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CD4 T Cell Responses in *Mycobacterium tuberculosis* and HIV Co-infection

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B.S., Saint Augustine's College, 2012

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An abstract of
A dissertation submitted to the Faculty of the
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Doctor of Philosophy in Immunology and Molecular Pathogenesis

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Abstract

CD4 T Cell Responses in *Mycobacterium tuberculosis* and HIV Co-infection

By Morgan S. Barham

Mycobacterium tuberculosis (*Mtb*) is the infectious agent that causes tuberculosis (TB) disease. TB is the leading cause of death due to a single infectious agent and has remained one of the top 10 causes of death worldwide for decades. In 2017, 10 million new cases of TB disease were reported, resulting in 1.6 million deaths. An estimated 1.7 billion people, representing nearly a quarter of the world's population, are latently infected with *Mtb* and therefore at risk for developing active TB disease. Infection with HIV induces immune suppression and depletion of CD4 T cells, which play a critical role in limiting *Mtb* bacterial growth and reducing progression to active TB disease.

Mtb-specific CD4 T cells in HIV-infected individuals exhibit elements of immune dysfunction, including impaired proliferative capacity, heightened immune activation and cell death, and reduced cytokine production capacity. Using cohorts of HIV-infected and HIV-uninfected individuals with latent and active TB in South Africa and Kenya, two high TB burden countries, we conducted a thorough examination of T cell inhibitory receptor expression profiles on antigen-specific CD4 and CD8 T cells and the upregulation of surface activation-induced markers (AIM) CD25, OX40, CD69 and CD40L on *Mtb*-specific CD4 T cells. Further, we extended our studies to analyze the effect of HIV co-infection on *Mtb*-specific cytokine production profiles.

Our findings provide novel evidence that the majority of *Mtb*-specific CD4 T cells do not co-express multiple inhibitory receptors, regardless of HIV infection status. Our findings also highlight a previously unrecognized role of BTLA expression on *Mtb*-specific CD4 T cells. In addition, our findings suggest that infection with HIV modifies *Mtb*-specific cytokine production profiles towards elevated TNF-α production and that AIM assays enable identification of *Mtb*-specific CD4 T cell responses in a cytokine-independent manner in HIV-uninfected and HIV-infected individuals. Overall, our findings provide a foundation for future studies to further define immune correlates of protection to *Mtb* and elucidate mechanisms of HIV-associated dysregulation of anti-mycobacterial immunity.

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"You don't get paid for A's. School is your job."

- *William "Pop Pop" Ramsey*

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CHAPTER I

INTRODUCTION

The Global Burden of TB

Tuberculosis (TB) is an ancient disease that remains a global public health threat, affecting almost every nation and ethnicity (1), yielding 22 high TB-burdened countries (Figure 1). TB disease is caused by an infectious agent discovered in 1882 by Robert Koch called *Mycobacterium tuberculosis* (*Mtb*), also known as Koch's bacillus (2). *Mtb* has the highest mortality rate worldwide due to a single infectious agent (3). Approximately 90% of *Mtb*-infected individuals are considered latently infected and do not exhibit clinical symptoms of TB disease. In 2017, 10 million people were reported to be infected with TB disease and 1.6 million reported deaths due to TB disease (3).

Tuberculosis disease incidence decreased approximately 5.8% each year, from 1953 to 1984, as a result of national surveillance and systematic reporting in the United States (4). Further, death rates decreased from 200 deaths per 100,000 death each year, to one death per 100,000 each year (4). Unfortunately, the emergence of multidrug resistant tuberculosis and the HIV epidemic lead to the reemergence of TB and declaration of TB as a global public health emergency by the World Health Organization (WHO) in 1993 (5). As a result, the Centers for Disease Control and Prevention (CDC) allocated funds to tuberculosis control programs in large cities and states (4), resulting in a 7.3% decrease in TB incidence annually by 2001. However, this decline was transient, leading to only a 2% decrease in TB incidence by 2015. As a result, the WHO propelled a 20-year strategy aiming to end the global TB epidemic called 'The End TB Strategy' (6, 7). The goals of the End TB Strategy were to end the TB epidemic nationally and internationally – zero deaths, disease and suffering (6, 7) (Figure 2).

The Global Burden of TB/HIV Co-infection

There is a substantial global burden of *Mtb* and HIV infections independently, as well as a very large global burden of *Mtb* and HIV co-infection (8). Although 90% of *Mtb* infected individuals are considered latently infected and do not exhibit clinical symptoms of TB disease, infection with HIV is the single greatest risk factor causing reactivation from LTBI to active TB disease TB. HIV-uninfected individuals have a 10% lifetime risk of development of TB, compared to immunocompromised HIV-infected individuals who have an 8-10% annual risk of developing TB disease (3, 9).

Worldwide, about 9% of new reported TB cases are in people living with HIV, and over 50% of new TB cases are in high burden countries, such as Kenya and southern Africa (3) (Figure 3). HIV-infected individuals with LTBI have above a 20% higher risk of developing active TB compared to HIV-uninfected persons (10), despite antiretroviral therapy (11). Of the 1.6 million reported deaths due to TB in 2017, approximately 300,000 of these deaths are among people living with HIV.

History of TB

The acid-fast, aerobic bacterium responsible for over a million deaths each year is *Mycobacterium tuberculosis* (*Mtb*). *Mtb* is a human pathogen that is thought to have originated from *Mycobacterium bovis*, the bovine strain of tuberculosis, yielded from the domestication of cattle. In the seventeenth century, the 'Great White Plague' spread from England to most of Western Europe, infecting most of the European population and killing one-fourth of the population over a span of 200 years.

In 1872, Dr. Edward L. Trudeau contracted TB and fled to the Adirondack Mountains to die. Contrary to his expected outcomes, he did not perish. As a result, he concluded that fresh air, exercise, and healthy living were the cure for TB. Thus, in 1874, he established the Trudeau laboratory, a sanitorium which became the popular method for treatment against TB before the discovery of antibiotics (12). On March 24, 1882, the German physicians and bacteriologist, Robert Koch discovered *Mtb*, the causative agent of TB. In 1890, he discovered tuberculin, a substance which he proposed could prevent bacterial growth (13, 14) and defined the criteria to establish a causative relationship between a microbial organism and a disease, now known as Koch's postulates (15), contributing to the vast advancements in the current TB field.

The Pathogen, Transmission, and Replication

Mtb is a nonmotile, aerobic, acid-fast bacilli. It is a rod-shaped bacterium that can range from 0.2 to 0.5 micrometers in length and are nonsporulating. The bacilli are surrounded by a waxy cell wall and have an envelope that contains mycolic acids that are thought to play a role in *Mtb* pathogenesis. The bacilli are slow-growing, reproducing within 24-48 hours and can take up to twenty-one days to culture. Unlike most mycobacteria, *Mtb* is pathogenic due to its loci RD1 through RD16, which is not present in nonpathogenic strains, such as *M. bovis* (16). In particular, *Mtb* contains the RD1 (ESX-1) locus, which is required for the full virulence of *Mtb*. RD-1 encodes for secretion of the prominent antigens, early secretory protein 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), and translocates into the cytosol of infected macrophages from the phagosome (17, 18).

Mtb is transmitted from person to person by aerosolized droplet nuclei (19). An individual with active TB disease can effortlessly infect other individuals by simply coughing, sneezing or speaking. The infectious bacilli are exhaled into the atmosphere and can remain airborne from hours to ten weeks. As little as a single bacilli can cause *Mtb* infection (20). Once *Mtb* droplet nuclei enter the airways of an *Mtb* uninfected individual, it can infect the alveolar macrophages in the lung, which may lead to development of causing pulmonary TB. Other forms of *Mtb* infection include disseminated TB, such as miliary TB, lymph node TB and TB meningitis, which affect sites of the body outside of the lung (21).

After inhalation, aerosolized *Mtb* enters the lungs and are phagocytosed by alveolar macrophages. *Mtb* interacts with phagocytic receptors on the surface of the

macrophage in order to enter the cell. Once in the cell, components of the *Mtb* cell wall prevent killing by the macrophage via inhibition of fusion of the phagosome and lysosome. Once *Mtb* has evaded the innate immune defenses, it replicates in alveolar macrophages and then spreads to tissues via the lymphatic system, activating the adaptive immune response, which asymptomatically controls the infection in approximately 90% on *Mtb*-infected individuals (16).

Diagnostics

Infection with *Mtb* can result in latent infection or development of active TB disease. There are multiple tests to determine *Mtb* infection; however, few can differentiate between LTBI and active TB disease. A common method used worldwide to test for *Mtb* infection is the Mantoux test, better known as the tuberculin skin test (TST). TST utilizes the intradermal administration of purified protein derivative (PPD), which is sterilized *Mtb* culture filtrate proteins, to measure a delayed hypersensitivity reaction within 48 – 72 hours. However, an individual that has been vaccinated with BCG may yield a false positive due to induction of cross-reactive anti-mycobacterial immune responses induced by live BCG vaccine; moreover, TSTs require at least two patient visits for injection of PPD and subsequent reading of induration. In recent years, new diagnostic test for *Mtb* have been developed utilizing whole blood, known as interferon-gamma (IFN-γ) release assays (IGRA) (22).

Currently, the two U.S. Food and Drug Administration (FDA)-approved and commercially available IGRAs are QuantiFERON®-TB test (QFT) and T-SPOT®.TB test (T-Spot). IGRAs measure IFN-γ production in whole blood or PBMC, supply a result within 24 hours and only require one patient visit. More importantly, IGRAs measure IFN-γ production in response to the *Mtb*-specific immunodominant antigens CFP-10 and ESAT-6, which are not present in BCG, and thus eliminating false positive results from individuals who are BCG-vaccinated but not infected with *Mtb*. QFT is an enzyme-linked immunosorbent assay (ELISA), whereas T-Spot is an enzyme-linked immunospot (ELISPOT)-based assay. Although both tests are highly effective in detecting *Mtb* infection, neither test is able to differentiate between LTBI and active TB disease.

In addition to a positive TST or IGRA, active TB disease can be confirmed via sputum smear microscopy, cultures, and chest x-rays or CT scans. Sputum smears are fixed and stained with an acid-fast stain to determine the presence of *Mtb*. Sputum smear microscopy is the most common method used for diagnosis of active TB disease (23, 24). Following smear microscopy, solid and/or liquid cultures can be performed to confirm the presence of live, replicating *Mtb*. Smears can often result in false positive results, since live and dead bacteria are not distinguished, and cultures can take up to 21 days or longer due to the slow-growing nature of *Mtb*. Chest x-rays or CT scans can visually depict granuloma formation, which displays as white spots.

More recently, one of the newest molecular systems to detect active TB is the GeneXpert *MTB*/RIF (Xpert) assay, which can simultaneously determine rifampin drug resistance (25). GeneXpert utilizes a sputum sample for real-time PCR to amplify the *Mtb* gene rpoB and can provide results within two hours. A next-generation Xpert assay has been released, Xpert Ultra, which provides superior sensitivity in detecting TB disease in patients with paucibacillary TB and in HIV-infected TB patients (26).

Spectrum of *Mycobacterium tuberculosis* infection

Mtb infection is characterized by the formation of cellular structures known as granulomas (27). Ninety percent of individuals with *Mtb* infection present no symptoms of infection and are considered to have latent tuberculosis infection (LTBI) (3). LTBI is not infectious and is characterized by the successful formation of granulomas. The remaining 5-10% of individuals infected with *Mtb* that exhibit symptoms such as fatigue, loss of appetite, coughing, chills, fever, weight loss, chest pain, hemoptysis, or night sweats are considered to have active TB disease (28). Pulmonary TB disease is infectious and characterized by failed immune cell aggregation to 'wall off' the infection (28), yielding to the spread of bacteria from person to person and a weakened immune system.

Though LTBI is commonly described in individuals whom present immunological responses to *Mtb* but manifest no clinical symptoms and TB disease associated with the presence of clinical symptoms, the spectrum of *Mtb* infection is far more complex. A host of scenarios can define an individual as latently infected, ranging from inactive *Mtb* contained in a granuloma to actively replicating *Mtb* but no clinical manifestations (29, 30). Incipient and subclinical TB lie in the gray areas between LTBI active TB. Incipient TB is described as an early stage of *Mtb* containment manifesting no clinical symptoms. Further along the spectrum, an individual is considered to have subclinical TB when they have actively replicating bacteria but present no clinical symptoms (Figure 4) (30, 31). Overall, stages of *Mtb* infection before development of active TB disease are commonly accepted to have some aspect of granuloma formation (29).

Granulomas are cellular structures that control *Mtb* infection by surrounding the bacteria with innate and adaptive immune cells preventing the spread from person to person (27). Necrotizing or caseous granulomas occur in most cases of LTBI and active TB. These granulomas consist of various innate and adaptive immune cells, including B cells, neutrophils, CD4 and CD8 T cells, fibroblasts, and macrophages. In the center of the immune cells lie free floating bacteria and infected macrophages. Fibrotic granulomas are mostly identified in individuals with LTBI, majorly consisting of fibroblasts and a few infected macrophages. Dissimilar, non-necrotizing or non-caseous granulomas are majorly comprised of macrophages and a few lymphocytes. In addition, these granulomas do not have a caseous center and are customarily seen in individuals active TB disease (32).

Other forms of extrapulmonary TB include miliary TB and TB meningitis. Miliary TB occurs when *Mtb* infects the bloodstream, affecting multiple organs simultaneously (33). TB meningitis manifests when *Mtb* infects the central nervous system instead of the lung (34). Both miliary TB and TB meningitis are active forms of TB that have high morbidity and mortality rates if not treated early. Further, an individual with LTBI can develop reactivation TB disease. Longitudinal epidemiological studies indicate that TB disease predominately occurs within a few months to two years after *Mtb* infection (35).

Vaccines and Treatment

The Bacille Calmette-Guerin (BCG) vaccine, which is a preventative vaccine, is the only licensed vaccine against TB. Administration of BCG varies depending on geographic location. The BCG vaccine is not commonly used in the United States, however, in high TB burden countries, this vaccine is ordinarily administered to infants as a preventative mode against disseminated *Mtb* infection. The BCG vaccine is a live, attenuated vaccine formulated from *M. bovis*, in which the virulent RD-1 locus has been deleted (36). It has been proven effective for the prevention of disseminated TB in infants, however results remains less effective against pulmonary TB disease in adults (37, 38).

Over the years, there has not been another vaccine licensed for prevention of *Mtb* infection or TB disease. However other vaccine candidates have provided insight to different immune correlates and a few are currently being developed and tested, with clinical trials completed on over a dozen novel TB vaccine candidates. Recombinant BCG vaccine candidates and live-attenuated BCG are currently being tested as a possible replacement for the current BCG vaccine given to infants at birth (39). The other TB vaccine candidates target adolescent and adult populations, which according to mathematical modeling reported by Harris *et al* are the target population to achieve the goals of the WHO End TB Strategy, potentially preventing 17 million TB cases over 10 years (40). These candidates utilize a host of approaches ranging from protein and adjuvant combinations to attenuated strains of BCG and *Mtb* to recombinant viral vector subunit vaccines. In addition, one of the recombinant BCG vaccine candidates,

VPM1002, is being evaluated as a therapeutic vaccine to prevent recurrence of disease in adults who have previously been treated for pulmonary TB (39).

