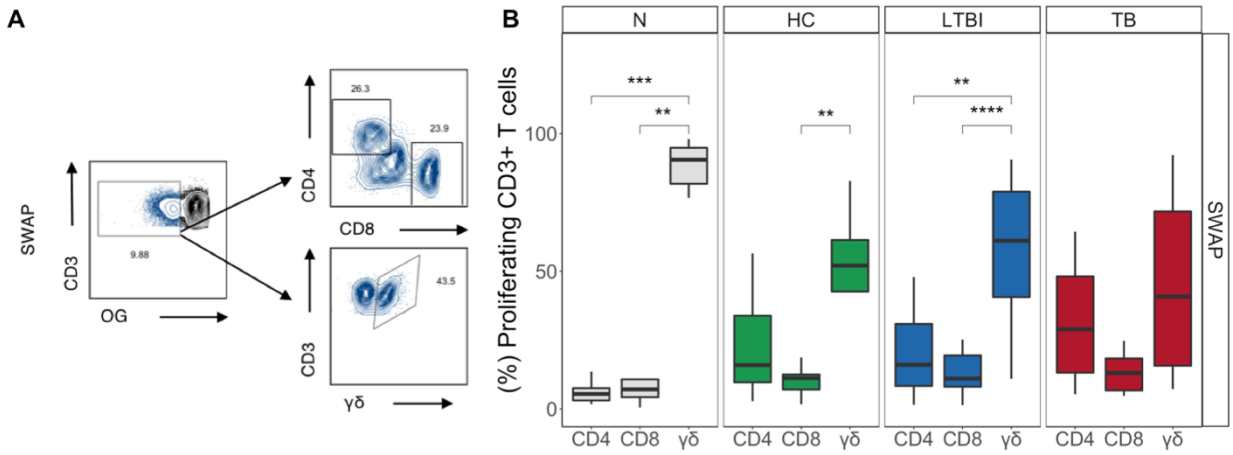


Figure 3-4: Proliferating SWAP-reactive T cells are predominantly γδ T cells.

Proliferating $CD3^+$ T cells from the SWAP conditions were evaluated by flow cytometry for expression of CD4, CD8 and γδ. **(A)** Representative flow cytometry data from an LTBI individual. Plots show the expression of CD4, CD8 and γδ on cells gated on live OG^{lo} $CD3^+$ lymphocytes. **(B)** The proportions of SWAP-reactive proliferating T cells that are CD4, CD8 and γδ T cells were evaluated in each Mtb and SM infection group. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each T cell population was assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. **** $p<0.0001$; *** $p<0.001$; ** $p< 0.01$

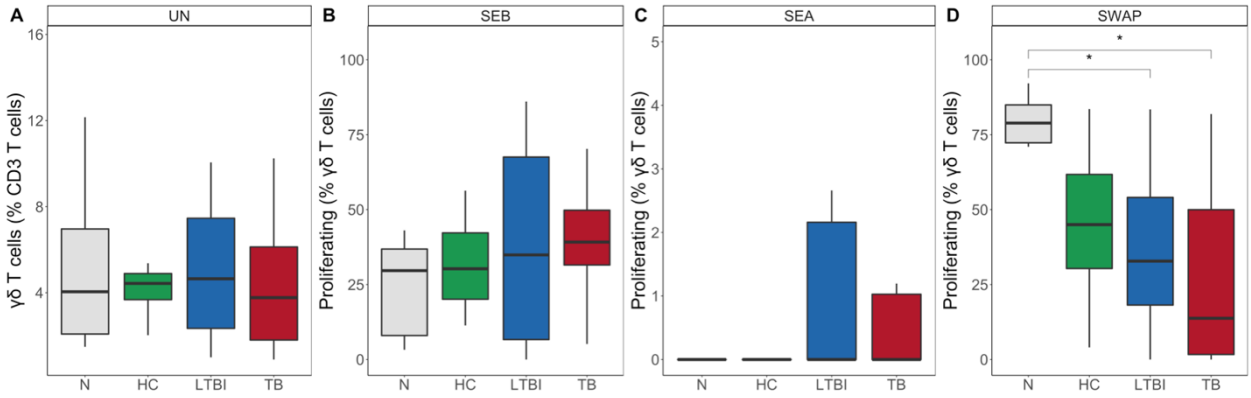


Figure 3-5: SWAP-reactive γδ T cells have lower proliferative capacity in individuals with LTBI and active TB.

Proliferation assays were performed as described in Figure 3. **(A)** Frequency of total γδ T cells in the unstimulated (UN) condition (N, n=10; HC, n=13; LTBI, n=24; TB, n=16). **(B-D)** Frequency of proliferating (OG^{lo}) γδ T cells to SEB **(B),** SEA **(C)** and SWAP **(D)**. Proliferation data are shown after subtraction of background proliferation in the UN condition. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each proliferating γδ T cell population between groups were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. * $p < 0.05$

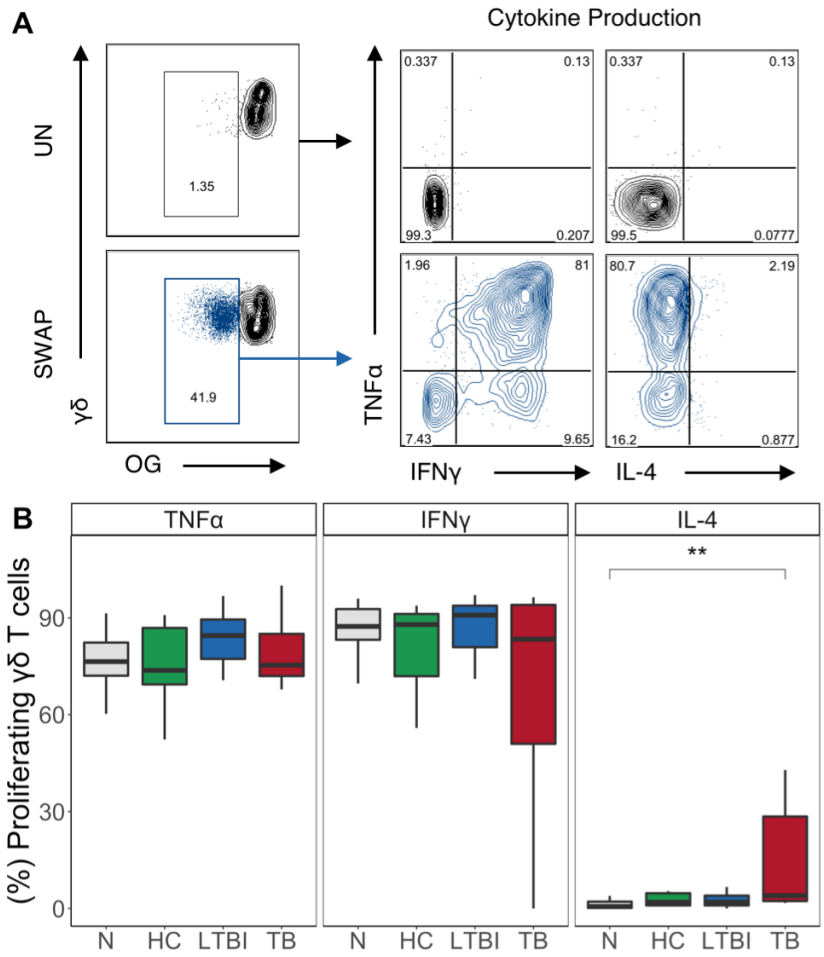
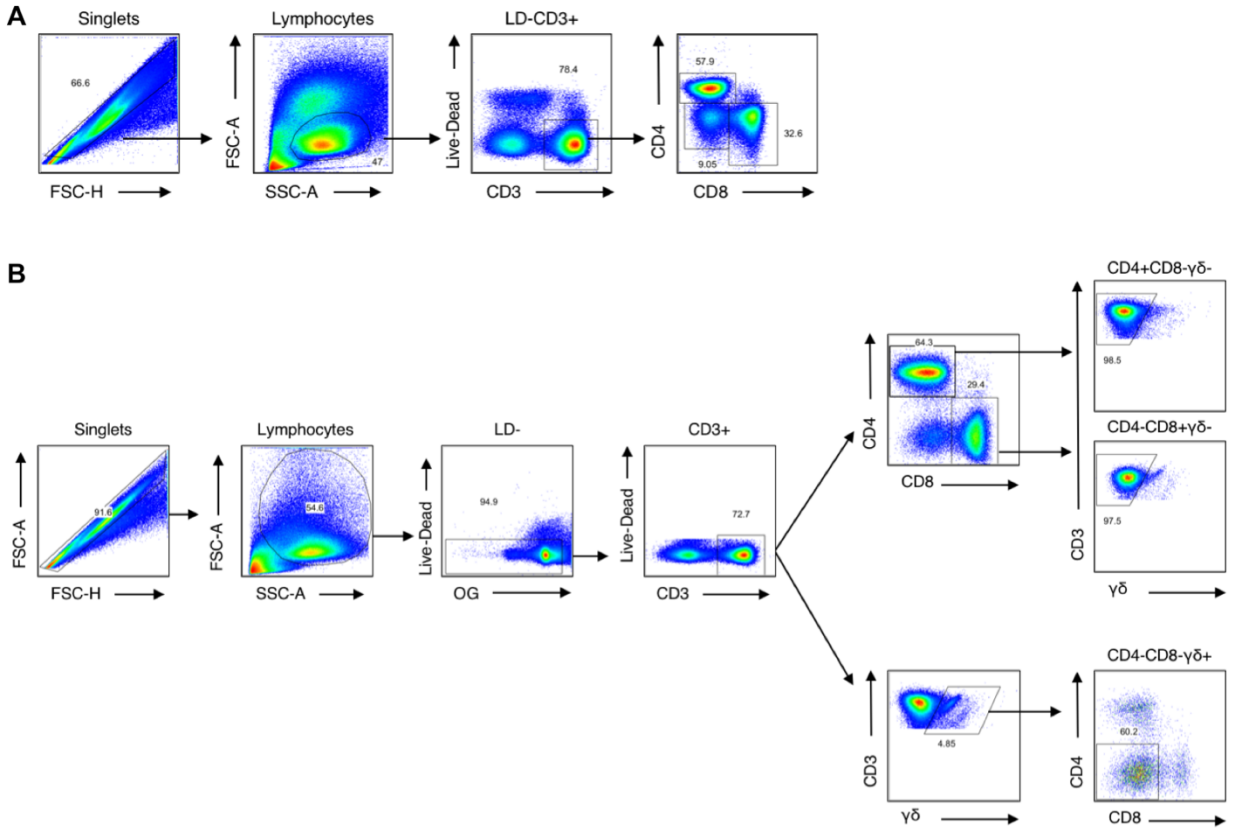


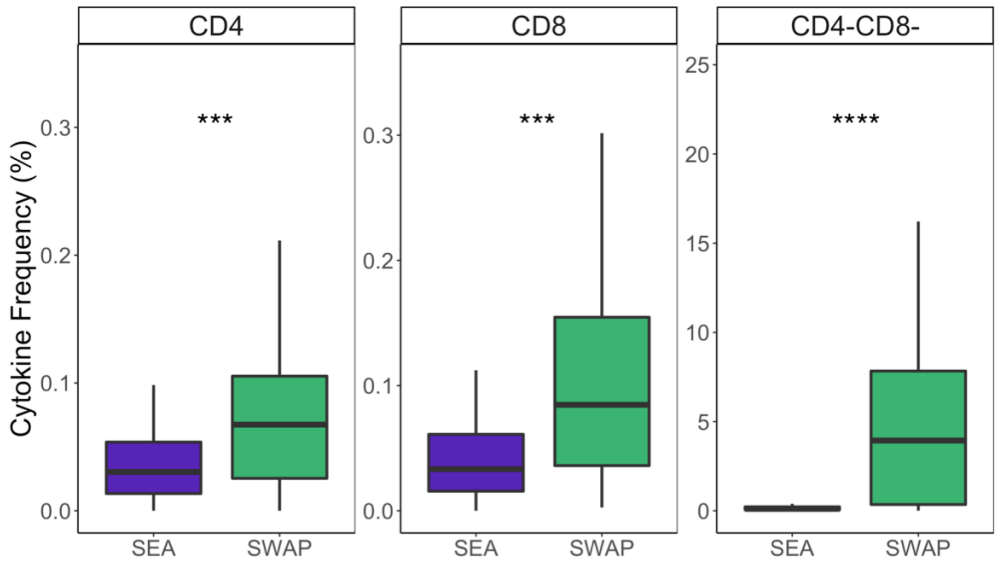
Figure 3-6: Individuals with active TB have higher frequencies of IL-4 producing SWAP-reactive γδ T cells.

On day 5 of the proliferation assay PBMCs were restimulated with PMA and ionomycin for 5 hours to induce cytokine production. Samples meeting the criteria for a positive proliferative response (see Materials and Methods) were evaluated for cytokine expression (N, n=8; HC, n=12; LTBI, n=21; TB, n=10). **(A)** Representative flow plots from an LTBI individual. Unstimulated samples (upper) show cytokine production on cells gated on live $CD3^{+}CD4^{-}CD8^{-}\gamma\delta^{+}$ lymphocytes. SWAP-stimulated samples (lower) show cytokine production and phenotypes on cells gated on live $OG^{lo}CD3^{+}CD4^{-}CD8^{-}\gamma\delta^{+}$ lymphocytes. **(B)** Frequency of $cytokine^{+}$ cells amongst proliferating γδ T cells. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each $cytokine^{+}$ γδ T cell population between groups were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. ** $p < 0.01$



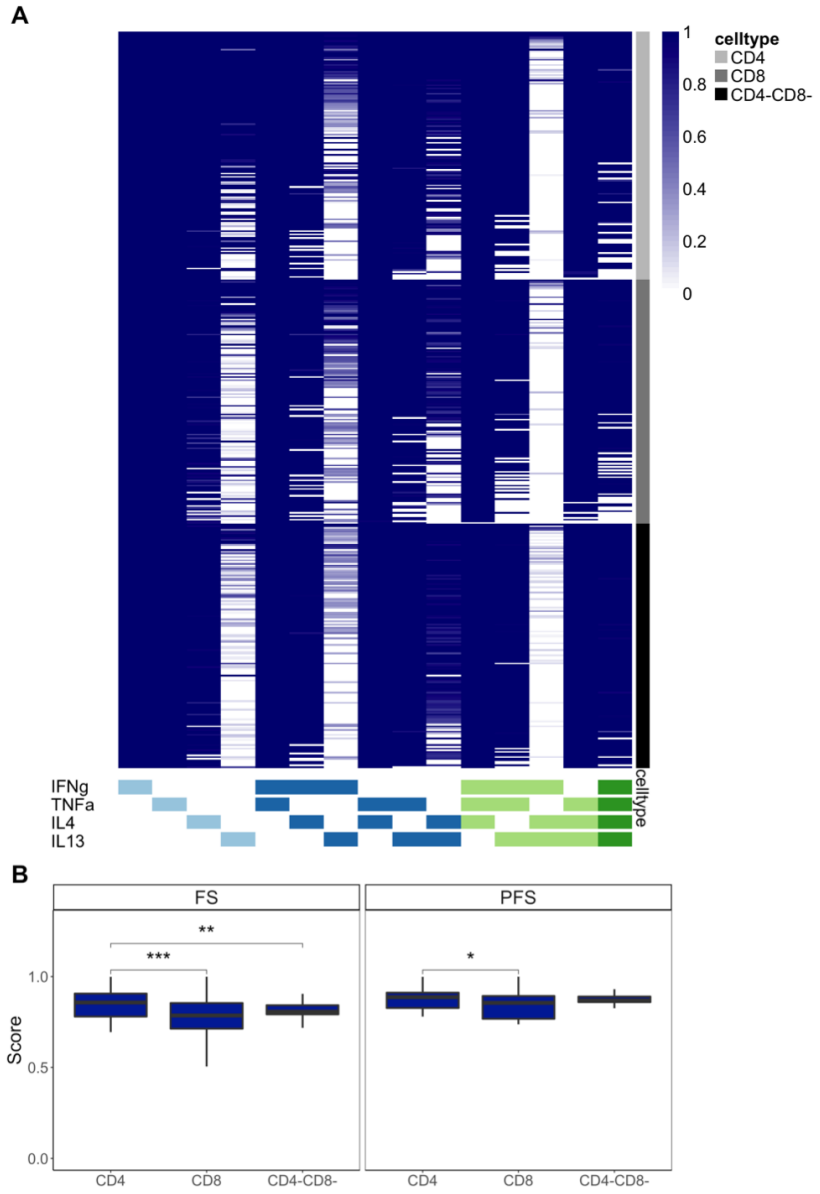
Supplementary Figure 3-1: Gating strategy for flow cytometry analysis.

(A) In this sample gating for the overnight ICS assay, cells were first gated for singlets (FSC-H vs. FSC- A) and lymphocytes (SSC-A vs. FSC-A). The lymphocyte gate is further analyzed for their uptake of the Zombie IR Live/Dead stain to determine live versus dead cells and their expression of CD3 (Zombie Near-IR^{lo}, CD3^{+}). CD4 and CD8 surface expression is then determined from this gated population. CD4 T cells were defined as CD3^{+}CD4^{+}CD8^{-} lymphocytes, CD8 T cells were defined as CD3^{+}CD4^{-}CD8^{+} lymphocytes, and a third population of T cells were defined as CD3^{+}CD4^{-}CD8^{-}lymphocytes (B) In this sample gating for the Proliferation ICS assay, cells were first gated for singlets (FSC-H vs. FSC- A) and lymphocytes (SSC-A vs. FSC-A). The lymphocyte gate is further analyzed for their uptake of the Zombie IR Live/Dead stain to determine live versus dead cells (Zombie Near-IR^{lo}). Live cells are then gated for their expression of CD3 (CD3^{+}). CD4, CD8 and γδ surface expression is then determined from this gated population. CD4 T cells were defined as CD3^{+}CD4^{+}CD8^{-}γδ^{-} lymphocytes, CD8 T cells were defined as CD3^{+}CD4^{-}CD8^{+}γδ^{-} lymphocytes, γδ T cells were defined as CD3^{+}CD4^{-}CD8^{-}γδ^{+} lymphocytes



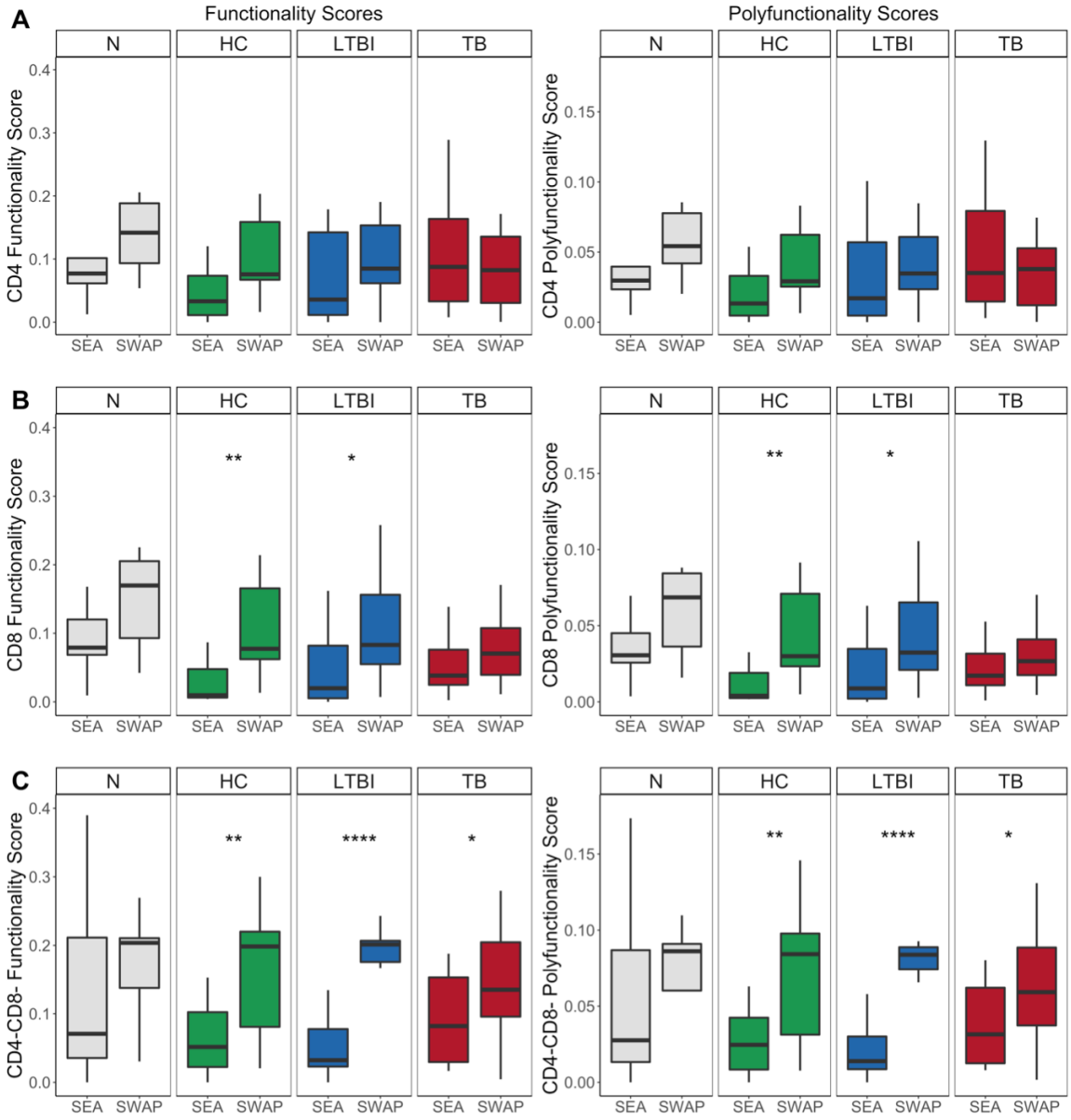
Supplementary Figure 3-2: Cytokine Frequencies are higher following SWAP stimulation than SEA Stimulation.