Treatment of *Mtb* also varies depending on disease state and geographic location. Within the United States, treatment of LTBI with antibiotics is recommended in order to keep TB disease controlled. However, in high TB burden countries, due to the high probability of reinfection, individuals with LTBI usually remain untreated and are considered healthy unless immunocompromised (41). Worldwide, individuals with latent and active TB disease are currently treated with antibiotics.

Latently infected individuals are treated with the antibiotics isoniazid (INH), rifapentine (RPT), rifampin (RIF), or a combination. Treatment is generally for three to 9 months. However, the recommended treatment from the Center for Disease Control and Prevention (CDC) is a weekly dosage of INH and RPT for 12 weeks (42). Similarly, active TB disease is treated with the antibiotics, however, for a longer period of time – six to nine months, and with a combination of antibiotics. There are multiple antibiotics approved for the treatment of active TB disease, however the four predominantly used drugs in combination are isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA). Typically, all 4 antibiotics are given together for the first two months as an intensive treatment phase, followed by 4 months of INH and RIF in the continuation phase (3, 43).

It is particularly imperative to monitor TB treatment vigilantly in order to maintain antibiotic effectiveness. Incomplete antibiotic regimens, inappropriate dosage and time, as well as recurrent treatments can lead to drug resistant TB, which has become increasingly problematic. An *Mtb* strain that is resistant to one or more of the first-line anti-TB drugs is known as drug-resistant TB. *Mtb* strains resistant to INH,

RIF, and an additional anti-TB drug is known as multidrug-resistant TB (MDR-TB). Worse, *Mtb* strains resistant to INH, RIF, any fluoroquinolone, and one of the second-line drugs (amikacin, kanamycin, or capreomycin) is known as extensively drug-resistant TB (XDR-TB) (3, 43).

There currently lies a challenge to the treatment of *Mtb*-infected individuals due to the lack of compliance of completing a prescribed TB regimen in combination with the lack of a cure. In order to fulfill the goals that have been set by the WHO's 'End TB strategy', a more effective vaccine targeting pulmonary TB in adolescents and adults is needed (44).

The Immune Response

The immune system is the mechanism of defense for a host against the outside world. It is comprised of a network of cells and small molecules capable of recognizing and responding to invasion, such as pathogenic organisms or altered self antigens. The immune system constantly surveys the host searching for foreign entities. Recognition of non-self antigens initiates an immune response, leading to elimination of the foreign agent. The immune system is divided into two main branches, the innate and adaptive immune systems, that cover a spectrum of possible dangers (Figure 5).

The initial responses of the immune system constitute the innate immune response. Innate immunity is considered nonspecific and functions in creating physiological and anatomical barriers that protect the host in response to any perceived threat. The innate system responds rapidly after an invading pathogen is encountered (45). In this event, an inflammatory response is initiated that causes vasodilation and increased vascular permeability to allow for the recruitment of non-specific phagocytic cells, such as monocytes, macrophages and neutrophils. The next line of defense is the adaptive immune response which is responsible for antigen specificity. The adaptive immune response recognizes and responds to specific foreign entities. It has the ability to distinguish self from non-self as well as immunological memory, which yields a robust response to antigens previously encountered. Cells involved in adaptive immunity include T cells, B cells and antigen presenting cells (APCs) (45).

The Immune Response to *Mtb*

Protection against *Mtb* infection is comprised of both the innate and adaptive immune responses (46). Once *Mtb* enters the airways, an immune response is initiated via toll-like receptors, most commonly toll-like receptor 2 (TLR-2) and toll-like receptor 4 (TLR-4) which recognize mycobacterial ligands and has been associated with apoptosis induction. TLR-2 and TLR-4 mediate a downstream signal via MyD88 for production of pro-inflammatory cytokines such as interlukin-12 (IL-12), interlukin-1-beta (IL-1β), tumor necrosis factor alpha (TNF- α), and interlukin-6 (IL-6) (47), which induce recruitment of the first responders of the immune system, neutrophils, and additional macrophages. After responding to the site of infection, ninety percent of *Mtb* infected individuals have successful granuloma formation (3), which is aided by direction of cytokines (48).

Macrophages, dendritic cells, myeloid cells and NK cells play important roles in controlling TB infection. However, *Mtb*-specific CD4 and CD8 T cells play critical roles in limiting *Mtb* bacterial growth and reducing progression to active disease via activating macrophages and cultivating granuloma formation (46, 49). CD4 T helper 1 (Th-1) cytokines IFN-γ and TNF-α, and IL-2 are required for these processes (50-55). In mice, studies have shown that CD4 T cell depletion yields increased susceptibility to *Mtb* infection (56-58). In addition, CD4 T cell depletion in non-human primates with LTBI resulted in reactivation to active TB (59, 60). CD8 T cells also play critical roles in the containment of *Mtb* via their cytolytic activity and production of cytotoxic molecules (61-63). Chen *et al* demonstrated the important role of CD8 T cells in BCG-vaccinated rhesus macaques. Depletion of CD8 T cells in these animals led to decreased vaccine

induced immunity as well as a loss of anti-tuberculosis immunity after reinfection with *Mtb* (64). Furthermore, studies have been shown that vaccine candidates that enhance CD8 T cell responses, in addition to CD4 T cell responses, provide more adequate protection against *Mtb* (65, 66).

The Immune Response to *Mtb*/HIV Co-infection

With treatment and uncompromised immunity, especially CD4 T cells, *Mtb* infection can result in satisfactory outcomes. However, a simultaneous *Mtb* and HIV infection, the single greatest risk factor for reactivation of LTBI, often results in progression to TB disease (3). HIV-infected individuals have an increased risk of developing TB disease within the first year of HIV infection and this risk continues to increase as HIV depletes CD4 T cells (67). While the introduction of antiretroviral therapy (ART) has greatly decreased the incidence of TB disease, the risk of TB disease still remains higher in HIV-infected individuals compared with those that are not infected with HIV (11, 68).

Aside from the various immunological effects of *Mtb*-HIV co-infection, simply diagnosing *Mtb* within HIV infected individuals is challenging in itself. HIV-infected individuals often have difficulty producing sputum for sputum-based diagnostic tests. In addition, individuals with *Mtb*-HIV co-infection frequently have paucibacillary disease or disseminated disease and may present false negative results on sputum testing (69).

In addition to diagnostic challenges, HIV infection induces immune suppression and depletion of CD4 T cells, which are critical for protection against *Mtb* (70-72). In addition to CD4 T cell depletion, HIV impairs cytokine production, the induction of apoptosis and phagosome-lysozyme fusion in macrophages (73, 74). Cytokine-producing *Mtb*-specific T cells have been detected in HIV-infected individuals, these cells are dysfunctional, including impaired proliferative capacity and induced CD4 T cell death (75). Preferential infection of CD4 T cells by HIV and rapid depletion of *Mtb*-specific CD4 T cells within one year of HIV seroconversion have been reported (76, 77).

In addition, in the setting of chronic viral infections such as HIV, T cells can become dysfunctional. These dysfunctional cells overexpress inhibitory receptors, downregulate pro-inflammatory cytokines, have altered T cell receptors, and express different genes and transcription factors than effector T cells (78).

Furthermore, HIV interrupts the formation and integrity of granulomas that contain *Mtb* in individuals with latent infection (79). Diedrich *et al* reports an association between higher bacterial load and lower CD4 T cell count with poor granuloma formation (80, 81), yielding a correlation between granuloma integrity and absolute CD4 T cell count (Figure 6). As HIV depletes CD4 T cells, macrophage activation and differentiation are diminished along with the presence of epithelial cells that surround the bacilli, leading to increased neutrophil infiltration, necrosis, and higher bacillary load (69, 80, 81). Further, *Mtb*-HIV infection leads to higher bacterial loads in the tissues, which in turn enhances HIV replication. Therefore, a better understanding of the immunological mechanisms that are modulated in *Mtb*-HIV infection is required to develop strategies that augment immune control of *Mtb* in HIV-infected individuals (69).

Thesis Overview

The work described in this dissertation assesses the effect of HIV on antigen-specific T cell responses across a spectrum of *Mtb* infection states, including IGRA-negative individuals, IGRA-positive individuals with LTBI, and patients with pulmonary TB disease. To do so, we conducted analysis of antigen-specific CD4 T cells utilizing peripheral blood mononuclear (PBMC) samples collected from individuals residing in high TB and HIV burden settings in South Africa and Kenya. In addition, studies were completed to assess the effect of HIV on the *Mtb*-specific CD4 T cells activation induced marker profile within IGRA-negative individuals and individuals with LTBI, with and without HIV infection. Furthermore, we analyzed the effect of HIV infection on cytokine production profiles within IGRA-negative individuals and individuals with LTBI, with and without HIV infection. Overall, we defined phenotypic and function profiles of *Mtb*-specific T cells that are associated with HIV infection, across a spectrum of *Mtb* infection states. These findings contribute to a better understanding of HIV-associated perturbations of T cell immunity to *Mtb* infection and open up new avenues of research in developing immunotherapeutic interventions to enhance T cell-mediated immune control of *Mtb* infection in HIV-infected individuals.

Figures

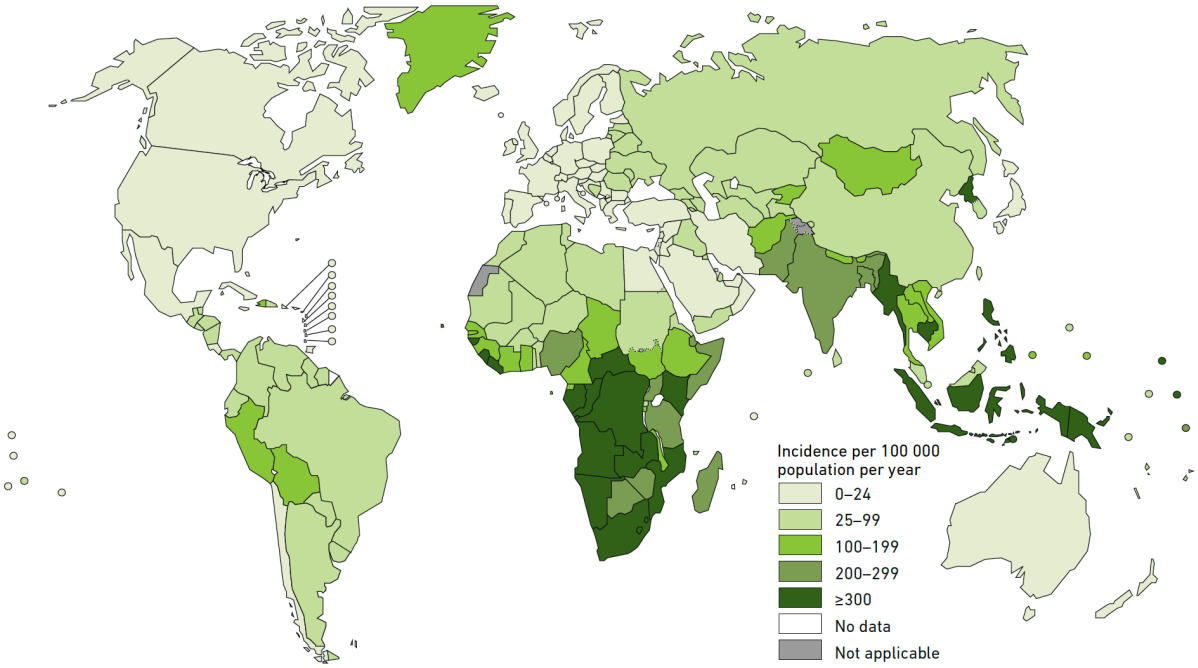


Figure 1. Estimated TB incidence rates in 2017 (3).

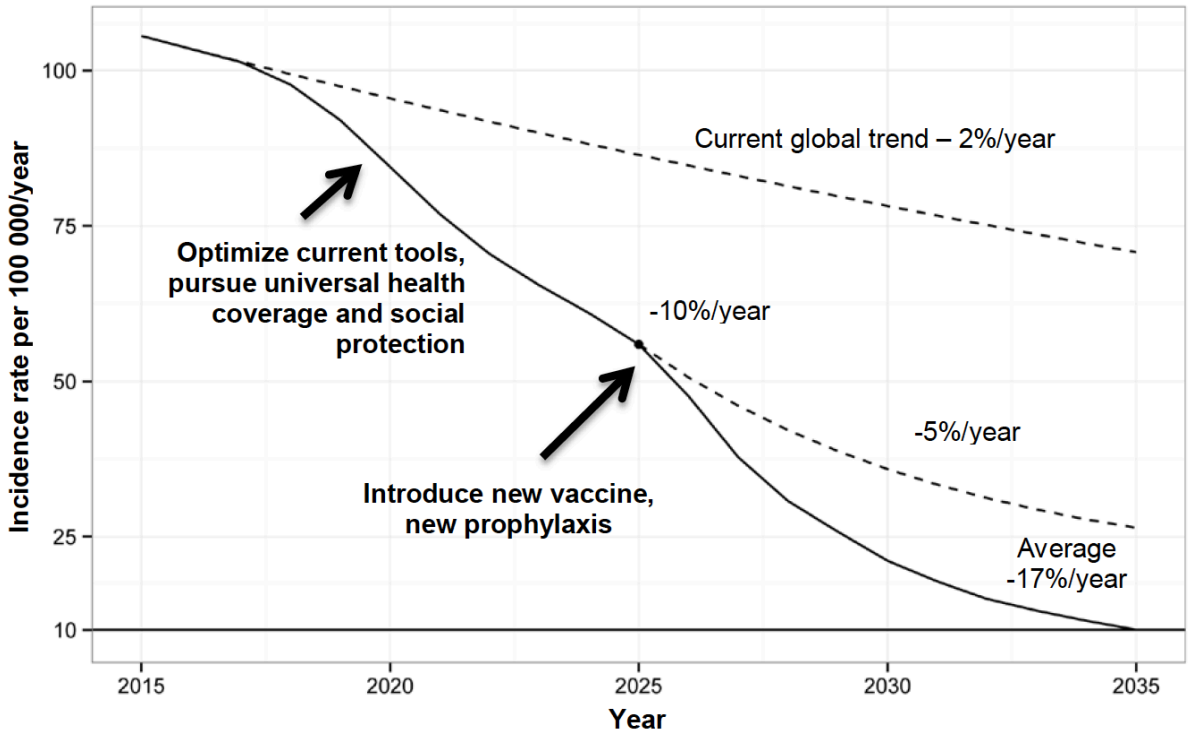


Figure 2. Projected acceleration in the decline of global tuberculosis incidence rated to target levels (7).