PBMCs from individuals in each group were stimulated and analyzed by flow cytometry as described in Figure 3-1. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry. Frequency of cytokine$^+$ cells within each designated celltype. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the cytokine frequency between SEA and SWAP were assessed using a Mann Whitney U test. *** $p<0.001$; ** $p< 0.01$; * $p< 0.05$



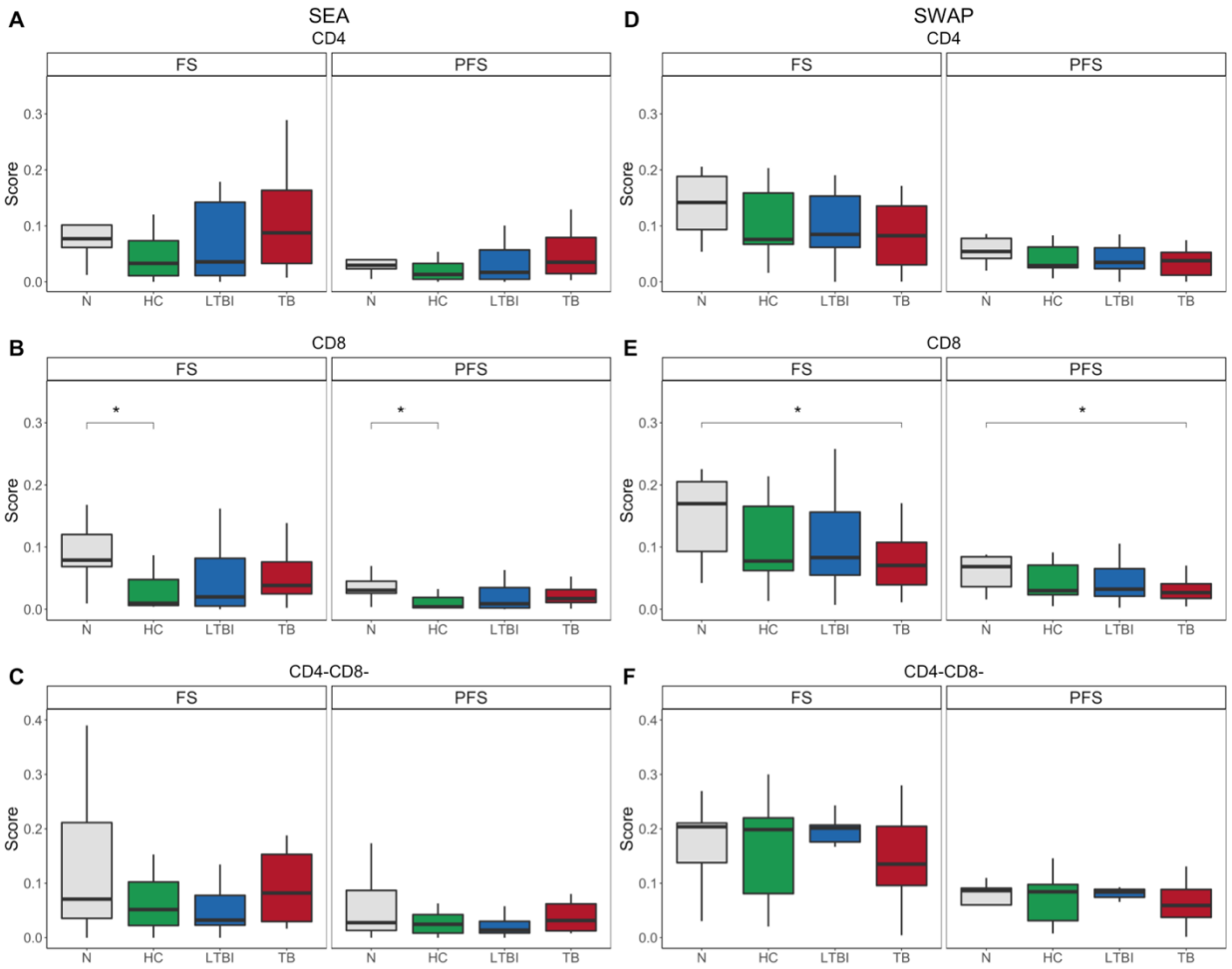
Supplementary Figure 3-3: CD4 T cells have a greater functional response to PMA than CD8 and CD4$^-$CD8$^-$ T cells.

PBMCs from individuals in each group were stimulated with PMA and analyzed by flow cytometry as described in Figure 3-1. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry. (A) ICS data were analyzed using COMPASS and the results from each cytokine subset are displayed as a heatmap. Rows represent study subjects and columns represent cytokine combinations. The intensity of shading represents the probability of detecting a response above background. (C) Subject-specific COMPASS results were summarized for 63 individuals using the functionality and polyfunctionality scores. Scores from CD4, CD8 and CD4$^-$CD8$^-$ T cell subsets were aggregated across all groups. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences between the scores of each T cell subset were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. *** $p<0.001$; ** $p< 0.01$; * $p< 0.05$



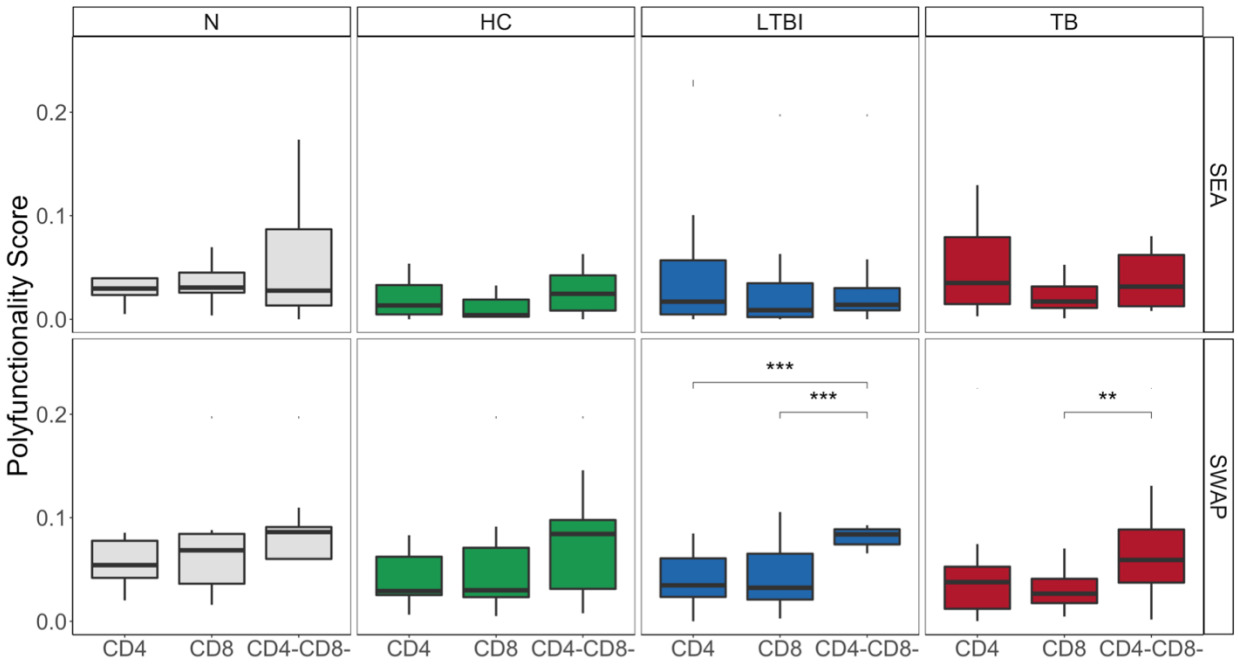
Supplementary Figure 3-4: SWAP functionality and polyfunctionality scores are higher than SEA responses in HC and LTBI groups.

PBMC samples obtained from individuals in each of four groups defined by TB and SM infection status (N, n=12; HC, n=12; LTBI, n=23; TB, n=15) were incubated for 18 h in media alone (negative control) or stimulated with SEA or SWAP. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry and data were analyzed using COMPASS. (A-C) Functionality and polyfunctionality scores for CD4 (A), CD8 (B), and $CD4^{-}CD8^{-}$ (C) T cells. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the scores of each T cell subset were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. **** $p<0.0001$; *** $p<0.001$; ** $p< 0.01$; * $p< 0.05$



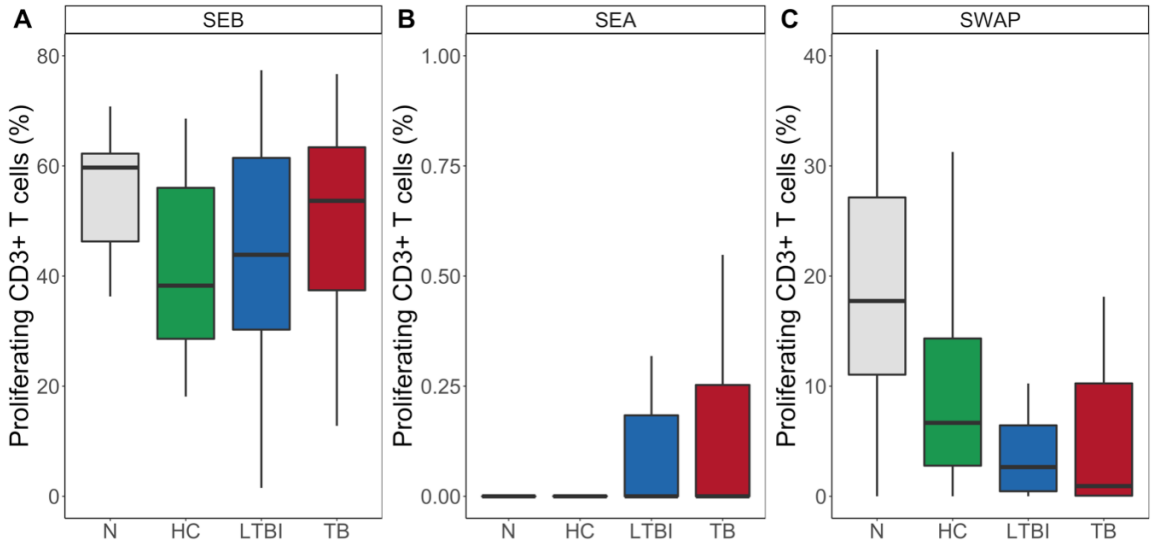
Supplementary Figure 3-5: CD8 T cell responses to both SEA and SWAP are higher in Naïve individuals