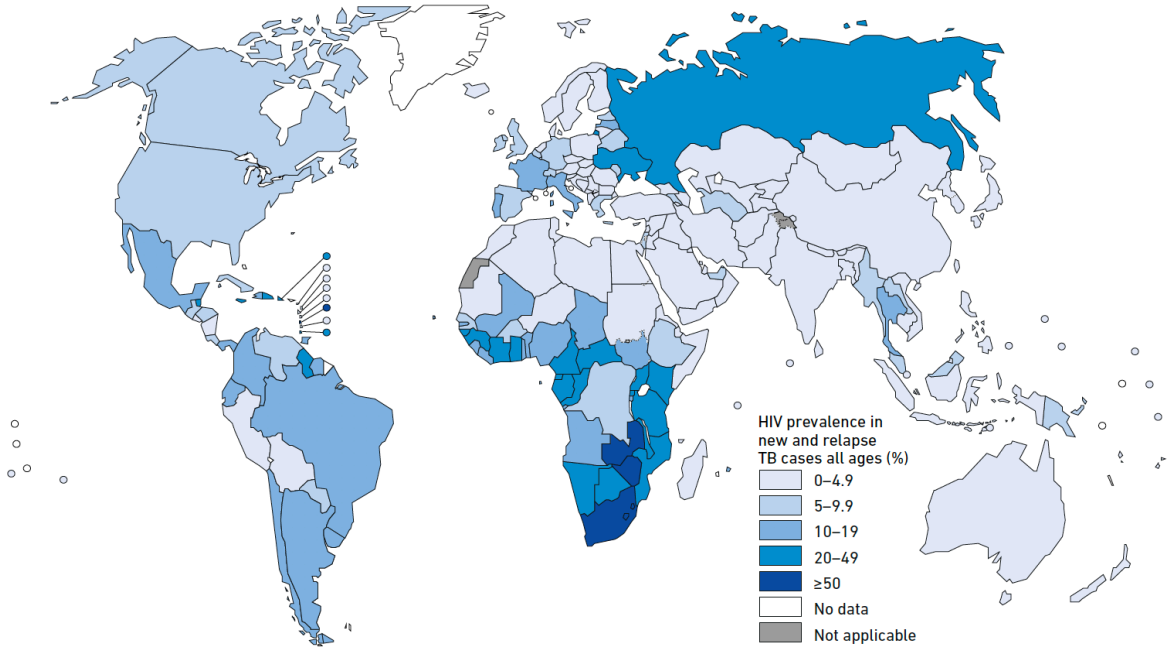
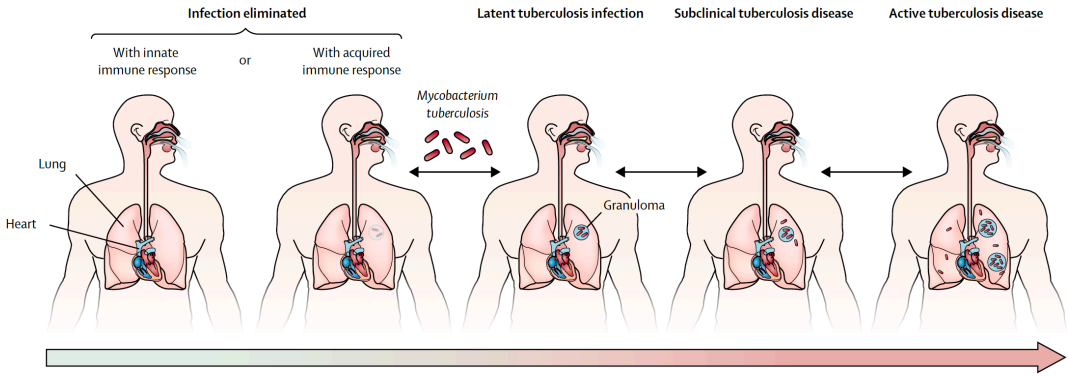


Figure 3. Estimated HIV prevalence in new and relapse TB cases in 2017 (3).



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 4. Spectrum of *Mtb* infection and TB disease (30).

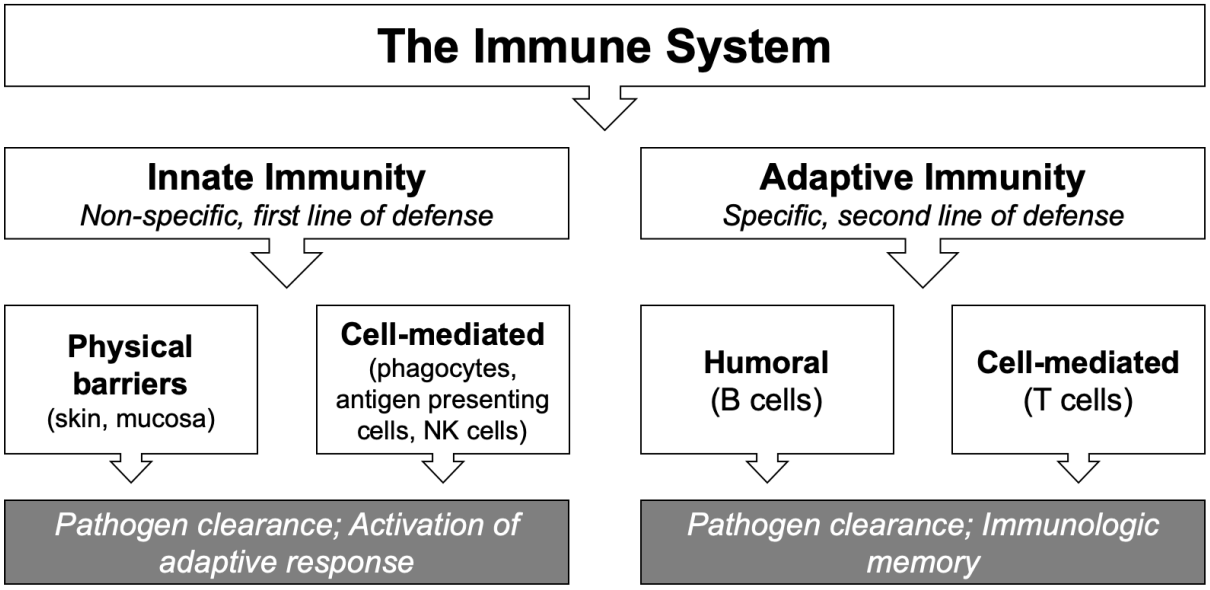


Figure 5. Simplified flow chart of immune functions.

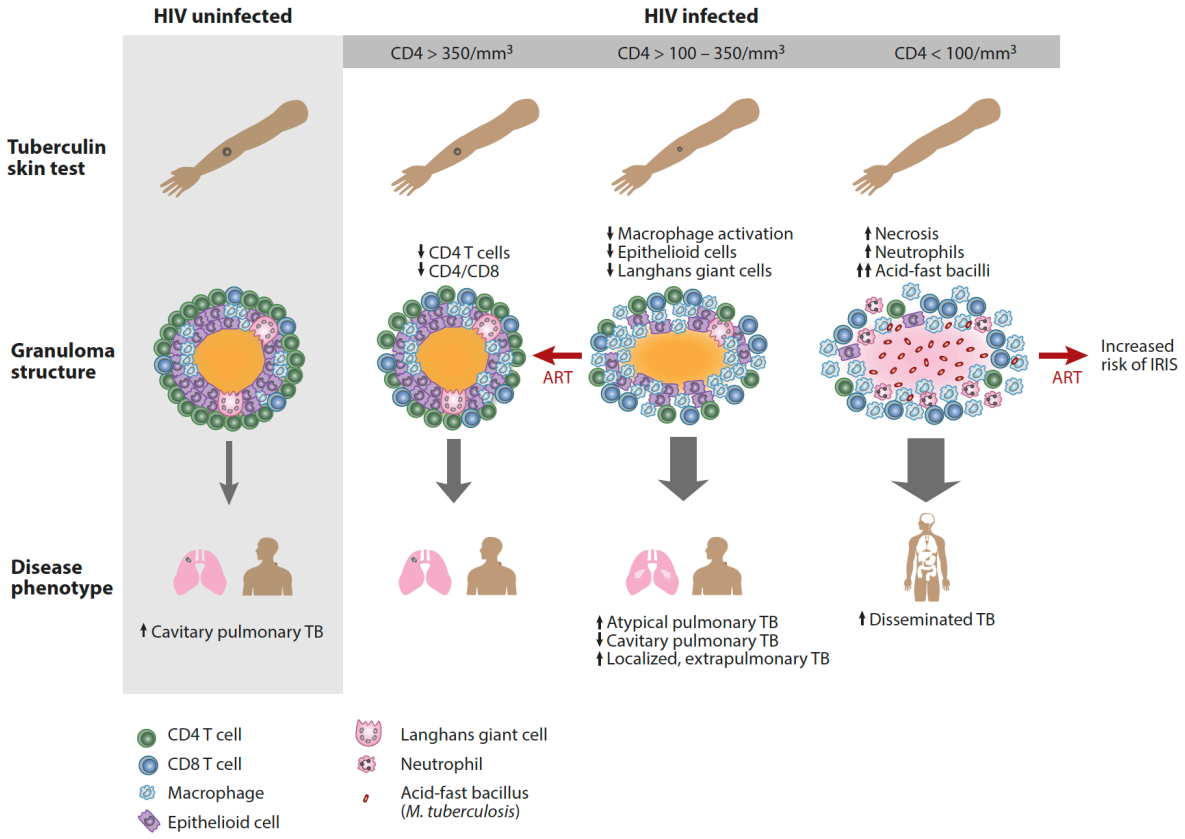


Figure 6. The impact of HIV co-infection on granuloma formation (69).

CHAPTER II

HIV infection is associated with downregulation of BTLA expression on *Mycobacterium tuberculosis*-specific CD4 T cells in active tuberculosis disease

Abstract

Nearly a quarter of the global population is infected with *Mycobacterium tuberculosis* (*Mtb*), with 10 million people developing active tuberculosis (TB) annually. Co-infection with HIV has long been recognized as a significant risk factor for progression to TB disease, yet the mechanisms whereby HIV impairs T cell-mediated control of *Mtb* infection remain poorly defined. We hypothesized that HIV infection may promote upregulation of inhibitory receptors on *Mtb*-specific CD4 T cells, a mechanism that has been associated with antigen-specific T cell dysfunction in chronic infections. Using cohorts of HIV-infected and HIV-uninfected individuals with latent *Mtb* infection (LTBI) and with active TB disease, we stimulated peripheral blood mononuclear cells (PBMC) for 6 hours with *Mtb* peptide pools and evaluated co-expression profiles of the inhibitory receptors BTLA, CTLA-4, and PD-1 on IFN-γ^+/TNF-α^+ *Mtb*-specific CD4 T cells. *Mtb*-specific CD4 T cells in all participant groups expressed predominately either one or no inhibitory receptors, unlike cytomegalovirus- and HIV-specific CD4 T cells circulating in the same individuals, which were predominately CTLA-4^+PD-1^+. There were no significant differences in inhibitory receptor expression profiles of *Mtb*-specific CD4 T cells between HIV-uninfected and HIV-infected individuals with LTBI. Surprisingly, BTLA expression, both alone and in combination with CTLA-4 and PD-1, was markedly downregulated on *Mtb*-specific CD4 T cells in HIV-infected individuals with active TB. Together, these data provide novel evidence that the majority of *Mtb*-specific CD4 T cells do not co-express multiple inhibitory receptors, regardless of HIV infection status; moreover, they highlight a previously unrecognized role of BTLA expression on *Mtb*-specific CD4 T cells that could be further explored as a potential

biomarker of *Mtb* infection status, particularly in people living with HIV, the population at greatest risk for development of active TB disease.

Introduction

Mycobacterium tuberculosis (*Mtb*) is the infectious agent that causes tuberculosis (TB) disease (3). TB is the leading cause of death due to a single infectious agent and has remained one of the top 10 causes of death worldwide for decades (3). In 2017, 10 million new cases of TB disease were reported, resulting in 1.6 million deaths (3). An estimated 1.7 billion people, representing nearly a quarter of the world's population, are latently infected with *Mtb* and therefore at risk for developing active TB disease (82). Although the precise immune correlates of protection against TB have not been defined, co-infection with human immunodeficiency virus (HIV) is the single greatest risk factor for reactivation from latent *Mtb* infection (LTBI) to active TB disease (3, 83). Worldwide, approximately 9% of new reported TB cases occur in people living with HIV, of which 72% live in Africa (3).

Infection with HIV induces immune suppression and depletion of CD4 T cells, which play a critical role in limiting *Mtb* bacterial growth and reducing progression to active TB disease (49). *Mtb*-specific CD4 T cells in HIV-infected individuals exhibit elements of immune dysfunction, including impaired proliferative capacity, heightened immune activation and cell death (75), and intermediate differentiated effector memory profiles (51). IL-2 producing *Mtb*-specific CD4 T cells have been inversely correlated with HIV viral load in individuals with LTBI (51), and decreased frequencies of cytokine-producing *Mtb*-specific CD4 T cell subsets in HIV-infected individuals (75, 84-87). Other studies have demonstrated that *Mtb*-specific CD4 T cells are depleted early after HIV seroconversion (77) and that *Mtb*-specific CD4 T cells may be preferentially infected by HIV (76). Although HIV co-infection clearly disrupts protective immunity to

Mtb, the precise mechanisms whereby HIV impairs *Mtb*-specific T cell immunity and accelerates progression to TB disease have not been fully elucidated.

Ag-specific T cell dysfunction is a well described feature of chronic infections, including HIV, with upregulation of negative regulatory receptors on Ag-specific T cells described as one mechanism contributing to inhibition of T cell activation and effector functions such as cytokine production, cytotoxicity and proliferation (88). In mice with chronic lymphocytic choriomeningitis virus (LCMV) infection, transcriptional profiling of dysfunctional or 'exhausted' LCMV-specific CD8 T cells identified inhibitory receptors with sustained expression at high levels on dysfunctional T cells, including PD-1, CTLA-4, 2B4, CD160, and LAG-3 (78, 89). While T cell dysfunction in chronic infections was initially described in Ag-specific CD8 T cells, Ag-specific CD4 T cells also exhibit functional impairment and high expression of inhibitory receptors in the setting of persistent Ag stimulation (90). Similar to CD8 T cells, Ag-specific CD4 T cell in chronic infections express high levels of PD-1 and CTLA-4, as well as B and T lymphocyte attenuator (BTLA), which is upregulated on Ag-specific CD4 T cells but not Ag-specific CD8 T cells in chronic infection (91, 92). Moreover, co-expression of multiple inhibitory receptors is associated with greater severity of Ag-specific T cell dysfunction (91), thus providing evidence of the additive and detrimental effect of co-expression of multiple inhibitory receptors on Ag-specific T cell function.