PBMC samples obtained from individuals in each of four groups defined by TB and SM infection status (N, n=12; HC, n=12; LTBI, n=23; TB, n=15) were incubated for 18 h in media alone (negative control) or stimulated with SEA or SWAP. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry and data were analyzed using COMPASS. (A-C) SEA-specific Functionality and polyfunctionality scores for CD4 (A), CD8 (B), and $CD4^{-}CD8^{-}$ (C) T cells. (D-F) SWAP-specific Functionality and polyfunctionality scores for CD4 (D), CD8 (E), and $CD4^{-}CD8^{-}$ (F) T cells. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the scores of each T cell subset were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. * $p < 0.05$



Supplementary Figure 3-6: $CD4^{-}CD8^{-}$ T cells have a greater polyfunctional response to SWAP than CD4 and CD8 T cells in LTBI and TB individuals.

PBMC samples obtained from individuals in each of four groups defined by TB and SM infection status (N, n=12; HC, n=12; LTBI, n=23; TB, n=15) were incubated for 18 h in media alone (negative control) or stimulated with SEA or SWAP. Intracellular expression of IFNγ, TNFα, IL-4 and IL-13 was measured by flow cytometry. COMPASS functionality scores among each T cell subset. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the scores of each T cell subset were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons. *** $p<0.001$; ** $p< 0.01$



Supplementary Figure 3-7: Proliferation of total CD3⁺ T cells does not differ by Mtb and SM infection status.

Proliferation assays were performed using PBMC samples obtained from individuals in each of four groups defined by Mtb and SM infection status (N, n=10; HC, n=13; LTBI, n=24; TB, n=16). Cells were labeled with Oregon Green (OG) and incubated for 5 days under the following conditions: media alone (negative control), SEB (positive control), SEA or SWAP. (A-C) Frequency of OG^{lo} (proliferating) $CD3^+$ T cells to SEB (A), SEA (B) and SWAP (C). Data are shown after subtraction of background proliferation in the unstimulated negative control condition. Boxes represent the median and interquartile ranges; whiskers represent the 1.5*IQR. Differences in the frequency of each cytokine⁺ γδ T cell population between groups were assessed using a Kruskal Wallis test with Nemenyi correction for multiple pairwise comparisons.

Chapter 4 *Schistosoma mansoni* Infection is Associated with a Higher Probability of Tuberculosis Disease in HIV$^+$ Adults in Kisumu, Kenya

This chapter is reproduced with minor edits from: McLaughlin TA, Khayumbi J, Ongalo J, et al. *Schistosoma mansoni* Infection is Associated with a Higher Probability of Tuberculosis Disease in HIV$^+$ Adults in Kisumu, Kenya. Clinical Infectious Diseases (In preparation)

4.1 Abstract

Helminth infections can modulate immunity to *Mycobacterium tuberculosis* (Mtb). However, the effect of helminths, including *Schistosoma mansoni* (SM), on Mtb infection outcomes is less clear. Furthermore, HIV is a known risk factor for tuberculosis (TB) disease and has been implicated in SM pathogenesis. Therefore, it is important to evaluate whether HIV modifies the association between SM and Mtb infection. Adults in Kisumu, Kenya were enrolled into three categories based on Mtb infection status: Mtb-uninfected healthy controls, latent TB infection (LTBI), and active TB disease. Participants were subsequently evaluated for infection with HIV and SM. Targeted minimum loss estimation and super learning were utilized to estimate a covariate-adjusted association between Mtb infection outcomes and SM. HIV status was further evaluated as an effect modifier of this association. SM was not associated with differences in baseline demographic or clinical features of participants in this study, nor with additional parasitic infections. Covariate-adjusted analyses indicated that infection with SM was associated with a 4% higher estimated proportion of active TB cases in HIV^- individuals and a 14% higher estimated proportion of active TB cases in HIV^+ individuals. There were no differences in estimated proportions of LTBI cases. We provide evidence that SM infection is associated with a higher probability of active TB disease, particularly in the setting of HIV.

4.2 Introduction

Mycobacterium tuberculosis (Mtb) is a serious public health concern, infecting a quarter of the world, leading to 10 million cases of tuberculosis (TB) disease and resulting in 1.5 million deaths annually (8). Infection with Mtb leads to a spectrum of clinical states ranging from complete clearance, to latent infection (LTBI), to subclinical and active TB disease (28). The mechanisms that lead to these disparate outcomes are not well understood; however, coinfections, such as with human immunodeficiency virus (HIV), and comorbidities, such as diabetes, are known risk factors (8). Helminths, which are known to be immunomodulatory, are co-endemic with Mtb (47,44,43). As such, determining the impact of helminths on Mtb infection outcomes is important and may identify potential risk factors for Mtb infection and the development of TB disease.

While the impact of helminths on immunity to Mtb is well documented (43,111), the consequence of helminth infection on TB disease is less clear. Some studies have reported higher prevalence of intestinal helminth infections in individuals with active TB as compared with healthy controls (206–208). Others, however, have found no significant differences in helminth infections between individuals with TB and healthy controls (209,210). In addition, two longitudinal studies found no increase in TB incidence among individuals infected with intestinal helminths in either HIV^- (126) or HIV^+ individuals (211). This variation is particularly evident in a recent meta-analysis of epidemiological studies of individuals co-infected with Mtb and intestinal helminths, which found that prevalence of co-infection as well as measured associations between helminth and Mtb infections varies greatly between studies, as well as between helminth species (128). These findings may indicate that associations for a single helminth species are not broadly applicable to all helminth species. Moreover, it is not clear that these results apply when considering helminths that do not reside in the intestine, such *Schistosoma mansoni* (SM) (53).

Infection with SM or other schistosome worms leads to the clinical disease schistosomiasis, which is the second most common form of helminthiasis and affects 240 million people globally (118). In Sub-Saharan Africa, approximately one quarter of the population is estimated to be infected with SM (61). Schistosome infections are estimated to cause 280,000 deaths in Sub-Saharan Africa per year (62). Despite this high disease burden, few studies have measured the association between SM and TB disease. Some evidence suggests that individuals with SM are at higher risk of developing TB disease and that SM infection may impact the clinical manifestations of TB disease (210,211). However, others have reported decreased odds of TB disease in individuals infected with SM (207). Importantly, there has not been a comprehensive analysis of SM infection on Mtb infection outcomes that includes both LTBI and active TB.

There are also limited data on the impact of SM and HIV coinfection on Mtb infection outcomes. This is of particular interest, not only because HIV is the greatest risk factor for TB disease (212), but also due to the bi-directional effects of SM and HIV. Schistosomiasis is suspected to play a role not only in susceptibility to HIV, but also in higher viral load set point and worse HIV disease progression (213). HIV in turn has been shown to reduce egg shedding from SM adult worms, which not only makes detecting infection more difficult but can also result in increased pathogenesis due to higher internal egg burden (214). Thus, the combination of these three infections could have distinct clinical outcomes.

The inability to perform randomized controlled trials of SM infection makes it challenging to definitively evaluate the impact of SM on Mtb infection outcomes. Nevertheless, we used data from an observational cohort coupled with modern causal inference and machine learning methodology to estimate a covariate-adjusted association between SM and Mtb infection

outcomes. We further evaluated whether HIV infection modified this association. Our analysis suggests that SM infection is associated with a higher probability of active TB but not LTBI, and that this is exacerbated in HIV^+ individuals.

4.2 Materials and Methods

Study Population:

This study was part of a larger case-control study analyzing cellular immune responses to Mtb. Participants ≥18 years old were recruited in Kisumu, Kenya as described previously (141,180). Participants were screened and enrolled based on Mtb infection status and then evaluated for HIV and SM infection. Of 1109 participants screened for this study, 941 had complete Mtb, HIV and SM study records and were included in the analysis (Figure 4-1).

Laboratory Results:

Mtb infection outcomes were defined as Healthy Controls (HC), latent TB infection (LTBI), and active TB disease (TB) based on the following criteria. Healthy asymptomatic individuals with no previous history of TB disease or treatment were evaluated by QuantiFERON®-TB Gold In-Tube (QFT) assay. HC were defined as those with a negative QFT result (TB Ag-Nil <0.35 IU IFNγ/mL). LTBI were defined as those with a positive QFT result (TB Ag-Nil >0.35 IU IFNγ/mL). TB participants were initially screened as referrals from satellite public health clinics in Kisumu who had an initial positive sputum smear or GeneXpert MTB/RIF result. Upon enrollment in the study, another sputum sample was collected and evaluated for Mtb by fluorescence microscopy, GeneXpert MTB/RIF, HAIN MTBDR, and MGIT culture. Individuals with active TB were evaluated within the first 7 days of initiating the standard 6-month course of TB treatment, which was provided according to Kenyan national health guidelines.

Helminth infection was determined using standard Kato Katz microscopy. Two thick Kato Katz smears were prepared from stool samples collected on two separate days. Slides were analyzed by experienced lab technicians who recorded the presence of eggs belonging to SM,

hookworm, *Ascaris lumbricoides*, and *Trichuris trichuria*. SM^+ individuals were defined as having at least one egg present in at least one slide examined. The number of SM eggs were counted and recorded as eggs per gram (epg) for SM^+ individuals.

Serologic testing for HIV antibodies was done for all individuals using the Diagnostic Kit for HIV (1+2) Antibody V2 (*KHB*® Shanghai Kehua Bio-engineering Co., Ltd). Plasma HIV viral load and CD4 T cell counts were measured for HIV^+ individuals.

Women of childbearing age were tested for pregnancy using test strips for detection of hCG in urine. Individuals were tested for malaria using a malaria rapid diagnostic test (SD BIOLINE Malaria Ag P.f/Pan test from Abbott).