T cell dysfunction associated with increased expression of inhibitory receptors has been well described in chronic HIV infection (93), and expression of inhibitory receptors, including PD-1 and CTLA-4, on HIV-specific T cells correlates with viral load and absolute CD4 T cell count (94, 95), important parameters of HIV disease progression. Moreover, HIV-specific CD4 T cells co-express combinations of PD-1,

CTLA-4 and LAG-3 (95-97), which correlates with viral load and decreases after suppression of HIV viremia by antiretroviral therapy (96). Blockade of PD-1 and CTLA-4 signaling in HIV-specific CD4 T cells enhances cytokine production and proliferation (94, 95, 97, 98), thus providing further evidence of the relationship between inhibitory receptor expression and HIV-specific CD4 T cell dysfunction. Although systemic immune activation and upregulation of inhibitory receptors on HIV-specific CD4 T cells are well-described in HIV-infected individuals, it is currently unclear whether HIV infection is also associated with increased expression of inhibitory receptors on CD4 T cells specific for other co-infections, such as *Mtb*, which may subsequently impair T cell-mediated control of infection.

Given that HIV infection is associated with upregulation of inhibitory receptors on CD4 T cells, and that the risk of developing active TB disease is more than 20-fold higher in HIV-infected individuals, compared with HIV-uninfected individuals (3, 83), we sought to determine if inhibitory receptors are upregulated on *Mtb*-specific CD4 T cells in individuals co-infected with *Mtb* and HIV, which may ultimately contribute to impairment of *Mtb*-specific CD4 T cell functional capacity and progression to active TB disease. Using cohorts of HIV-infected and HIV-uninfected individuals with LTBI and active TB in South Africa and Kenya, two high TB burden countries (3), we conducted a thorough examination of inhibitory receptor expression on Ag-specific CD4 T cells. We evaluated expression patterns of BTLA, CTLA-4, and PD-1, three inhibitory receptors on T cells that are members of the immunoglobuin (Ig) superfamily and have reported to be upregulated on Ag-specific CD4 T cells in chronic infections (92, 95-97). Furthermore, we compared inhibitory receptor expression profiles of *Mtb*-specific CD4

T cells to that of human cytomegalovirus (HCMV)- and HIV-specific CD4 T cells within the same individual.

Materials and Methods

Study participants and sample collection: Blood samples were collected from individuals ≥18 years of age living in Western Kenya and in the Western Cape province of South Africa. Study participants included HIV-uninfected and HIV-infected adults with latent *Mtb* infection (LTBI) or active TB disease (75, 99). Individuals with LTBI included in the study had a positive QuantiFERON-TB Gold (QFT) test result and no previous history of active TB; LTBI participants were asymptomatic for TB, with no cough, weight loss, night sweats, or fever. Participants with active TB disease were acid-fast bacilli (AFB) sputum smear-positive or Xpert *MTB*/RIF assay-positive, with pulmonary TB disease confirmed by a positive mycobacterial sputum culture. Blood was collected from patients with TB disease within the first 7 days of starting the standard 6-month course of TB treatment. Serologic testing for HIV antibodies was done for all individuals using the Alere Determine HIV-1/2 Ag/Ab Combo test. Plasma HIV viral load and CD4 T cell counts were measured for HIV-infected individuals; viral load results were not available from three HIV-infected participants with active TB. All HIV-infected individuals with LTBI had >200 CD4 T cells/μl. With the exception of 4 individuals with active TB disease, all HIV-infected participants were antiretroviral therapy-naïve at the time of analysis. Blood samples from all participants were collected in sodium heparin tubes for isolation of peripheral blood mononuclear cells (PBMC) for analysis of Ag-specific CD4 T cells, as described below.

Ethics statement. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. All subjects provided written informed consent for

participation in the study, which was approved by the Human Research Ethics Committee at the University of Cape Town, the Western Cape Province Department of Health, the Kenya Medical Research Institute Scientific and Ethics Review Unit, and the Emory University Institutional Review Board.

PBMC isolation and antigen stimulation: Within 4 hours of blood collection, PBMC were isolated from heparinized whole blood via density gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich) and then cryopreserved and stored in LN_2 until use. Cryopreserved PBMC 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 centrifuged at 2,000 RPM for 5 minutes at 25°C, resuspended in RPMI, and centrifuged again under the same conditions. Cells were 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 4 hours 37°C and 5% CO_2. After resting, cells were stimulated with the following antigens: pooled, overlapping 15-mer peptides corresponding to the sequences of CFP-10 (22 peptides, 1 μg/ml/peptide), ESAT-6 (21 peptides, 1 μg/ml/peptide), and human CMV (HCMV) pp65 (138 peptides, 1 μg/ml/peptide). Pooled, overlapping 15-mer peptides comprising HIV-1 consensus A Gag (122 peptides, 1 μg/ml/peptide) and HIV-1 consensus C Gag (121 peptides, 1 μg/ml/peptide) were used to stimulate PBMC from Kenyan and South African HIV-infected individuals, respectively. The CFP-10 and ESAT-6 peptide pools were obtained through BEI Resources, NIAID, NIH (catalog numbers NR-50712 and NR-50711, respectively). The HCMV pp65 peptide pool was obtained from the National Institutes of Health AIDS Reagent Program, Division of

AIDS, NIAID, NIH (100-102). The HIV Gag peptide pools were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Subtype C (DU422) Gag Peptide Set and HIV-1 Consensus A Gag Peptides – Complete Set. PBMC were stimulated with staphylococcal enterotoxin B (SEB; 1 μg/ml; Sigma-Aldrich) as a positive control. PBMC incubated with no Ag were used as a negative control. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added after a 1-hour incubation at 37°C and the incubation continued for an additional 5 hours at 37°C.

Antibodies and intracellular cytokine staining (ICS) assay: Stimulated cells were washed with PBS and stained for 20 minutes at room temperature with Zombie NIR™ Fixable Viability Dye (BioLegend). Cells were surface stained for 30 minutes at room temperature with anti-CD3 Brilliant Violet 711 (UCHT1; BD Horizon), anti-CD4 Brilliant Violet 570 (RPA-T4; BioLegend), anti-CD8 PerCP-Cy5.5 (SK1; BioLegend), anti-PD-1 PE (EH12.2H7; BioLegend), and anti-BTLA APC (MIH26; BioLegend). Cells were washed with PBS containing 1% FCS and fixed with FACS Lysing Solution (BD Biosciences), then washed with Perm/Wash Buffer (BD Biosciences). Cells were stained intracellularly for 30 minutes at room temperature with anti-IFN-γ FITC (B27; BD Pharmingen), anti-TNF-α Brilliant Violet 421 (Mab11; BioLegend), and anti-CD152 PE-CF594 (BNI3; BD Horizon), washed with Perm/Wash Buffer (BD Biosciences) and suspended in PBS prior to acquisition.

Flow cytometry and data analysis: Cells were acquired on a BD LSRII flow cytometer with BD FACSDiva software (v8.0) and analyzed using FlowJo software (v9.7.6; Tree Star). Compensation was calculated using single-stained anti-mouse Ig,κ beads (BD

Biosciences). Doublet cell populations were excluded by plotting forward scatter area versus forward scatter height. Viable lymphocytes were defined as Zombie NearIR-low cells. Combinations of cytokine-producing cells were determined using Boolean gating in FlowJo. The flow cytometry gating strategy is indicated in Figure S1 in Supplementary Material.

Data analysis and statistics: Only individuals with a positive response to a given Ag were included in the phenotypic analyses of Ag-specific CD4 T cells. CD4 T cells producing TNF-α and/or IFN-γ were used to determine Ag-specific responses via the Bayesian mathematical model, MIMOSA (Mixture Models for Single Cell Assays) (103) to account for cell counts and background cytokine secretion. TNF-α- and IFN-γ-producing CD4 T cells with a positive response rate >75% and a false discover rate (fdr/q-value) <3% were considered positive responses. R programming software was used to perform statistical analyses of frequencies and phenotype of Ag-specific CD4 T cells (104). Differences between two groups were evaluated using a non-parametric Mann-Whitney test. Differences between three or more groups were first evaluated using a non-parametric Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *P*-values of less than 0.05 was considered to be statistically significant.

Results

Study participants.

Blood samples were collected from 32 HIV-uninfected and 22 HIV-infected participants with LTBI, and from 37 HIV-uninfected and 19 HIV-infected participants with pulmonary TB disease enrolled in Western Kenya and in the Western Cape Province of South Africa (Table 1). The median CD4 T cell count of HIV-infected participants with LTBI was 562 cells/μl; the median viral load was 11,710 HIV RNA copies/ml plasma. The median CD4 T cell count of HIV-infected participants with active TB disease was 420 cells/μl; the median viral load was 6,350 HIV RNA copies/ml plasma.

Frequencies of *Mtb*-specific CD4 T cells producing IFN-γ and/or TNF-α are similar in HIV-infected and HIV-uninfected individuals.

Infection with HIV is associated with CD4 T cell depletion and dysfunction. To further characterize the effect of HIV infection on the frequency of *Mtb*-specific CD4 T cells, we used a flow cytometry-based ICS assay to measure Ag-specific CD4 T cell responses in HIV-infected and HIV-uninfected individuals with LTBI and with active TB disease. PBMCs were stimulated for 6 hours with the immunodominant *Mtb* Ags CFP-10 and ESAT-6 and viral Ags (HCMV pp65 and HIV Gag peptide pools); Ag-specific CD4 T cells were identified by production of IFN-γ and TNF-α (Figure 1A). No significant differences in the frequency of CFP-10/ESAT-6-specific CD4 T cells producing IFN-γ and/or TNF-α were observed between HIV-uninfected and HIV-infected individuals

with LTBI (Figure 1B, C) or active TB disease (Figure 1D, E). The frequencies of CD4 T cells producing IFN-γ and/or TNF-α following stimulation with HCMV pp65 and SEB were also similar between HIV-infected and HIV-uninfected individuals with latent and active TB (Figure S2 in Supplementary Material). These data indicate that circulating CFP-10/ESAT-6-specific CD4 T cells with the capacity to produce the Th1 effector cytokines IFN-γ and TNF-α are not significantly depleted in HIV-infected individuals with either LTBI or active TB, compared with HIV-uninfected individuals.

Active TB disease is associated with reduced inhibitory receptor co-expression on total CD4 T cells

In addition to the frequency of cytokine-producing T cells, expression profiles of multiple types of receptors are indicative of functional status of Ag-specific T cells. Given that infection with HIV has been shown to lead to modulation of T cell phenotypic profiles (105), we next determined the effect of HIV infection on the total CD4 T cell expression profiles of BTLA, CTLA-4, and PD-1 in individuals with LTBI and with active TB, using a Boolean gating strategy (Figure 2). In individuals with LTBI, the predominant population consisted of CD4 T cells expressing BTLA alone, regardless of HIV infection status (Figure 2A). In individuals with active TB, CD4 T cells predominately express either BTLA alone or PD-1 alone (Figure 2B).

Although there were no significant differences in the frequency of CD4 T cell subsets expressing each combination of BTLA, CTLA-4, and PD-1 between HIV-infected and HIV-uninfected individuals (Figure 2A, B), we did observe significant differences in the overall total number of inhibitory receptors expressed by CD4 T cells in individuals with

LTBI and active TB (Figure 2C). Individuals with active TB had significantly lower frequencies of CD4 T cells expressing either two or three inhibitory receptors, compared with individuals with LTBI. The decrease in CD4 T cells expressing two or three inhibitory receptors in individuals with active TB corresponded to a significant increase in their proportion of CD4 T cells lacking co-expression of the three inhibitory receptors measured (Figure 2C). Importantly, the expansion of CD4 T cells expressing no inhibitory receptors in active TB, compared with LTBI, was evident in both HIV-infected and HIV-uninfected individuals. Taken together, these data suggest that TB disease status impacts CD4 T cell inhibitory receptor expression profiles and is associated with expansion of circulating CD4 T cells that lack co-expression of BTLA, CTLA-4 and PD-1.

Mtb-specific CD4 T cell inhibitory receptor expression profiles are modulated in HIV-infected individuals with active TB

Next, we sought to determine whether co-infection with HIV significantly modifies inhibitory receptor expression profiles of *Mtb*-specific CD4 T cells. To do so, we used our ICS assay to analyze expression of BTLA, CTLA-4, and PD-1 on CD4 T cells producing IFN-γ and/or TNF-α following stimulation with CFP-10 and ESAT-6 peptide pools (Figure 3A, D). MIMOSA was used to define cytokine-positive *Mtb*-specific CD4 T cell responses for further phenotypic analysis of BTLA, CTLA-4, and PD-1 expression (103).

We first determined the total number of inhibitory receptors expressed by *Mtb*-specific CD4 T cells in individuals with LTBI. In both groups of individuals with LTBI, the majority of *Mtb*-specific CD4 T cells expressed either no inhibitory receptors or only

one inhibitory receptor (Figure 3B). There were no significant differences in the proportion of *Mtb*-specific CD4 T cells expressing three, two, one, or no inhibitory receptors in HIV-infected individuals, compared with HIV-uninfected individuals with LTBI (Figure 3B). Similar to individuals with LTBI, the majority of *Mtb*-specific CD4 T cells in individuals with active TB disease expressed either no or only one inhibitory receptor (Figure 3E). However, HIV-infected individuals with active TB disease had a significantly lower proportion of *Mtb*-specific CD4 T cells co-expressing all three inhibitory receptors, compared with HIV-uninfected patients with active TB.

We next evaluated the proportion of *Mtb*-specific CD4 T cells expressing each combination of BTLA, CTLA-4, and PD-1. Among *Mtb*-specific CD4 T cells that expressed between one and three inhibitory receptors, there was no single dominant subset represented in either HIV-infected or HIV-uninfected individuals with LTBI; moreover, there was no significant difference in the proportion of *Mtb*-specific CD4 T cells expressing each combination of inhibitory receptors between HIV-infected and HIV-uninfected individuals with LTBI (Figure 3C). By contrast, HIV infection of individuals with active TB disease was associated with a significant decrease in three subsets of *Mtb*-specific CD4 T cells, compared with HIV-uninfected individuals: $BTLA^{+}CTLA\text{-}4^{+}PD\text{-}1^{+}$, $BTLA^{+}CTLA\text{-}4^{+}PD\text{-}1^{-}$, and $BTLA^{+}CTLA\text{-}4^{-}PD\text{-}1^{-}$ cells (Figure 3F). Together, these data indicate that *Mtb*-specific CD4 T cell inhibitory receptor expression profiles are significantly modified in the setting of HIV co-infection with active TB, but not LTBI.