Ethics Statement:

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All participants gave written informed consent for the study, which was approved by the KEMRI/CDC Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

Statistical Analysis:

Participants' demographic and clinical characteristics were summarized using descriptive statistics based on SM and Mtb infection status. Study groups were compared using a Kruskal-Wallis test for continuous data, with pairwise comparisons adjusting for multiple testing using the Benjamini & Hochberg method in the case of three or more groups. Comparisons of categorical data were made using either a χ^2 or Fisher's exact test.

Our primary analysis examined differences in Mtb infection outcomes over the entire study population, and separately amongst HIV^+ and HIV^- individuals. To control for potential confounders of SM and Mtb outcomes, we adjudicated a pre-specified list of potential confounders. We used these data to estimate the covariate-adjusted probability of outcomes under each SM infection state using targeted minimum loss estimation (TMLE). TMLE is a doubly-robust method that requires estimates of the probability of SM infection as a function of covariates (a propensity score) and the probability of each Mtb infection outcome as a function of SM status and covariates (215). These estimates were both obtained using SuperLearner. SuperLearner requires pre-specifying multiple candidate regression models and uses cross-validation to build a combination of the various models that provides the best estimated fit to the observed data (216). Pre-specified regressions and further details of this approach are indicated in Supplementary Figure 4-1. We used Wald tests with a significance level of 0.05 to test the null hypothesis of no difference in proportion of Mtb outcomes between SM groups. Standard error estimates were obtained using estimated influence functions and the delta method.

Our secondary analysis evaluated whether HIV modified the effect of SM infection on Mtb infection outcomes. We tested the null hypothesis that the difference in the probability of each Mtb infection outcome between SM^- and SM^+ groups was equivalent between HIV^- and HIV^+ groups. Furthermore, we performed a sensitivity analysis to evaluate the impact of adjusting for CD4 T cell counts, measured amongst HIV^+ individuals. All primary and secondary statistical analyses were carried out with R software, using the SuperLearner (216) and drtmle (217) packages.

4.3 Results

Of the 941 individuals included in the analysis 177 (18.81%) tested positive for SM (Table 4-1). The median egg burden for SM^+ individuals was 48 epg, with 122 individuals categorized as having a light intensity infection (1-99 epg), 41 a moderate intensity infection (100-399 epg) and 14 a heavy intensity infection (>399 epg) according to WHO standards (118).

Participants in each SM group were similar with regard to demographic and clinical parameters (Table 4-1). Few participants had additional parasitic infections, the majority of which were hookworm infections. Furthermore, the frequency of both malaria and additional helminth infections was equivalent between SM^- and SM^+ groups. The proportion of HIV^+ individuals in each group was equivalent and had similar clinical features with regard to viral load and CD4 count between SM^- and SM^+ individuals. There were no differences in median quantitative QFT response or the percentage of abnormal chest x-rays between SM^- and SM^+ individuals.

Participants in Mtb infection groups were different with regard to both clinical and demographic measures (Table 4-2). HC individuals were younger than individuals in both the LTBI and TB group. TB participants had lower hemoglobin levels compared to LTBI and HC individuals. In addition, there were fewer females and more participants from Kisumu in the TB group compared to the LTBI and HC group. The LTBI group had the lowest frequency of HIV^+ individuals, the lowest viral load, and the highest CD4 T cell counts. All HIV^+ HC and LTBI individuals were ART treatment naïve at the time of screening. A smaller frequency of TB participants had additional helminth infections compared to LTBI and HC, particularly with regard to hookworm infection.

To estimate a covariate-adjusted association between SM and Mtb infection outcomes, we utilized machine learning to build regressions for both the propensity score and the outcome

regression. These models were built using the combined HIV^- and HIV^+ data sets and pre-specified regressions with the SuperLearner package in R (Supplementary Figure 4-1). We then used TMLE to evaluate the impact of SM infection on Mtb infection outcomes in HIV^- and HIV^+ individuals. The estimated probability of HIV^- individuals with active TB was modestly higher in SM^+ as compared to SM^- (SM^+: 19.7%; SM^-: 15.8%), although the distribution of Mtb infection outcomes was not statistically different between SM groups (Table 4-3, p-value = 0.486). By contrast, within the HIV^+ group, the estimated distribution of Mtb infection outcomes differed significantly between SM^+ and SM^- groups (Table 4-3, p-value = 0.0018). The difference was driven largely by higher estimates of active TB in the SM^+ group when compared to the SM^- group (SM^+: 41.3%; SM^-: 27.4%). Interestingly, the estimated proportion of HIV^+ individuals with LTBI was similar between groups (SM^+: 38.31%; SM^-: 36.65%). Sensitivity analysis including adjustment for CD4 count led to a modest attenuation of the association of SM on the probability of active TB; however, the estimated distribution of Mtb infection outcomes between SM^- and SM^+ groups still differed significantly among HIV^+ individuals (Table 4-4).

We then evaluated whether HIV modifies the probability of Mtb outcomes attributable to SM infection. The difference in the proportions of Mtb infection outcomes beween SM^+ and SM^- groups was indeed significantly different in HIV^+ individuals compared to HIV^- individuals (Table 4-3, p-value = 0.0002). In particular, amongst HIV^- individuals there was only a 3.9% higher probability of TB attributable to SM infection, whereas in the HIV^+ group, SM^+ individuals had a 13.9% higher probability of TB than SM^- individuals. This suggests that the combination of HIV and SM coinfection is associated with altered Mtb infection outcomes, compared with either infection alone.

4.4 Discussion

In this study we used a robust machine learning-based strategy to evaluate the association of SM infection on Mtb infection outcomes amongst HIV^+ and HIV^- individuals in western Kenya. Using machine learning approaches, we determined that SM infection perturbs the distribution of Mtb infection outcomes and was associated with a higher proportion of active TB disease, with this difference most pronounced in in HIV^+ individuals.

Our data provide evidence that SM is associated with higher probability of active TB disease, after controlling for common covariates and additional helminth infections. This is consistent with findings from previous observational studies which evaluated SM as well as gastro-intestinal helminths, despite differences in study design and setting (210,211). While helminths in general have been shown to impair Mtb immunity by compromising TH1 responses (43,111), this has not been observed for SM in humans (180). As such, the means by which SM might contribute to active TB disease remains unclear. Further studies need to be conducted to ascertain the potential mechanism by which this occurs, particularly in the setting of HIV coinfection.

The strong association of SM with active TB amongst HIV^+ but not HIV^- individuals suggests that coinfection with HIV and SM may be a greater risk factor than either infection alone. This is consistent with a study conducted in Uganda that reported an increased incidence of active TB amongst SM^+ HIV^+ individuals compared with SM^- HIV^+ individuals (211). Thus, treatment of SM amongst HIV^+ individuals should be considered in the clinical management of TB as well as in TB control programs.

The main strengths of this study are a large sample size and the use of a robust analytic method. While this cohort had a large number of coinfected individuals, SM was likely underdiagnosed in the HIV^+ group. HIV is known to reduce egg burden in stool, which was the

diagnostic criterion utilized in this study (70). Despite this, our analysis strategy was able to detect the effect of HIV on the association between SM and active TB. In contrast, traditional analysis methods, such as logistic regression, may have suffered from a lack of power after stratifying Mtb infection outcomes by both SM and HIV infection status. Our findings therefore support alternative testing methods, such as circulating cathodic antigen (CCA) tests, to evaluate SM infection amongst HIV^+ individuals in future studies. Not only is this test more sensitive than microscopy (57,74), but it has been shown to perform well in HIV^+ individuals with low egg counts (70).

While we found little evidence of confounding by measured characteristics, the observational nature of the study leaves open the possibility of bias by unmeasured confounding such as socioeconomic status, occupational risk, and previous SM infection and/or treatment. This study was also limited by the inability to determine the order or timing of each infection, which would be crucial for deriving any causal conclusions about the direction of effects in this setting. Epidemiologically, people living in western Kenya often become infected with SM early in their childhood with prevalence reaching a peak of around 60% in early adolescence (158). While mass drug administration has been very successful in this region, reinfection does occur in both children and adults (159). This suggests that individuals likely experience repeated SM infections prior to HIV and Mtb infection. One study in Uganda which reported increased TB incidence in SM^+ individuals compared with SM^- individuals supports the notion that prior SM infection can increase the risk of active TB disease (211). Future studies, however, would benefit from evaluating Mtb infection outcomes over time amongst SM^+ individuals with and without HIV infection. This would be of particular interest in the setting of anthelminthic treatment with praziquantel.

The interpretation of our results is influenced by the definitions used for Mtb infection outcomes, which are based on imperfect diagnostic tools. First and foremost, there is no gold standard for diagnosing LTBI (218). The test utilized in this study, QuantiFERON-TB Gold (QFT), is an IFNγ release assay (IGRA) and is based on an immune response to Mtb antigens (30). A positive IGRA therefore does not differentiate LTBI from subclinical or active TB. Furthermore, there is significant within-subject variability that can lead to discordant test results during serial testing (218). We only performed one IGRA test to stratify individuals into HC and LTBI. The diagnosis of active pulmonary TB relies on a combination of symptoms and microbiological validation. These microbiological assays include microscopy, culture-based methods and molecular tests, the sensitivity of which have sensitivities ranging from 50-90% (33). Culture-based tests are the gold standard test for active TB, however they require a high enough bacterial burden and a quality sputum sample for optimal performance (33). The diagnosis of LTBI and active TB are even more challenging in HIV^+ individuals who are more likely to have indeterminate IGRA results and negative microbiological tests (219,220).

In high pathogen burden regions such as western Kenya, individuals may be infected with multiple microbes at any given time. It is therefore important to understand the interplay between these infections, not only to appropriately treat each individual, but also to manage public health initiatives. We provide evidence from machine learning-based approaches that SM infection is associated with altered Mtb infection outcomes in HIV^+ individuals. Further research is needed to establish the underlying mechanisms by which this occurs, and to compare SM-induced immune regulation in HIV^- and HIV^+ individuals. This finding, however, if supported by a prospective study, could have relevance to the control of TB in SM-endemic areas of the world. Our findings suggest that treatment of SM with praziquantel should be considered in the clinical management

of HIV^+ TB patients. Furthermore, regular praziquantel treatment of HIV^+ individuals who are at risk for SM infection could be used as an additional low-cost component of TB control programs. Such consideration when designing treatment and prevention strategies could reduce the burden of TB disease at the population level.