BTLA expression is downregulated on *Mtb*-specific CD4 T cells HIV-infected individuals with active TB

A common feature of the three phenotypic subsets of *Mtb*-specific CD4 T cells that are significantly decreased in HIV-infected individuals with active TB is expression of BTLA, which has previously been reported to be downregulated on T cells in HIV-infected individuals, compared with HIV-uninfected individuals (106). Therefore, we next evaluated whether CD4 T cell expression of BTLA expression alone, independent of PD-1 and CTLA-4 expression, was different between HIV-infected and HIV-uninfected individuals (Figure 4A). We found that BTLA expression was downregulated on total CD4 T cells from HIV-infected individuals with active TB, compared with HIV-uninfected individuals with active TB and with HIV-infected individuals with LTBI (Figure 4B). Although BTLA expression on *Mtb*-specific CD4 T cells was similar in HIV-infected and HIV-uninfected individuals with LTBI (Figure 4C, D), it was significantly downregulated on *Mtb*-specific CD4 T cells in HIV-infected individuals with active TB, compared with HIV-uninfected individuals with active TB (Figure 4D). Furthermore, BTLA expression on *Mtb*-specific CD4 T cells in HIV-infected individuals with active TB was significantly lower than *Mtb*-specific CD4 T cells in HIV-infected individuals with LTBI (Figure 4D). By contrast, evaluation of CTLA-4 alone and PD-1 alone did not indicate any significant differences in expression levels on either total or *Mtb*-specific CD4 T cells in HIV-infected and uninfected individuals with either LTBI or active TB (Figure S3 in Supplementary Material). Together, these data provide further evidence that HIV infection is associated with downregulation of BTLA expression on CD4 T cells; moreover, they identify BTLA as a phenotypic marker on *Mtb*-specific CD4 T cells

that is specifically downregulated in HIV-infected individuals with active TB disease, but not LTBI.

To determine whether CD4 T cell expression of BTLA is associated with parameters of HIV disease progression, we analyzed the relationship between CD4 T cell BTLA expression and HIV viral load (Figure S4 in Supplementary Material) and absolute CD4 T cell count in individuals with LTBI and with active TB (Figure S5 in Supplementary Material). We found no significant correlation between HIV viral load and the proportion of either total CD4 or *Mtb*-specific CD4 T cells expressing BTLA (Figure S4 in Supplementary Material). Additionally, we found no significant correlation between absolute CD4 T cell count and BTLA expression on either total CD4 or *Mtb*-specific CD4 T cells (Figure S5 in Supplementary Material). These data indicate that CD4 T cell downregulation of BTLA expression in HIV-infected individuals is not directly related to HIV disease progression. Moreover, they suggest that marked downregulation of BTLA expression on *Mtb*-specific CD4 T cells occurs particularly in the setting of concurrent active TB disease and HIV infection.

Inhibitory receptor expression on *Mtb*-specific CD4 T cells differs from HCMV- and HIV-specific CD4 T cells

Previous studies have indicated that expression of PD-1 and CTLA-4 is upregulated on HIV-specific CD4 T cells (95), yet we did not find evidence that HIV co-infection is associated with upregulation of PD-1 and CTLA-4 expression on *Mtb*-specific CD4 T cells, compared with HIV-uninfected individuals. To further define inhibitory receptor profiles of *Mtb*-specific CD4 T cells in the setting of HIV infection,

we directly compared expression of BTLA, CTLA-4, and PD-1 on circulating *Mtb*-, HCMV-, and HIV-specific CD4 T cells within the same individual (Figure 5A). HCMV- and HIV-specific CD4 T cells predominately co-expressed CTLA-4 and PD-1 (Figure 5B). By contrast, *Mtb*-specific CD4 T cells from the same individuals consistently exhibited markedly lower proportions of $CTLA\text{-}4^{+}PD\text{-}1^{+}$ cells and PD-1 single-positive cells, compared with virus-specific CD4 T cells in both HIV-infected and HIV-uninfected individuals with LTBI and active TB (Figure 5B, D). A significantly higher proportion of *Mtb*-specific CD4 T cells from all four participant groups were CTLA-4 single-positive, compared with HCMV-specific CD4 T cells in the same individuals (Figure 5B, D). With the exception of HIV-infected individuals with active TB disease, in who BTLA is downregulated (Figure 4D), a significantly higher proportion of *Mtb*-specific CD4 T cells were also BTLA single-positive, compared with HCMV- and HIV-specific CD4 T cells (Figure 5B, D).

Since expression of multiple inhibitory receptors on HIV-specific CD4 T cells has been associated with HIV disease progression (96), we also evaluated the proportion of *Mtb*-, HCMV- and HIV-specific CD4 T cells expressing three, two, one, or no inhibitory receptors in individuals with LTBI and active TB (Figure 5C, E). In all 4 participant groups, the majority of *Mtb*-specific CD4 T cells expressed either no or one inhibitory receptor, whereas the majority of HCMV- and HIV-specific CD4 T cells co-expressed two inhibitory receptors (predominately CTLA-4 and PD-1). Overall, HCMV- and HIV-specific CD4 T cells exhibit similar inhibitory receptor expression profiles, which differ considerably from *Mtb*-specific CD4 T cells, regardless of HIV infection status (Figure 5).

In addition to our analysis of inhibitory receptor co-expression profiles, we also considered the differential expression of total BTLA, CTLA-4, and PD-1 on *Mtb*-, HCMV- and HIV-specific T cells (Figure S6 in Supplementary Material). With the exception of HIV-infected individuals with active TB disease, *Mtb*-specific CD4 T cells from all groups expressed significantly higher levels of BTLA, compared to HCMV-specific CD4 T cells. By contrast, HCMV- and HIV-specific CD4 T cells were consistently characterized by significantly higher expression of PD-1, compared with *Mtb*-specific CD4 T cells, regardless of *Mtb* or HIV infection status (Figure S6 in Supplementary Material). Taken together, these data indicate that while virus-specific CD4 T cells co-express high levels of CTLA-4 and PD-1, *Mtb*-specific CD4 T cells within the same individuals do not upregulate expression of multiple inhibitory receptors simultaneously.

Discussion

A state of T cell dysfunction, including dampened effector functions, upregulated inhibitory receptor expression and decreased proliferation has been described during chronic infections (107), yet the relationship between HIV infection and the phenotype and function of *Mtb*-specific CD4 T cell responses has not been fully elucidated. This study was conducted to evaluate the effect of HIV on the concurrent expression of the Ig superfamily T cell inhibitory receptors BTLA, CTLA-4, and PD-1 on total and Ag-specific CD4 T cells from individuals with LTBI and active TB disease. We demonstrated that active TB disease in both HIV-uninfected and HIV-infected individuals is associated with reduced proportions of total CD4 T cells co-expressing three inhibitory receptors, compared with LTBI. Surprisingly, we found no evidence of increased expression of BTLA, CTLA-4, or PD-1, either alone or in combination, on *Mtb*-specific CD4 T cells in HIV-infected individuals, compared with HIV-uninfected individuals. Moreover, expression of BTLA was significantly lower on *Mtb*-specific CD4 T cells from HIV-infected individuals with active TB, compared with the other three participant groups. By directly comparing inhibitory receptor phenotypes of *Mtb*-, HCMV-, and HIV-specific CD4 T cells circulating concurrently within the same individual, we provide compelling evidence that, by contrast with virus-specific CD4 T cells, *Mtb*-specific CD4 do not co-express inhibitory receptors at high levels, regardless of HIV infection status.

Mtb-specific CD4 T cell production of the Th1 cytokines IFN-γ and TNF-α is important for activating macrophages and promoting formation of granulomas in the lung for containment of *Mtb* (108). By stimulating whole blood with PPD, we have previously found that HIV-infected individuals with LTBI had lower frequencies of Th1

cytokine-producing CD4 T cells than HIV-uninfected individuals with LTBI (75), consistent with previous reports (76, 77). While PPD contains greater than 150 Ags (109, 110), in this study we used peptide pools spanning two immunodominant Ags to identify *Mtb*-specific CD4 T cells. By focusing on CFP-10/ESAT-6-specific CD4 T cells, we found similar frequencies of IFN-γ and/or TNF-α-producing CD4 T cells in HIV-infected and HIV-uninfected individuals with either LTBI or active TB disease, consistent with previous studies (111-113). In addition to Ag specificity, another important consideration in determining whether or not *Mtb*-specific CD4 T cells are depleted in HIV-infected individuals is the number and type of cytokines measured. While production of Th1 cytokines, including IFN-γ, TNF- α and IL-2 are commonly evaluated in studies of Ag-specific CD4 T cells, we did not include IL-2 in this study to identify *Mtb*-specific CD4 T cells for phenotypic analysis. IL-2 is important for T cell survival and is one of the first cytokines that is lost by dysfunctional Ag-specific T cells in chronic infections (107). A recent study of individuals with LTBI and active TB in Tanzania revealed that HIV infection is associated with reduced proportions of Th2 and IL-2-producing *Mtb*-specific CD4 T cells (87), thus highlighting the importance of evaluating multiple different cytokines to more comprehensively evaluate the effect of HIV co-infection on *Mtb*-specific T cell immune function. It is possible that future studies measuring CD4 T cells specific for Ags other than CFP-10 and ESAT-6, and that include measurement of additional cytokines, such as additional Th1 cytokines as well as Th2 and Th17 cytokines, may reveal additional information on the frequency and phenotype of *Mtb*-specific CD4 T cells that differentiate HIV-infected and HIV-uninfected individuals.

Although we did not find evidence of preferential depletion of CFP-10/ESAT-6-specific CD4 T cells in HIV-infected individuals, evaluation of phenotypic markers, such

as the inhibitory receptors BTLA, CTLA-4, and PD-1, provide additional insight into the functional nature of Ag-specific T cells. Multiple inhibitory receptors have been implicated as key contributors to the loss of immune control in cancer and chronic viral infections (93, 114), yet the role of inhibitory receptors immune control of *Mtb* is less clear. Given that HIV-infected individuals are at substantially higher risk of developing active TB, compared with HIV-uninfected individuals, we initially hypothesized that inhibitory receptors may be upregulated on *Mtb*-specific CD4 T cells in HIV-infected individuals, thus identifying a possible mechanism contributing to *Mtb*-specific CD4 T cell dysfunction in HIV infection. We were specifically interested in determining whether *Mtb*-specific CD4 T cells co-expressed multiple inhibitory receptors in HIV-infected individuals, as concurrent expression of multiple inhibitory receptors on the same Ag-specific T cell has been previously associated with greater T cell dysfunction (91, 96). While previous studies have reported co-expression of up to three inhibitory receptors on HIV-specific CD4 T cells (95-97), co-expression of multiple inhibitory receptors has not been thoroughly investigated on *Mtb*-specific CD4 T cells. Contrary to our initial hypothesis, we found that co-expression of all three inhibitory receptors was significantly lower on *Mtb*-specific CD4 T cells in HIV-infected individuals with active TB disease, compared with HIV-uninfected individuals with active TB. Interestingly, HIV infection did not have a significant impact on inhibitory receptor expression profiles of *Mtb*-specific CD4 T cells among individuals with LTBI. These data suggest that the combination of active TB disease and HIV infection together modify the phenotypic profiles of *Mtb*-specific CD4 T cells. However, it is important to note that we defined *Mtb*-specific CD4 T cells as those cells producing IFN-γ and/or TNF-α following short-term peptide stimulation, and it is possible that other approaches to defining *Mtb*-

specific T cells (i.e., different Ag specificity, different effector functions, or direct MHC tetramer staining without peptide stimulation) may reveal differences in inhibitory receptor expression by *Mtb*-specific T cells than we observed in this study. Furthermore, increasing evidence indicates that age, sex, and genetics contribute significantly to heterogeneity in immune response profiles in humans (115, 116), thus additional differences in *Mtb*-specific T cell responses may emerge in studies utilizing cohorts from diverse geographical regions.

Through Boolean analysis of BTLA, CTLA-4, and PD-1 expression on *Mtb*-specific CD4 T cells, we identified three distinct BTLA-expressing subsets that are significantly lower in HIV-infected active TB patients, compared with HIV-uninfected TB patients: $BTLA^+CTLA\text{-}4^+PD\text{-}1^+$, $BTLA^+CTLA\text{-}4^+PD\text{-}1^-$, and $BTLA^+CTLA\text{-}4^-PD\text{-}1^-$ cells. We found that BTLA expression is downregulated on bulk CD4 T cells in HIV-uninfected and HIV-infected individuals with active TB, a finding that is consistent with a previous report that BTLA expression is progressively downregulated on CD4 and CD8 T cells in chronic HIV infection (106). However, when evaluating *Mtb*-specific CD4 T cells, we found that BTLA was markedly downregulated only in HIV-infected patients with active TB, compared with all three other study groups. Thus, these data identify a novel phenotype of *Mtb*-specific CD4 T cells that is particular to the dual combination of active TB and HIV infection. The identification of potential biomarkers that can distinguish individuals with latent and active TB is particularly important in the setting of HIV infection, where HIV-infected individuals are less likely to have cavitary TB disease and less likely to be smear-positive for *Mtb*, compared with HIV-uninfected individuals (117), thus making microbiologically confirmed diagnosis of active TB disease particularly challenging in people living with HIV. Larger prospective, longitudinal

studies of HIV-infected individuals are warranted to determine whether downregulation of BTLA expression on *Mtb*-specific CD4 T cells is a prognostic indicator of active TB disease, or whether BTLA expression is downregulated only after exposure to high levels of *Mtb* Ag in the context of HIV infection.

The role of BTLA expression in T cell-mediated immune control of *Mtb* infection has not been investigated and requires further study. BTLA was defined as an inhibitory receptor containing two immunoreceptor tyrosine-based inhibition motifs (ITIMs) in its cytoplasmic tail that is functionally and structurally similar to CTLA-4 and PD-1 (118). BTLA is expressed on multiple immune cell types and interacts with the costimulatory molecule herpes virus entry mediator (HVEM) (119, 120), which is also widely expressed on immune cells. Increasing evidence indicates bidirectional signaling occurs through the interaction between BTLA and HVEM, with HVEM ligation of BTLA leading to inhibitory signals through phosphorylation of ITIMs and recruitment of the tyrosine phosphatases SHP1 and SHP2 (118, 121), and BTLA ligation of HVEM leading to proinflammatory signals through activation of NF-κB (122), thus providing a unique opportunity for BLTA and HVEM engagement to balance resulting immune responses. Studies in mice indicate that BTLA expression is necessary to prevent prolonged inflammation in the lung (123) and that BTLA-deficient CD4 T cells have altered expression of genes involved in effector function and memory differentiation, including decreased expression of CD127, granzyme B, MIP-1α and MIP-1β (124). Moreover, generation of protective memory Ag-specific CD8 T cell responses was significantly impaired in vaccinia virus-infected mice deficient in either BTLA or HVEM (125). Taken together, these data suggest co-signaling of BTLA and HVEM may regulate inflammatory responses in tissues and may also be necessary for generation of long-

lived Ag-specific memory T cell responses. Further studies are thus warranted to determine whether lack of BTLA expression on *Mtb*-specific CD4 T cells in HIV-infected individuals with active TB disease is associated with a sustained state of inflammation in these individuals, and/or with impaired ability to generate robust *Mtb*-specific memory CD4 T cell responses in HIV-infected individuals. Future studies are also necessary to determine whether modulation of BTLA/HVEM co-signaling can fine tune the immune response to *Mtb* to promote durable control of infection and prevent progression to TB disease.