4.5 Tables and Figures

Table 4-1: Characteristics of study participants

	SM^- n=764	**SM^+ n=177**
Age: median (IQR)‡	30 (23 - 40)	30 (23 - 36)
Sex: n (%)‡		
Female	472 (61.8%)	95 (53.7%)
Male	292 (38.2%)	82 (46.3%)
Race: n (%)		
Black African	764 (100%)	177 (100%)
Recruitment Site: n (%)‡		
Kisumu (urban)	426 (59%)	103 (60%)
Kombewa (rural)	296 (41%)	68 (40%)
Clinical Information		
Hemoglobin g/dL: median (IQR)‡	12.7 (11.4 - 13.9)	13.0 (11.7 - 14.1)
Pregnant: n (%)‡	1 (0.23%)	0 (0%)
Malaria: n (%)‡	2 (0.37%)	0 (0%)
Any Helminth (not including SM): n (%)‡	77 (10.1%)	19 (10.7%)
Ascaris‡	13 (1.7%)	1 (0.6%)
Trichuris‡	16 (2.1%)	5 (2.8%)
Hookworm‡	52 (6.8%)	14 (7.9%)
Helminth Species (not including SM): n (%)‡		
0	687 (89.9%)	158 (89.3%)
1	73 (9.6%)	18 (10.2%)
2	4 (0.52%)	1 (0.56%)
SM Features		
epg: median (IQR)		48 (24 - 144)
Light Intensity (1-99 epg)): n (%)		122 (68.9%)
Moderate Intensity (100-399 epg)): n (%)		41 (23.2%)
Heavy Intensity (400+ epg): n (%)		14 (7.9%)
HIV Features		
Positive: n (%)‡	301 (39.4%)	67 (37.9%)
Amongst Positive		
Viral Load: median (IQR)‡	30058 (1775 - 150826)	18879 (2442 - 101875)
CD4 Count (cells/mL): median (IQR)	395 (184 - 600)	467 (299 - 646)
Tuberculosis Features		
QFT IFNγ IU/mL: median (IQR)		
HC	0.00 (0.00 - 0.05)	0.00 (0.00 - 0.04)
LTBI	7.00 (2.12 - 9.57)	7.07 (1.76 - 9.53)
Abnormal Chest x-ray: n (%)		
HC	3 (1.3%)	0 (0%)
LTBI	8 (2.5%)	2 (2.7%)

‡: Included in propensity score and outcome regression models
Abbreviations: IQR- Interquartile Range; epg- Eggs Per Gram; QFT- QuantiFERON-TB Gold

Table 4-2: Characteristics of Study Participants by Mtb Infection Status

	HC n=315	LTBI n=432	TB n=194	p-value
Age (years): median (IQR)‡	25.0 (20.0 - 34.0)	31.0 (24.0 - 41.2)	33.0 (26.2 - 40.0)	<0.001[a]
Sex: n (%)‡				<0.001[b]
Female	212 (67.3%)	276 (63.9%)	79 (40.7%)	
Male	103 (32.7%)	156 (36.1%)	115 (59.3%)	
Race: n (%)				1[b]
Black African	315 (100%)	432 (100%)	194 (100%)	
Recruitment Site: n (%)‡				<0.001[b]
Kisumu (urban)	150 (50%)	250 (59.2%)	129 (75.4%)	
Kombewa (rural)	150 (50%)	172 (40.8%)	42 (24.6%)	
Clinical Information				
Hemoglobin (g/dL): median (IQR) ‡	12.9 (11.8 - 13.9)	13.2 (11.9 - 14.2)	11.2 (9.80 - 12.3)	<0.001[a]
Pregnant: n (%)‡	0 (0%)	1 (0.38%)	0 (0%)	1[c]
Malaria: n (%)‡	1 (0.47%)	1 (0.38%)	0 (0%)	1[c]
Any Helminth (not including SM): n (%)‡	39 (12.4%)	46 (10.6%)	11 (5.67%)	0.048[b]
Ascaris‡	2 (0.63%)	11 (2.55%)	1 (0.52%)	0.060[c]
Trichuris‡	11 (3.49%)	5 (1.16%)	5 (2.58%)	0.087[c]
Hookworm‡	28 (8.89%)	33 (7.64%)	5 (2.58%)	0.020[c]
Number of Helminth Species (not including SM): n (%)‡				0.131[c]
0	276 (87.6%)	386 (89.4%)	183 (94.3%)	
1	37 (11.7%)	43 (9.95%)	11 (5.67%)	
2	2 (0.63%)	3 (0.69%)	0 (0%)	
HIV Clinical Features				
Positive: n (%)‡	131 (41.6%)	132 (30.6%)	105 (54.1%)	<0.001[b]
Among Positive				
Viral Load (copies/mL): median (IQR) ‡	52004 (6100 - 145968)	14856 (1810 - 54166)	41728 (355 - 376951)	0.022[a]
CD4 Count (cells/μL): median (IQR)	466 (292 - 646)	518 (356 - 720)	172 (78 - 386)	<0.001[a]
Tuberculosis Clinical Features				
QFT IFNγ IU/mL: median (IQR)	0.00 (0.00 - 0.04)	7.07 (2.09-9.57)	ND	<0.001[a]
Abnormal Chest x-ray: n (%)	3 (1.05%)	10 (2.5%)	ND	<0.001[a]

‡: Included in propensity score and outcome regression models
a: Kruskall-Wallis
b: $\chi 2$ test
c: Fisher's exact test

Abbreviations
IQR: Interquartile Range
QFT: QuantiFERON
MGIT: Mycobacterial Growth Indicator Tube
ND: Note Done

Table 4-3: Estimated Distribution[a] of Mtb Infection Outcomes

	HIV⁻		**HIV⁺**	
	SM⁻	**SM⁺**	**SM⁻**	**SM⁺**
HC	32.0%	27.7%	36.0%	20.4%
LTBI	52.2% (47.8 - 56.6)	52.6% (44.4 - 60.8)	36.7% (31.1 - 42.2)	38.3% (28.3 - 48.3)
TB	15.8% (12.3 - 19.4)	19.7% (13.1 - 26.3)	27.4% (22.7 – 32.0)	41.3% (32.93 - 49.6)

[a]: Estimated probabilities are derived from TMLE estimation and are reported as estimated proportions (95% CI). The distribution of Mtb infection outcomes between SM^- and SM^+ did not differ in HIV^- individuals (p-value = 0.4860). The distribution of Mtb infection outcomes between SM^- and SM^+ differed in HIV^+ individuals (p-value = 0.0018). The difference in the proportions of Mtb infection outcomes between SM^+ and SM^- groups differs between HIV^+ and HIV^- individuals (p-value = 0.0002).

Table 4-4: Sensitivity Analysis of Estimated Distribution[a] of Mtb Infection Outcomes

	HIV$^-$		HIV+	
	SM$^-$	**SM+**	**SM$^-$**	**SM+**
HC	32.1%	29.2%	35.6%	31.1%
LTBI	52.2% (47.8 - 56.7)	52.5% (44.2 - 60.9)	36.9% (31.3 - 42.4)	34.8% (24.2 - 45.3)
TB	15.6% (12.3 – 19.0)	18.3% (13.5 - 23.0)	27.5% (22.7 - 32.3)	34.14% (26.2 - 42.1)

[a]: Estimated probabilities are derived from TMLE estimation including CD4 T cell counts as a covariate and are reported as estimated proportions (95% CI). The distribution of Mtb infection outcomes between SM^- and SM^+ differed (P-value < 0.05).

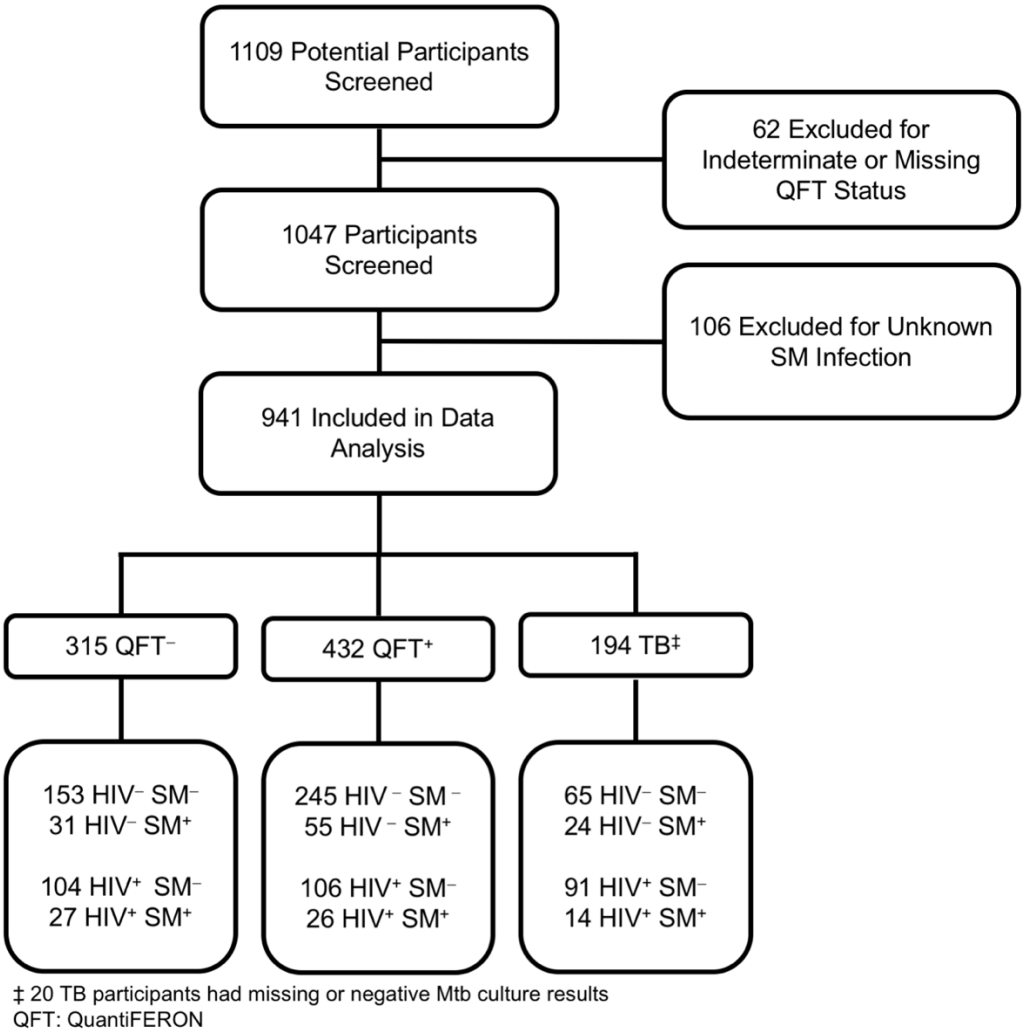


Figure 4-1: Enrollment diagram

Abbreviations:
Mtb, Mycobacterium tuberculosis
SM, Schistosoma mansoni
QFT, QuantiFERON-TB Gold