While we found compelling evidence that BTLA expression is downregulated on *Mtb*-specific CD4 T cells in HIV-infected patients with active TB, we found no evidence that CTLA-4 and PD-1 are upregulated in *Mtb*-specific CD4 T cells in HIV-infected individuals, unlike previous studies that have reported upregulation of CTLA-4 and PD-1 on HIV-specific CD4 T cells in HIV-infected individuals (126). To further substantiate our *Mtb*-specific CD4 T cell inhibitory receptor profiles in the context of the literature on inhibitory receptor profiles in HIV infection, we directly compared expression profiles of BTLA, CTLA-4, and PD-1 on *Mtb*-, HIV-, and HCMV-specific CD4 T cells within the same individual. Consistent with previous reports (95-97), HIV- and HCMV-specific CD4 T cells co-express CTLA-4 and PD-1, in both groups of LTBI and active TB, at significantly higher levels than *Mtb*-specific CD4 T cells. The direct comparison of inhibitory receptor profiles across multiple Ag-specific CD4 T cells circulating in peripheral blood provides compelling and novel evidence that the majority of *Mtb*-specific CD4 T cells do not upregulate co-expression of multiple inhibitory receptors, regardless of HIV infection status, and that Ag-specific CD4 T cells circulating in HIV-

infected individuals display markedly different phenotypic profiles, depending on the Ag specificity.

A limitation to our study was the use of overlapping peptide pools to stimulate IFN-γ and/or TNF-α production to identify Ag-specific CD4 T cells, which precludes our ability to detect *Mtb*-specific cells that do not produce these cytokines and thus may display different inhibitory receptor profiles than Ag-specific CD4 T cells that maintain Th1 cytokine production capacity. Nonetheless, our findings are consistent with a previous study using MHC class II tetramers bearing CFP-10 and ESAT-6 peptides, which demonstrate low levels of PD-1 expression on tetramer$^+$ *Mtb*-specific CD4 T cells in HIV-infected and HIV-uninfected individuals with LTBI and with active TB (113). An additional limitation was evaluation of inhibitory receptor expression on *Mtb*-specific CD4 T cells circulating in peripheral blood, and not at the site of *Mtb* infection in the lung. While analysis of lung-resident T cells is technically challenging in humans, a recent study has evaluated expression of inhibitory receptors on T cells isolated from granulomas of *Mtb*-infected macaques. Consistent with our findings in peripheral blood of humans, expression of the inhibitory receptors CTLA-4, PD-1, and LAG-3 was very low on T cells isolated from granulomas of *Mtb*-infected macaques and did not correlate with *Mtb* bacterial load (127). Furthermore, while we evaluated expression of BTLA, CTLA-4 and PD-1 in this study, there are additional inhibitory receptors, such as T cell immunoglobulin and mucin domain-containing molecule 3 (TIM-3) and Lag-3, that could be differentially expressed on *Mtb*-specific CD4 T cells in HIV-infected and HIV-uninfected individuals. Interestingly, expression of TIM-3 on CD4 and CD8 T cells in the lungs of *Mtb*-infected mice has been associated with T cell exhaustion (128), whereas TIM-3 expression on CD4 and CD8 T cells in PBMCs from patients with active

TB disease exhibited greater Th1 cytokine production capacity and cytotoxic molecule production, compared with T cells lacking TIM-3 expression (129). These studies thus highlight the variability in the functional significance of inhibitory receptor expression in *Mtb* infection, which can differ depending on the particular inhibitory receptor, cell populations evaluated, and the model system used. Future studies employing RNA sequencing of *Mtb*-specific CD4 T will be necessary to more comprehensively define CD4 T cell signatures of latent and active TB, and further define how co-infection with HIV impairs protective T cell immunity to *Mtb* infection. An additional important consideration in evaluating the effect of HIV co-infection on *Mtb*-specific T cell immunity is HIV disease state. While the HIV-infected participants in our cohorts had relatively preserved CD4 T cell counts, it is possible that more substantial differences in inhibitory receptor expression on *Mtb*-specific CD4 T cells would be apparent in HIV-infected individuals with CD4 T cell counts <200 cells/μl and more advanced HIV disease.

In summary, by evaluating concurrent expression of the Ig superfamily inhibitory receptors BTLA, CTLA-4, and PD-1, we determined that these inhibitory receptors are not upregulated on *Mtb*-specific CD4 T cells in peripheral blood of HIV-infected individuals, compared with HIV-uninfected individuals, in the context of either LTBI or pulmonary TB disease. These data suggest that the increased risk of developing active TB disease in HIV-infected individuals is not due solely to upregulation of inhibitory receptors and subsequent immune exhaustion of *Mtb*-specific CD4 T cells, a mechanism of Ag-specific T cell dysfunction that has been well described in other persistent infections (93, 114). Moreover, we provide evidence that BTLA is markedly downregulated on *Mtb*-specific CD4 T cells in HIV-infected individuals with active TB,

thus highlighting a previously unrecognized role of BTLA expression levels on *Mtb*-specific CD4 T cells as a potential biomarker of active TB disease, particularly in people living with HIV. Together these data provide new insights into the phenotype of *Mtb*-specific CD4 T cells in the setting of co-infection with *Mtb* and HIV and provide rationale for future studies to evaluate the utility of targeting BTLA and HVEM signaling pathways to enhance protective immunity to *Mtb*.

Acknowledgements

We thank many additional members of the South African Tuberculosis Vaccine Initiative (SATVI) team and the Kenya Medical Research Institute (KEMRI)/Centers for Disease Control and Prevention (CDC) team who helped with enrollment and evaluation of participants, and the participants themselves.

Tables

Table I. Characteristics of study participants

Participant Group	*n*	Age, y[a] (IQR)	Sex (% Male)	CD4 Count, Cells/μl[b] (IQR)	HIV Viral Load, Copies RNA/ml plasma[b] (IQR)
LTBI/HIV–	32	32 (20–41)	38	N/A	N/A
LTBI/HIV+	22	35 (28–44)	18	562 (432–595)	11,710 (2,855–30,586)
TB/HIV–	37	31 (22–37)	68	N/A	N/A
TB/HIV+	19	36 (29–42)[c]	42	420 (261–614)	6,350 (256–36,368)[d]

[a] value denotes median age in years
[b] values denote median
[c] $p < 0.05$, compared with TB/HIV–
[d] Viral load not available for 3 TB/HIV+ participants
IQR, interquartile range; N/A, not applicable

Figure Legends

Figure 1. Similar frequencies of *Mtb*-specific T cells producing IFN-γ and TNF-α in HIV-infected and HIV-uninfected individuals with latent and active TB. PBMCs from HIV-uninfected (n=32) and HIV-infected (n=22) individuals with LTBI and HIV-uninfected (n=37) and HIV-infected (n=19) individuals with active TB were incubated for 6 hours in media alone (negative control) or stimulated with Ags (CFP-10/ESAT-6 peptide pools, HCMV pp65 peptide pool, HIV Gag peptide pool, and SEB). Intracellular expression of IFN-γ and TNF-α was measured by flow cytometry. (A) Representative flow cytometry data from an HIV-uninfected individual with LTBI (top row), and an HIV-infected individual with LTBI (bottom row). Plots are shown gated on live CD3+CD4+ lymphocytes. (B) Total frequency of cytokine+ *Mtb*-specific CD4 T cells from HIV-uninfected and HIV-infected individuals with LTBI. (C) Frequencies of the indicated cytokine+ subsets of *Mtb*-specific CD4 T cells from HIV-uninfected and HIV-infected individuals with LTBI. (D) Total frequency of cytokine+ *Mtb*-specific CD4 T cells from HIV-uninfected and HIV-infected individuals with active TB. (E) Frequencies of the indicated cytokine+ subsets of *Mtb*-specific CD4 T cells in HIV-uninfected and HIV-infected individuals with active TB. Horizontal lines represent the median. Data are shown after subtraction of background cytokine production in the unstimulated negative control condition. Differences in the frequencies of each cytokine+ T cell population between HIV-uninfected and HIV-infected individuals were assessed using a Mann Whitney U test.

Figure 2. Active TB disease is associated with reduced inhibitory receptor co-expression on total CD4 T cells. PBMC from HIV-uninfected and HIV-infected individuals with LTBI (n=32 and n=22, respectively) and active TB (n=37 and n=19, respectively) were analyzed by flow cytometry for expression of the inhibitory receptors BTLA, CTLA-4, and PD-1 on total CD4 T cells. (A, B) The frequencies of total CD4 T cells expressing each combination of BTLA, CTLA-4 and PD-1 are shown for individuals with LTBI (A) and active TB (B). (C) Summary data representing the proportion of total CD4 T cells expressing three, two, one, or no inhibitory receptors from HIV-uninfected and HIV-infected individuals with LTBI and active TB. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the proportion of CD4 T cells expressing each inhibitory receptor population between HIV-uninfected and HIV-infected individuals (A, B) were assessed using a Mann-Whitney *U* test. Differences among groups in the number of inhibitory receptors expressed by CD4 T cells in panel C were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *p< 0.05; **p<0.01; ***p< 0.001; ****p<0.0001.

Figure 3. Modulation of *Mtb*-specific CD4 T cell inhibitory receptor expression profiles in HIV-infected individuals with active TB. PBMCs from HIV-uninfected and HIV-infected individuals were stimulated with CFP-10 and ESAT-6 peptide pools and evaluated for expression of IFN-γ and TNF-α by flow cytometry, as described in Figure 1. *Mtb*-specific CD4 T cells meeting the criteria for a positive response (see Materials and Methods) were evaluated for expression of BTLA, CTLA-4,

and PD-1. (A, D) Representative flow cytometry data of BTLA, CTLA-4, and PD-1 expression are shown from an HIV-uninfected and HIV-infected individual with LTBI (A) and active TB (D). Flow plots are shown gated on live CD3+CD4+ lymphocytes. Gray dots represent the total cytokine-negative CD4 T cell population; blue and red dots represent IFN-γ and/or TNF-α producing CD4 T cells from individuals with LTBI and TB, respectively. (B, E) Composite data from individuals with LTBI (B) and TB (E) indicating the proportion of CFP-10/ESAT-6-specific CD4 T cells expressing three, two, one or no inhibitory receptors. (C, F) Composite data from individuals with LTBI (C) and TB (F) indicating the proportion of CFP-10/ESAT-6-specific CD4 T cells expressing the indicated combinations of BTLA, PD-1 and CTLA-4 (LTBI/HIV–, n=27; LTBI/HIV+, n=17; TB/HIV–, n=35; TB/HIV+, n=16). Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the proportions of *Mtb*-specific CD4 T cells expressing the indicated inhibitory receptors between HIV-uninfected and HIV-infected individuals were assessed using a Mann-Whitney U test. *** $p < 0.001$, after applying the Bonferroni correction for multiple comparisons.

Figure 4. HIV infection is associated with downregulation of BTLA expression on total CD4 T cells and *Mtb*-specific CD4 T cells in active TB. . PBMC from HIV-uninfected and HIV-infected individuals with LTBI and TB were evaluated for BTLA expression by flow cytometry. (A) Flow plots are shown gated on live $CD3^+CD4^+$ lymphocytes. (B) Summary of BTLA expression on total unstimulated CD4 T cells from HIV-uninfected and HIV-infected individuals with LTBI and active TB. (C) Representative flow data of PBMC following stimulation with CFP-10/ESAT-6 peptide pools, as described in Figure 1. Cells were gated on live $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells, then

evaluated for BTLA expression. (D) Summary of BTLA expression on CFP-10/ESAT-6-specific CD4 T cells from HIV-uninfected and HIV-infected individuals with LTBI and active TB. Boxes in panels B and D represent the median and interquartile ranges; whiskers represent the 5^{th} and 95^{th} percentiles. Differences in BTLA expression among groups in panels B and D were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 5. Differential inhibitory receptor expression profiles on *Mtb*-specific CD4 T cells, compared with HCMV- and HIV-specific CD4 T cells within the same individuals. PBMC were stimulated with CFP-10 and ESAT-6 peptide pools as described in Fig 1, as well as HCMV pp65 peptide pool and HIV Gag peptide pool. Ag-specific CD4 T cells meeting the criteria for a positive response (see Materials and Methods) were evaluated for expression of BTLA, CTLA-4, and PD-1. (A) Representative intracellular cytokine staining flow cytometry data from an HIV-infected individual with LTBI. Plots are shown gated on live $CD3^+CD4^+$ lymphocytes. Gray dots represent cytokine-negative CD4 T cells; blue dots represent IFN-γ^+ and/or TNF-α^+ CD4 T cells. (B, C) Summary data of the proportion of Ag-specific CD4 T cells expressing the indicated subsets of inhibitory receptors (B) and the proportion of Ag-specific CD4 T cells expressing three, two, one, or no inhibitory receptors (C) in individuals with LTBI (top row, HIV-uninfected LTBI; bottom row, HIV-infected LTBI). (D, E) Summary data of the proportion of Ag-specific CD4 T cells expressing the indicated subsets of inhibitory receptors (D) and the proportion of Ag-specific CD4 T cells expressing three,

two, one, or no inhibitory receptors (E) in individuals with active TB (top row, HIV-uninfected TB; bottom row, HIV-infected TB). Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences between *Mtb*- and HCMV-specific CD4 T cells in HIV-uninfected individuals (panels B and D) were assessed using a Mann-Whitney *U* test. Differences between *Mtb*-specific and HCMV- and HIV-specific CD4 T cells in HIV-infected individuals (panels B and D) were assessed using a Kruskal-Wallis test, with *p*-values adjusted for multiple comparisons using Dunn's post-test. *p< 0.05; **p< 0.01; ***p< 0.001; ****p<0.0001.

Figures

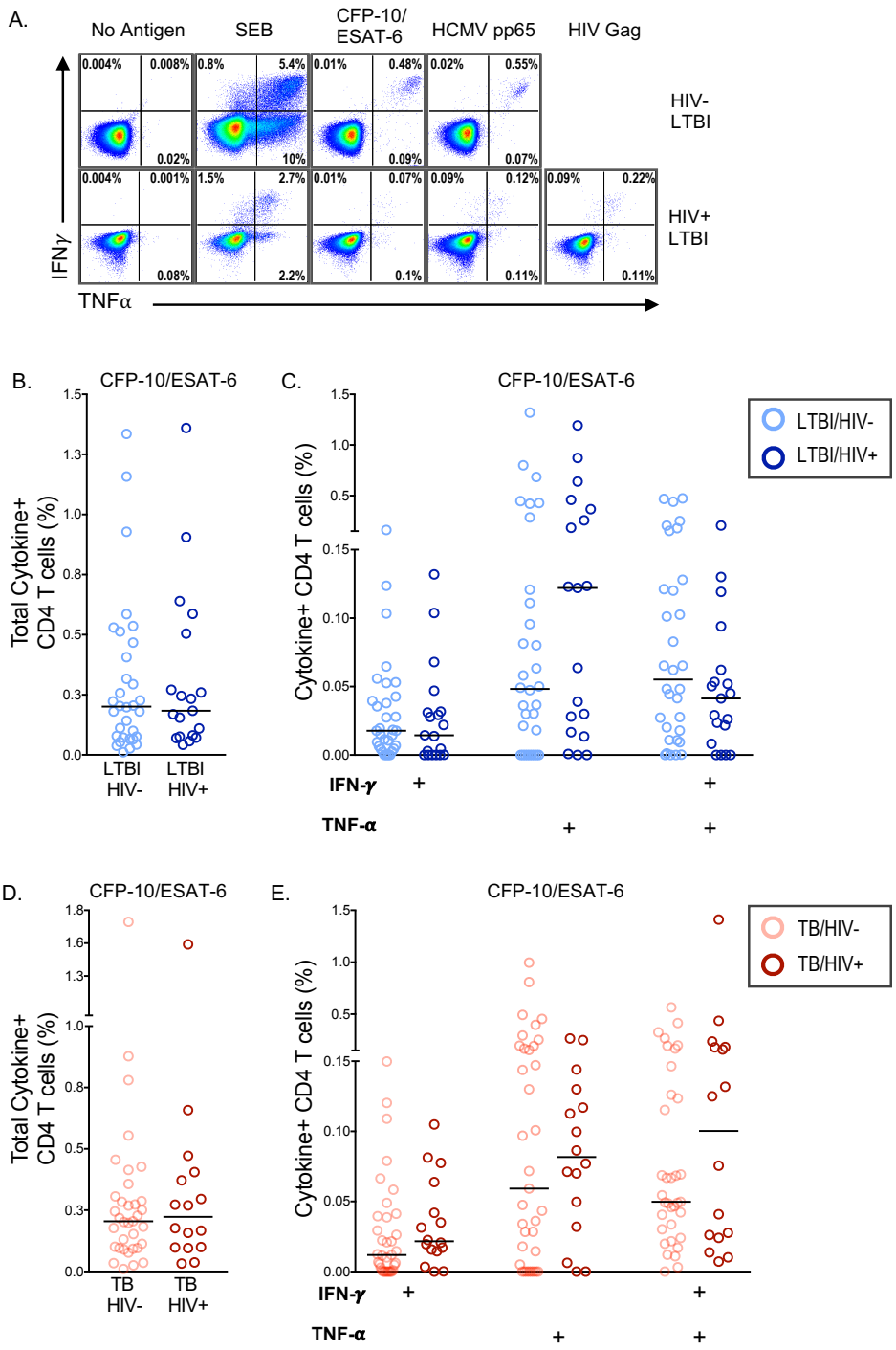


Figure 1. Similar frequencies of *Mtb*-specific T cells producing IFN-γ and TNF-α in HIV-infected and HIV-uninfected individuals with latent and active TB.

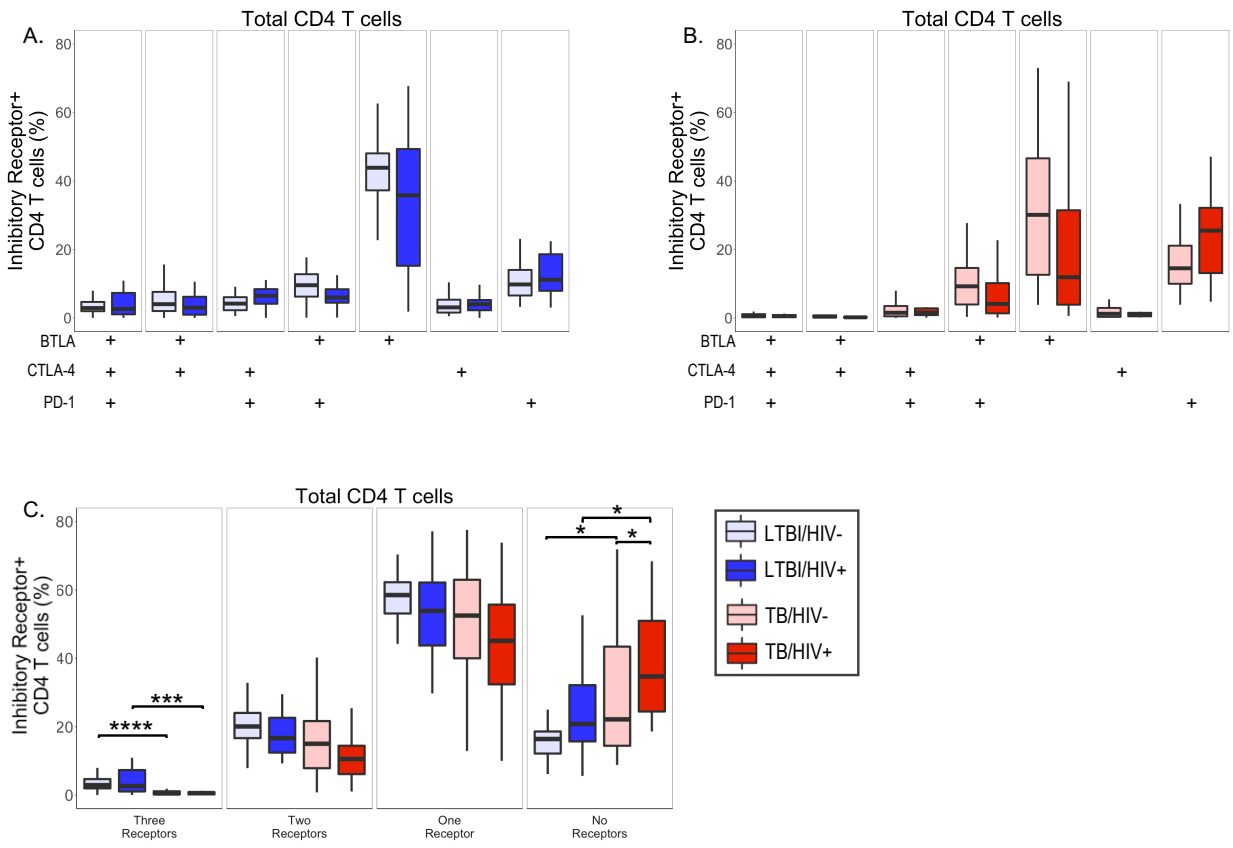


Figure 2. Active TB disease is associated with reduced inhibitory receptor co-expression on total CD4 T cells.

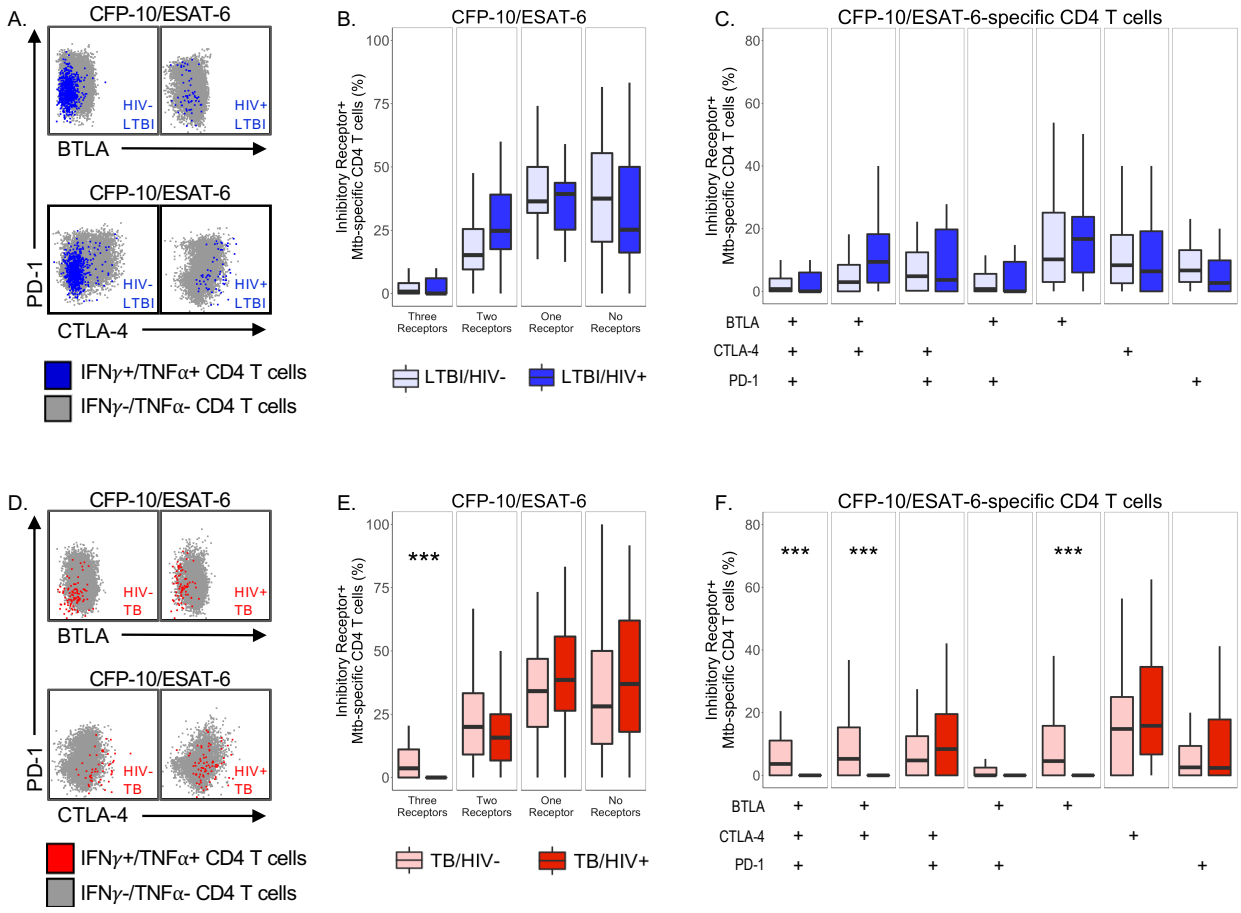


Figure 3. Modulation of *Mtb*-specific CD4 T cell inhibitory receptor expression profiles in HIV-infected individuals with active TB.

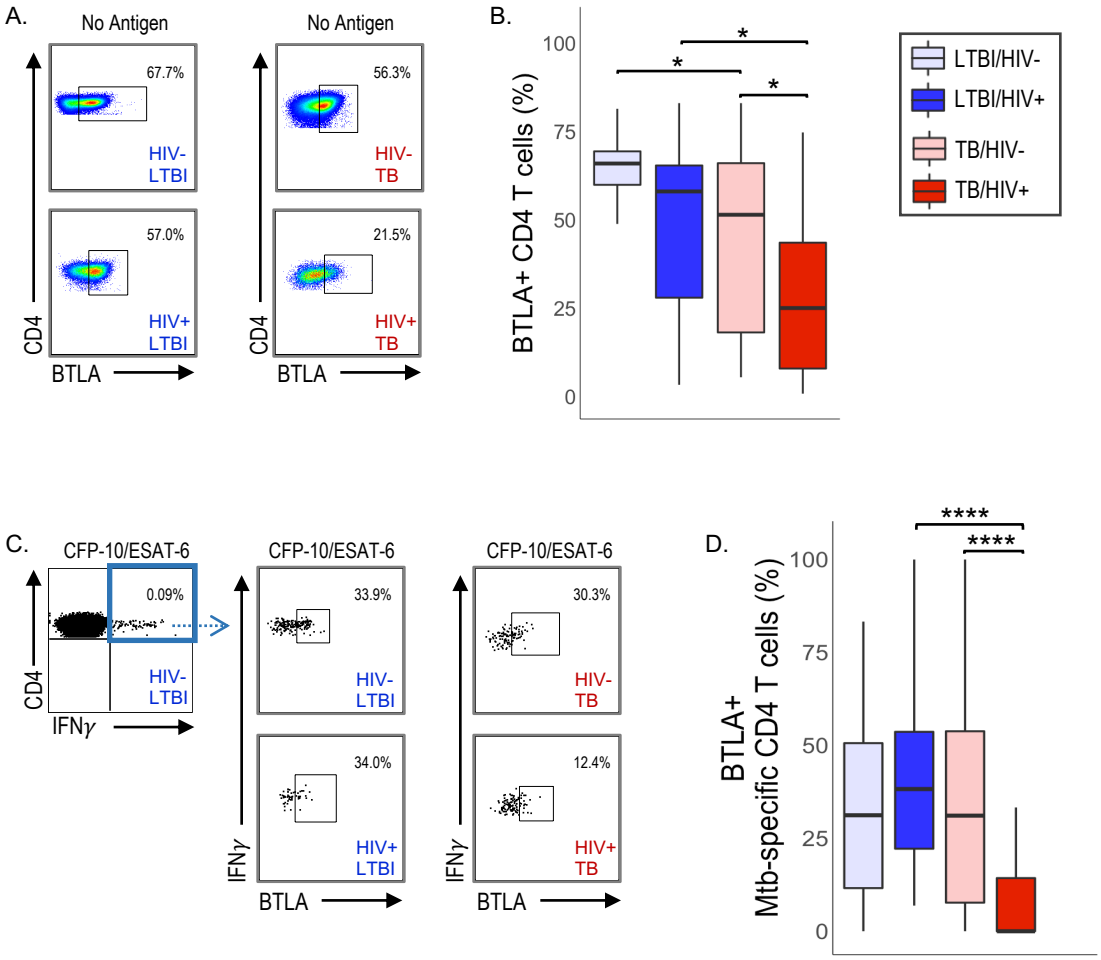


Figure 4. HIV infection is associated with downregulation of BTLA expression on total CD4 T cells and *Mtb*-specific CD4 T cells in active TB.