Standard	General Linear Models	Nonparametric
• Mean	• Including the infection status for each additional worm • Including total number of worm infection types • With splines for age, viral load, and hemoglobin • Step Forward • Step Forward + two-way interactions • glmnet + two-way interactions stratified by HIV status	• Ranger: Mtry (2, 4, 6) • Xgboost: Max depths (2, 4, 6) • rpartPrune • Cross Validated Earth (5 fold)

Supplementary Figure 4-1: SuperLearner Regressions

Chapter 5 Discussion

Mtb and SM are two of the greatest global health burdens facing scientists today. Not only do they cause a great deal of morbidity and mortality, but their immune correlates of protection are also not well defined. Both are complex organisms that have coevolved with humans for thousands of years (1,2). As such, they have developed ways of circumventing and manipulating the immune system. Mtb delays the immune response and then utilizes the very cells used to fight bacteria as its replicative niche (3). Each stage of the SM life cycle has developed strategies to similarly evade and exploit the immune system. SM worms coat themselves in host proteins to avoid detection while the eggs actually coopt the immune system to complete the SM life cycle. This occurs all while both worms and eggs are releasing immunoregulatory proteins that globally dampen the immune response (4,5). Because of their ability to modulate the immune system, it has been challenging to determine what an effective immune response looks like, let alone define correlates of protection to either infection. This challenge is further complicated by the fact that infections rarely occur in isolation. The areas of the world where tuberculosis is endemic are also areas where schistosomiasis is common and reoccurring (6,7). It is therefore important not only to understand the immune response to each individual pathogen, but also to understand what happens to the immune system during coinfection. This dissertation provides novel insights into the cellular immune response to both Mtb and SM, as well as into how these responses are maintained or impaired during coinfection. Lastly, this dissertation provides a population level perspective on the impact of coinfection on disease outcomes.

5.1 SM Does Not Impair TH1 Immunity

5.1.1 Summary

In Chapter 2, we examined the impact of SM infection on the phenotype and function of CD4 T cells to determine whether SM skewed TH1 responses towards a TH2 phenotype. We demonstrated that both total CD4 T cells and Mtb-specific CD4 T cells maintained TH1 cytokine responses in SM^+ individuals across a spectrum of Mtb infection states. In addition, we provided evidence that functional and phenotypic readouts of CD4 T cell lineage do not necessarily correlate. Indeed, both total and Mtb-specific CD4 T cells producing TH1 cytokines were found to express canonical TH2 lineage markers. Lastly, our data indicated that the impact of SM infection on Mtb-specific CD4 T cell phenotypes was dependent on Mtb infection status. The Mtb-specific TH1 cytokine producing cells from SM^+ active TB individuals, but not SM^+ LTBI individuals, had skewed TH2 phenotypic profiles compared to SM^- individuals. This indicates that unique combinations of SM and Mtb infection result in distinct immunological states.

5.1.2 Implications

The data in this chapter provide compelling evidence for a more flexible view of CD4 T cell subsets in human infectious diseases. In our study, CD4 T cells were able to maintain TH1 function in the face of competing TH2 stimuli. Furthermore, they preserved TH1 functional capacity despite phenotypic changes. Our findings are in line with recent studies that challenge the paradigm of terminally differentiated, lineage committed CD4 T cells, which were originally described in mouse models. These studies have opened up a dialogue surrounding the concept of CD4 T cell "plasticity" (8–10). Defining CD4 T cells by subset has been useful in interrogating

specific pathways involved in the response to distinct antigens. As the field uncovers new "lineages" and new combinations of existing lineages, however, it is becoming increasingly recognized that strictly dividing CD4 T cells into subsets is limiting outside of experimental settings. In reality, increasing evidence indicates that CD4 T cells are adaptable and dynamic cells capable of interpreting mixed signals and appropriately responding to distinct challenges.

The flexibility of CD4 T cells has broad implications in immunology because it acknowledges that the immune system is able to balance responses to multiple stimuli at once. Most of the time this balance is successful. Our data provide evidence that this is true in the setting of co-infection with Mtb and SM. Furthermore, the ability of CD4 T cells to maintain TH1 cytokine responses to Mtb antigens is likely the reason that HIV^- individuals in Chapter 4 did not have a higher probability of active TB during infection with SM. There may be specific combinations of infections that do indeed compromise the ability of the immune system to multitask. This may be why HIV^+ individuals in Chapter 4 do have a higher probability of active TB disease during infection with SM. Both SM and Mtb have circulated together in human populations for thousands of years. Both pathogens also require human hosts for their survival and transmission. It is possible that SM, Mtb and humans have reached an evolutionary stalemate, which is perturbed by HIV, a comparatively modern pathogen. Distinguishing between conditions that allow for preservation of immune function and conditions that result in dysregulation of the immune system could uncover risk factors for TB disease.

5.1.3 Future Directions

The tools available to evaluate the dynamic nature of immune cells have improved dramatically in recent years. In particular the advances in high dimensional flow cytometry and

the ability to do single cell sequencing have made it possible to interrogate CD4 T cells across all known lineage subsets and evaluate their functions at a more granular level. This will vastly improve studies of co-infection because it allows for an unbiased approach to characterize CD4 T cells and determine the full spectrum of antigen-specific responses. Furthermore, by interrogating CD4 T cell responses at a single cell level, we can better define immunological states associated with protection and disease.

An additional area of interest would be to interrogate CD4 T cell responses to additional Mtb antigens. The use of CFP-10 and ESAT-6 peptide pools, which are both immunodominant antigens known to elicit TH1 cytokine production, may have biased the results towards a TH1 phenotype (11). Recent studies have utilized peptide pools spanning 300 Mtb epitopes to evaluate the breadth and heterogeneity of the human immune response to Mtb (221). Using such an expansive peptide pool could uncover antigens more susceptible to TH2 bias. Moving beyond peptide antigens, it is also possible that CD4 T cell responses to more complex antigen preparations such as cell wall or even heat-killed Mtb would show more TH2 bias in SM^+ individuals.

Future studies would also benefit from expanding the combination of infections evaluated. SM and Mtb infection do not occur in isolation. This is particularly true with regards to other helminths and HIV infection. The modifying effect of HIV on the influence of SM on active TB outcomes observed in Chapter 4 highlights the possibility of a unique three-way interaction. Investigating the impact of HIV and SM co-infection on Mtb-specific CD4 T cells during active TB could uncover the reason for this increased probability. This is particularly interesting because of the interactions between all three pathogens. HIV is a known risk factor active TB disease, though the mechanisms for this risk are still not well understood (12). HIV has also been shown to reduce egg shedding from SM adult worms, which not only makes detecting infection more

difficult but can also result in increased pathogenesis due to higher internal egg burden (13). In turn, schistosomiasis is suspected to play a role, not only in susceptibility to HIV, but also in higher viral load set point and worse disease pathogenesis (14). Thus, the combination of these three infections could have a distinct impact on the immune response to each pathogen.

5.2 Mtb Impairs SWAP-reactive γδ T cells

5.2.1 Summary

The T cell response to SM is poorly understood, particularly with regard to CD8 and non-classical T cells. In addition, because of the complex life cycle of SM and the relative ease of studying specific antigens, there is a bias in the literature towards characterization of the T cell response to SM eggs. In Chapter 3 we presented data comparing the T cell response to SM eggs and SM worms and provided evidence that the response to worm antigen is more robust than to egg antigen. In addition, our data indicate that the magnitude of the T cell response to SM worm was largely due to γδ T cells. Lastly, we found that infection with Mtb impairs the intrinsic ability of peripheral γδ T cells to respond to worm antigen. Together, these data uncover a novel role for γδ T cells during co-infection with SM and Mtb.

5.2.2 Implications

The immune mechanisms involved in both acute schistosomiasis disease and immunopathology are fairly well characterized. Acute disease occurs only in non-immune individuals and is characterized by an early systemic TH1 responses to SM eggs that results in Katayama fever (5,15). In chronic disease settings, egg release results in a shift from TH1 to TH2 responses which limits acute disease but often results in fibrosis and tissue damage (5,16). Importantly, both disease states are the result of a strong CD4 T cell response to SM eggs. The majority of people in endemic settings therefore downregulate the immune response to eggs in order to limit pathology (17,18). The immune mechanisms involved in protection to SM, however, are still unknown. This may be due to our relatively sparse understanding of the cellular immune

response to SM during natural infection in humans. Our findings provide new insights into the T cell response to adult worms and fill a gap in our basic understanding of SM immunology.

There are a number of observations related to SWAP-specific responses in human studies of schistosomiasis that remain unresolved. First and foremost, many previous studies have concluded that while SEA-specific immune responses wane during chronic infection so as to limit immunopathology, SWAP-specific immune responses are preserved (18–20). These SWAP-specific immune responses are associated with less severe forms of disease, however it is unclear whether they truly provide a benefit to the host. Secondly, high SWAP-specific immune responses are thought to synergize with praziquantel treatment and provide resistance to reinfection, but the mechanism by which this happens is unclear (21,22). This lack of clarity can be attributed to the fact that the cells involved in the response to SWAP are not well characterized since most experiments utilized bulk PBMCs. Demonstrating that γδ T cells respond robustly to SWAP but not SEA therefore opens up a new avenue of research to determine the role of stage-specific immunity in protection. It will be interesting to determine what role, if any, γδ T cells have in mediating protection to SM particularly with regard to the development of SM treatments and vaccines.

Vγ2Vδ2 T cells in the blood respond to a well-defined group of related prenyl phosphate antigens produced by a variety of pathogens (23–25). Furthermore, they have recently been shown to have memory-like properties including phenotypic markers of memory T cells as well as shorter recall time and increased cytokine production following challenge (26–28). Lastly, they have been shown to bridge the innate and adaptive immune system through the production of cytokines which activate effector cells and improve the response of CD4 and CD8 T cells (28). As such, they are an attractive target for immunotherapies and vaccine development. The possibility of utilizing γδ

T cells to enhance SM responses in endemic settings is particularly appealing because γδ T cells essentially respond in an antigen-specific, but not pathogen-specific fashion. As such, they have the potential to provide cross-reactive immunological protection.

Our findings regarding the effect of Mtb infection on SWAP-reactive γδ T cells, however, calls into question the feasibility of such a strategy. Mtb infection in primates has been shown to recruit Vγ2Vδ2 T cells into the lung, depleting them from circulation (29). As such, Mtb infection may be drawing SWAP-reactive Vγ2Vδ2 T cells away from the site of SM infection. Specific Vγ2Vδ2 T cell clones, have been shown to be cross-reactive to HMBPP, produced by Mtb, and IPP, a putative SM product (30); however, unlike other γδ T cells, Vγ2Vδ2 T cells in the blood are actually polyclonal and are characterized by high junctional diversity (31). Whether the recruitment of Vγ2Vδ2 T cells out of circulation would be due to direct competition for the same pool of Vγ2Vδ2 T cells capable of recognizing both HMBPP and IPP, or bystander recruitment, is unclear. The efficacy of eliciting cross-reactive γδ T cell responses therefore may be based on the specific phosphoantigens produced by each pathogen and their anatomical location.

5.2.3 Future Studies

By identifying γδ T cells as SWAP-reactive, we can begin to translate observations from human studies of SM into testable hypotheses in animal models. First and foremost, however, further characterization of the γδ T cell response observed in this study is essential. Due to the exploratory nature of this study, we used a pan-γδ-TCR antibody. In humans γδ T cells are divided into two overarching subsets based on whether they are tissue resident or systemic (32,33). Each subset expresses a specific set of V chains that dictates what they respond to and their role in the

immune system. The majority of systemic γδ T cells express Vγ2Vδ2 (alternatively referred to as Vγ9Vδ2) and respond to phosphoantigens (27,34). We assume, due to the use of PBMCs in this study, that these cells are Vγ2Vδ2 cells, however confirming the V chain expression is a necessary first step.

Determining the mechanism by which γδ T cells respond to SWAP should also be a priority. To begin with, initial studies using proliferation as a read out, can determine whether the γδ T cell response to SWAP is the result of direct recognition of a cognate antigen or through indirect mechanisms such as cytokines produced by other cells within PBMCs. Previous studies have shown that Vγ2Vδ2 T cells can respond to prenyl phosphate antigen with or without assistance from antigen presenting cells (222). To evaluate whether this is true of SWAP-reactive γδ T cells, γδ T cells can be isolated from PBMCs and incubated with SWAP to determine if proliferation requires additional cell types. Conversely, removing specific cell-types from PBMCs will inform us as to which additional cell types are required in the γδ T cell response. Co-culture experiments separating γδ T cells from PBMCs using transwell plates can elucidate whether direct contact with antigen presenting cells is required. Lastly cytokines can be selectively neutralized during stimulation with SWAP to determine which, if any, are involved in the proliferative response of γδ T cells. If the γδ T cell response is due to direct antigen recognition, then further characterization of SWAP can be conducted to determine the antigen responsible.

While SWAP is a complex preparation there are a few candidate phosphoantigens that may be present in the mixture. One of the best characterized Vγ2Vδ2 T cell antigens is Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), an intermediate of the non-mevalonate pathway of isoprenoid synthesis (24,25,30). It is unknown whether SM utilizes this pathway, however because of the potency of HMBPP as an antigen it should be considered. There is evidence, however, that

SM does in fact use the mevalonate pathway which produces isopentenyl pyrophosphate (IPP) as an intermediate (35–37). IPP is structurally very similar to HMBPP and has also been shown to activate Vγ2Vδ2 T cells, though to a lesser degree than HMBPP (30). The specificity of SWAP-reactive γδ T cells to IPP therefore not only makes sense in terms of the worm's biology but would also explain the ability of Mtb to modulate SWAP-reactive γδ T cells, since Mtb produces HMBPP.

Once the antigen for SWAP-reactive γδ T cells is identified, a number of experiments can be done to determine the function of γδ T cells in vivo. Murine models can be utilized to determine whether γδ T cells, in general, are important in schistosomiasis. Mice do not, however, have a γδ T cell population equivalent to the Vγ2Vδ2 population in humans and non-human primates (223). Traditional experiments with knock-out animals and adoptive transfer of specific cell types therefore cannot be utilized to evaluate Vγ2Vδ2 T cells. This presents a challenge since this is the most likely subset of γδ T cells involved in the response to SWAP. To address this limitation, scientists studying TB have used HMBPP-expressing strains of listeria to specifically expand γδ T cells in primates. As a proof of concept, prior immunization with this listeria strain was able to provide protection against Mtb infection (28). This was associated not only with protection, but also a faster CD4 and CD8 T cell response in the lung. Furthermore, autologous adoptive transfers of Vγ2Vδ2 T cells can attenuate TB disease (224). A similar strategy utilizing IPP, for example, at different times – prior to infection, prior to egg shedding, or even during drug treatment – could elucidate the role of γδ T cells during specific stages of infection. This, however, is limited by the fact that the baboon is the best primate model of schistosomiasis (225). Studies involving baboons are hindered by the high cost of acquiring and maintaining baboons and a lack of reagents (225). Significant strides in the field of immunology need to be made before this model is feasible.

5.3 SM HIV Co-infection Increase Probability of TB

5.3.1 Summary

Due to the geographic overlap of helminthiasis and TB, there is a great deal of interest in evaluating the risk of TB due to helminth infections. In Chapter 4 we used modern statistical techniques to estimate the probability of TB disease attributable to SM and HIV infection. We found that SM infection alone was associated with a modest but statistically insignificant increase in the probability of TB. The combination of SM and HIV infection, however, was associated with a significant increase in the probability of TB disease. Furthermore, we confirmed that this difference was not simply due to HIV infection but was instead due to HIV modifying the effect of SM on TB disease.

5.3.2 Implications

A number of studies have attempted to measure the risk of TB during helminth infections and have come to very different conclusions. A recent meta-analysis of studies measuring the association between soil-transmitted helminths and TB reported a wide range of effect sizes and statistical significance (38). Even when considering a single helminth infection, schistosomiasis, studies have come to different conclusions. Two studies suggest that SM is associated with a higher risk of developing TB disease (39,40) while another actually reported lower odds of TB disease in SM^+ individuals (41). These findings are hard to interpret, and even harder to reconcile due to differences in study design, participant demographics, clinical definitions and the helminths themselves.

There are inherent challenges in analyzing data from observational studies. Two of the most important barriers to inference are confounding factors and interpretability. New statistical analysis strategies, like the one utilized in this dissertation, improve our ability to measure the

association between infections, extrapolate to larger populations and even evaluate cause and effect. In Chapter 4 we utilized the R packages “SuperLearner” and “drtmle” to minimize bias and improve interpretability.

While standard epidemiological analyses can “control” for confounding factors, it becomes hard to know what variables to include and how to select the “right” regression technique. This inherently introduces bias into the results by iteratively involving investigators in the model building process. Our strategy utilized pre-specification of regressions, careful variable selection, and an a priori statistical analysis plan to mitigate this bias. SuperLearner utilizes cross-validation and ensembling to ensure that the final regression best represents the data.

Many common statistical measures in epidemiology, such as odds ratios, are challenging to interpret. This is even more challenging in studies of Mtb infection because there is a spectrum of possible outcomes. By utilizing drtmle, we were able to estimate the probability of each disease outcome attributable to SM infection. As such we can make statements about the proportions of individuals within the entire population that would have active TB if they were SM^+. Due to the cross-sectional enrollment of our study, we were still limited to measuring associations. Prospective studies, however, can use this technique to perform causal inference. As such, this analysis strategy has major implications in the analysis of observational studies.

Lastly, by performing this analysis alongside the immunological studies performed in Chapter 2, we were able to link immunological phenotypes to epidemiological outcomes. We have provided evidence that SM infection alone neither disrupts the functional CD4 T cell response to Mtb nor increases the probability of TB disease. In doing so, were able to bridge basic immunology research to real world public health outcomes.

5.3.3 Future Studies

Our findings should be evaluated by utilizing the same methodology in additional cohorts across the world to determine how broadly applicable our results are. Furthermore, prospective studies evaluating the impact of SM and other helminths on TB infection must be conducted in order to validate the findings of this study. In an ideal world this would consist of a longitudinal study of SM^- and SM^+ LTBI individuals with and without HIV, evaluated over time to determine the incidence of active TB. There are a number of challenges in implementing and evaluating such a study including the requirement for a large sample size and extensive follow up. The main obstacle would be the feasibility of such a study given the relatively low rate of TB progression. Despite how common Mtb infection is, 5% or less of those infected with Mtb will develop active TB disease (42). For this reason, a large number of people would need to be followed for years in order to observe enough events for statistical analysis. The length of follow-up time in turn necessitates extensive monitoring of study participants. We fortunately have effective treatment for SM infection. Due to the lack of sterilizing immunity to SM, however, SM infection is reoccurring in endemic areas. As such, study participants would need to be evaluated regularly for SM infection using the cassette based CCA diagnostic. They would also need to be treated with praziquantel at each positive test. This poses a challenge in determining what the "exposure" variable is. A simple solution would be to have primary, secondary and tertiary definitions of exposure such as: a positive SM test at enrollment, any positive SM test, the number of positive SM tests. This, however, becomes difficult to interpret outside of analysis methods like the ones utilized in this dissertation. Indeed, the statistical strategies outline in Chapter 4 mitigate many of the challenges normally present in observational studies including interpretability as well as small sample size and missing data.

Our findings also warrant further investigation into the immunology of co-infection with SM, HIV and Mtb in order to determine the mechanism behind the increased probability of TB disease. Analysis of CD4 T cells would be a logical place to start, due to the depletion of CD4 T cells during HIV, which is known to affect both TB risk and SM pathology (12,13). In addition, there have been some studies indicating that HIV may replicate better in TH2 cells (13,14). The combination of HIV and SM may therefore have a different effect on the TH1/TH2 phenotype of CD4 T cells than SM alone.

5.4 Overall Conclusions

The immune response to infection is often thought of as a fine tuned highly specific set of cellular responses that, when disrupted, result in disease. For this reason, co-infection can be a nuanced and at times contradictory system to study. The data presented in this dissertation demonstrate a few key facets of this complexity. First, the effect of co-infection on the immune response is neither uniform nor bi-directional. While our data indicate that SM infection does not affect Mtb-specific CD4 T cell function, Mtb infection is able to impair functional T cell responses to SM. Second, each unique combination of infections results in a unique immunological state. In these studies, we were able to evaluate the cellular immune response during co-infection with only three pathogens and still found diversity in the response to a given pathogen and the associated infection outcomes. For example, the effect of SM on LTBI was not the same as the effect of SM on TB. Similarly, the probability of TB attributable to SM infection differed between HIV^- and HIV^+ individuals. Understanding these complex systems is necessary in determining appropriate public health strategies, including drug and vaccine development, to better control the global epidemics of TB, HIV, and schistosomiasis.

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