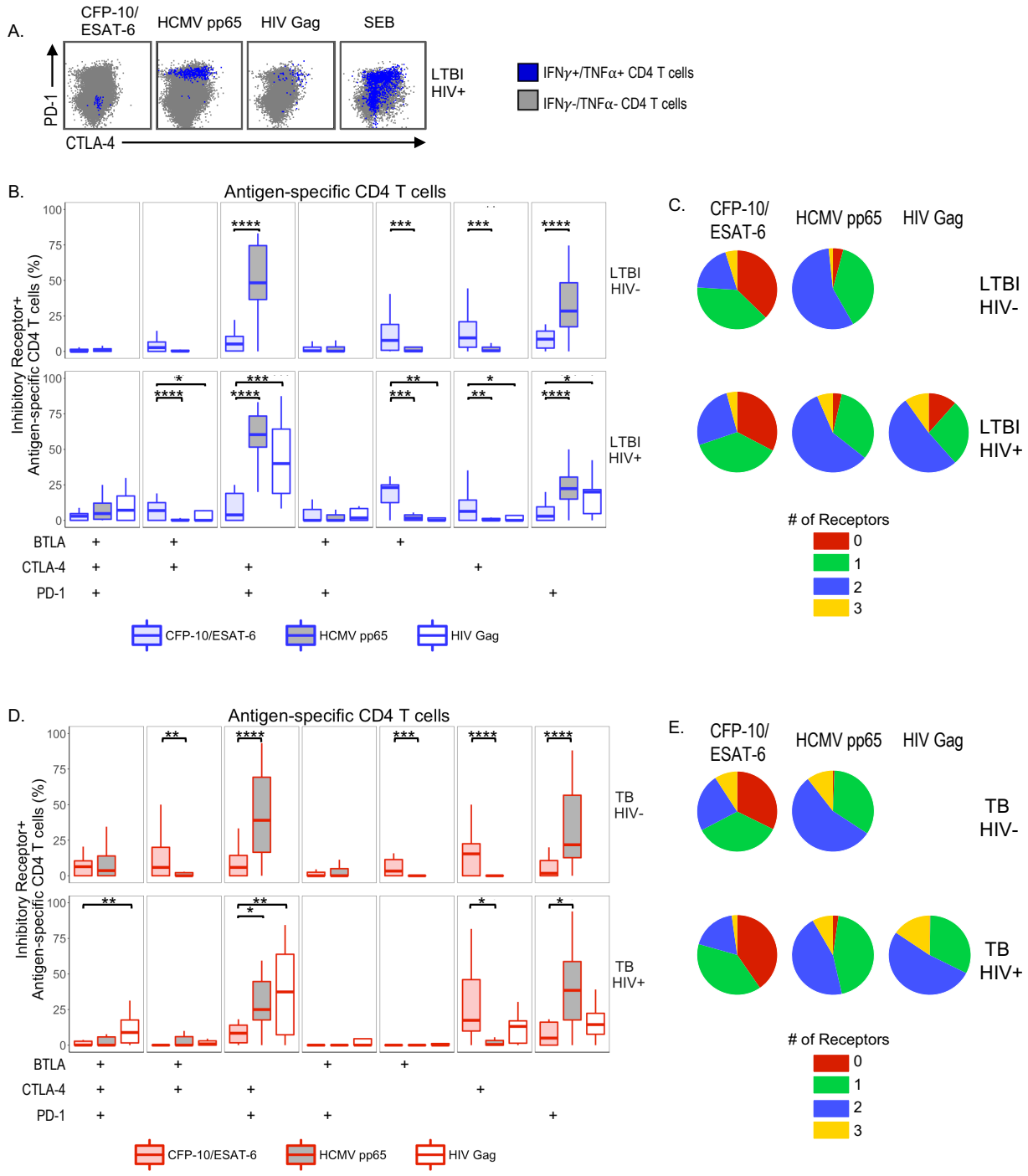


Figure 5. Differential inhibitory receptor expression profiles on *Mtb*-specific CD4 T cells, compared with HCMV- and HIV-specific CD4 T cells within the same individuals.

Supplementary Figure Legends

Figure S1. Gating strategy for flow cytometry analysis. In this sample gating, 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 NIR^{lo}, $CD3^{+}$). CD4 and CD8 surface expression is then determined from this gated population, followed by inhibitory receptor (BTLA, CTLA-4, and PD-1) expression and intracellular cytokine production (IFN-γ and TNF-α). Cytokine production is shown by CD4 T cells following stimulation with SEB.

Figure S2. Similar frequencies of SEB and HCMV-specific T cells producing IFN-γ and TNF-α in HIV-infected and HIV-uninfected individuals with latent and active TB. PBMCs from HIV-uninfected and HIV-infected individuals with LTBI and HIV-uninfected and HIV-infected individuals with active TB were incubated for 6 hours with SEB (positive control) or HCMV pp65 peptide pools. Intracellular expression of IFN-γ and TNF-α was measured by flow cytometry. Frequencies of the indicated cytokine+ subsets of (A) SEB-stimulated and (B) HCMV-specific CD4 T cells from HIV-uninfected and HIV-infected individuals with LTBI. Frequencies of the indicated cytokine+ subsets of (C) SEB-stimulated and (D) HCMV-specific CD4 T cells from HIV-uninfected and HIV-infected individuals with active TB disease. Horizontal lines represent the median. Data are shown after subtraction of background cytokine production in the unstimulated negative control condition. Differences in the frequencies of each cytokine+ T cell population between HIV-uninfected and HIV-

infected individuals were assessed using a Mann Whitney *U* test; no significant differences were found between HIV-uninfected and HIV-infected groups.

Figure S3. CTLA-4 and PD-1 expression on *Mtb*-specific CD4 T cells in latent and active TB. PBMCs from HIV-uninfected and HIV-infected individuals with LTBI and TB were either left unstimulated or stimulated with CFP-10 and ESAT-6 peptide pools as described in Figure 1. (A) Representative flow cytometry data from an HIV-uninfected individual with LTBI. Plots are shown gated on live $CD3^+CD4^+$ T cells. (B, C) Composite data of the percentage of CTLA-4 (B) and PD-1 (C) expression on total unstimulated CD4 T cells and CFP-10/ESAT-6-specific CD4 T cells from individuals with LTBI. (D, E) Composite data of the percentage of CTLA-4 (D) and PD-1 (E) expression on total unstimulated CD4 T cells and CFP-10/ESAT-6-specific CD4 T cells from individuals with active TB. Boxes represent the median and interquartile ranges; whiskers represent the 5^{th} and 95^{th} percentiles. Differences in the percentages between HIV-uninfected and HIV-infected individuals were assessed using a Mann-Whitney *U* test.

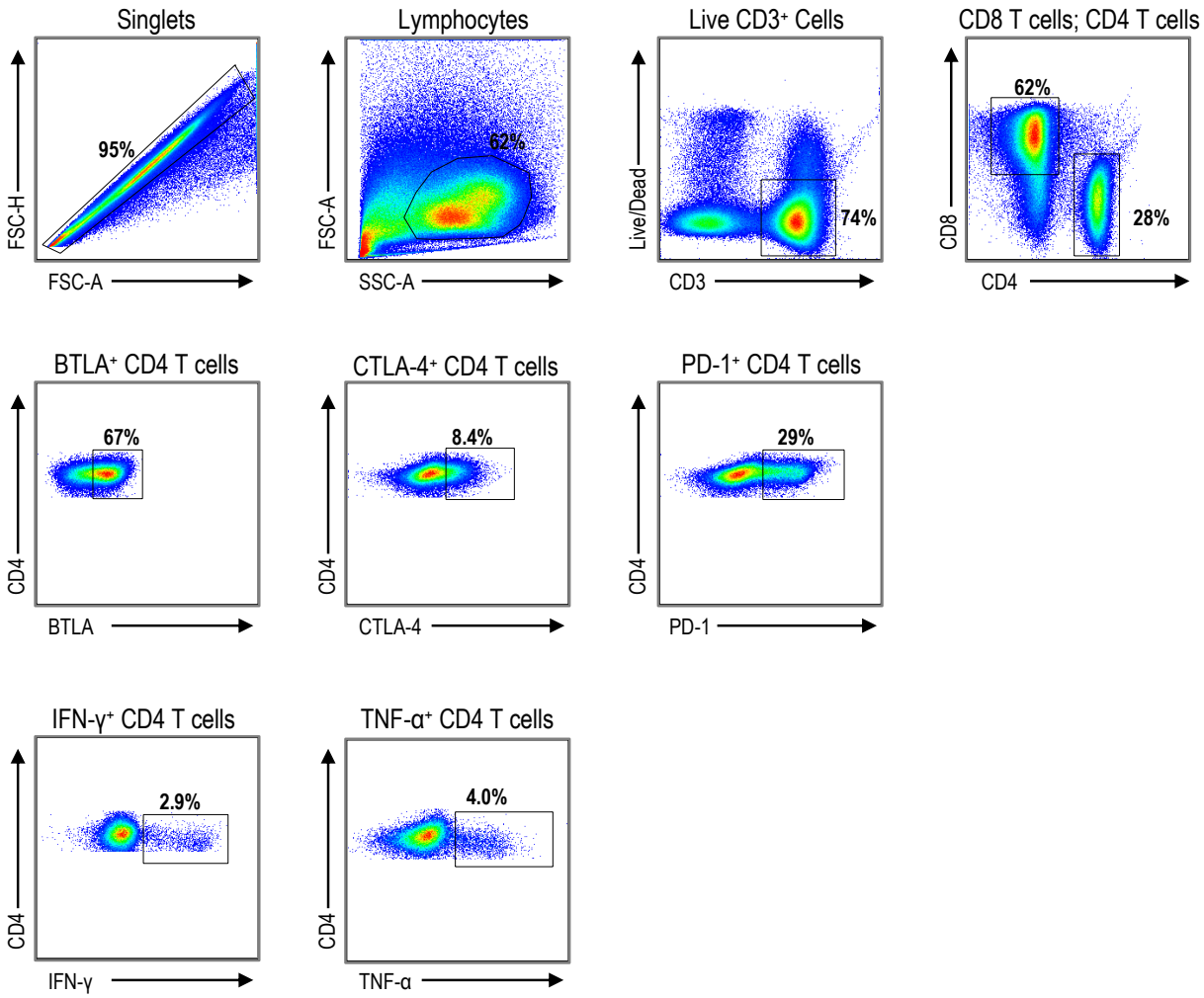
Figure S4. BTLA expression does not correlate with HIV viral load in HIV-infected individuals with LTBI and active TB. PBMCs from HIV-infected individuals with LTBI and TB were either left unstimulated or stimulated with CFP-10 and ESAT-6 peptide pools as described in Figure 1. (A, B) Correlation between HIV viral load and BTLA expression on bulk CD4 T cells (A) and *Mtb*-specific CD4 T cells (B) in HIV-infected individuals with LTBI. (C, D) Correlation between HIV viral load and BTLA expression on bulk CD4 T cells (A) and *Mtb*-specific CD4 T cells (B) in HIV-

infected individuals with active TB. Statistical significance was evaluated using the non-parametric Spearman rank test.

Figure S5. BTLA expression does not correlate with absolute CD4 T cell count in HIV-infected individuals with LTBI and active TB. PBMCs from HIV-infected individuals with LTBI and TB were either left unstimulated or stimulated with CFP-10 and ESAT-6 peptide pools as described in Figure 1. (A, B) Correlation between absolute CD4 T cell count and BTLA expression on bulk CD4 T cells (A) and *Mtb*-specific CD4 T cells (B) in HIV-infected individuals with LTBI. (C, D) Correlation between absolute CD4 count and BTLA expression on bulk CD4 T cells (C) and *Mtb*-specific CD4 T cells (D) in HIV-infected individuals with active TB. Statistical significance was evaluated using the non-parametric Spearman rank test.

Figure S6. *Mtb*-specific CD4 T cells express higher levels of BTLA and lower levels of CTLA-4 and PD-1, compared with HCMV- and HIV-specific CD4 T cells. PBMCs were stimulated with peptide pools as described in Figure 1. Expression levels of BTLA, CTLA-4, and PD-1 on Ag-specific CD4 T cells in HIV-uninfected and HIV-infected individuals with LTBI are shown in panels A, B, and C, respectively, and in HIV-uninfected and HIV-infected individuals with active TB in panels D, E, and F, respectively. Boxes represent the median and interquartile ranges; whiskers represent the 5^{th} and 95^{th} percentiles. Differences in the inhibitory receptor expression of HCMV- or HIV-specific CD4 T cells was compared with *Mtb*-specific CD4 T cells using a Mann-Whitney *U* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Supplementary Figures



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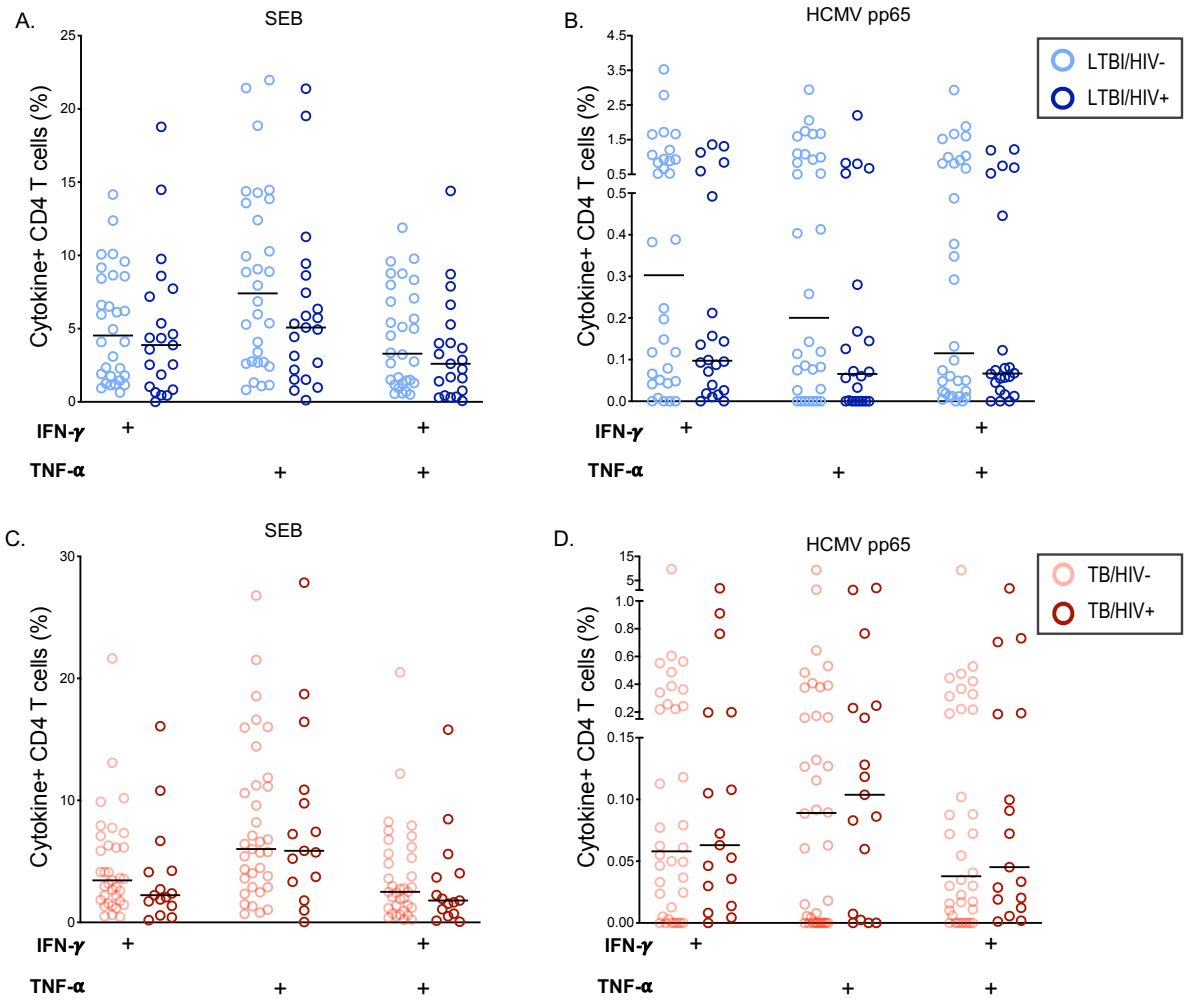

Figure S2. Similar frequencies of SEB and HCMV-specific T cells producing IFN-γ and TNF-α in HIV-infected and HIV-uninfected individuals with latent and active TB.

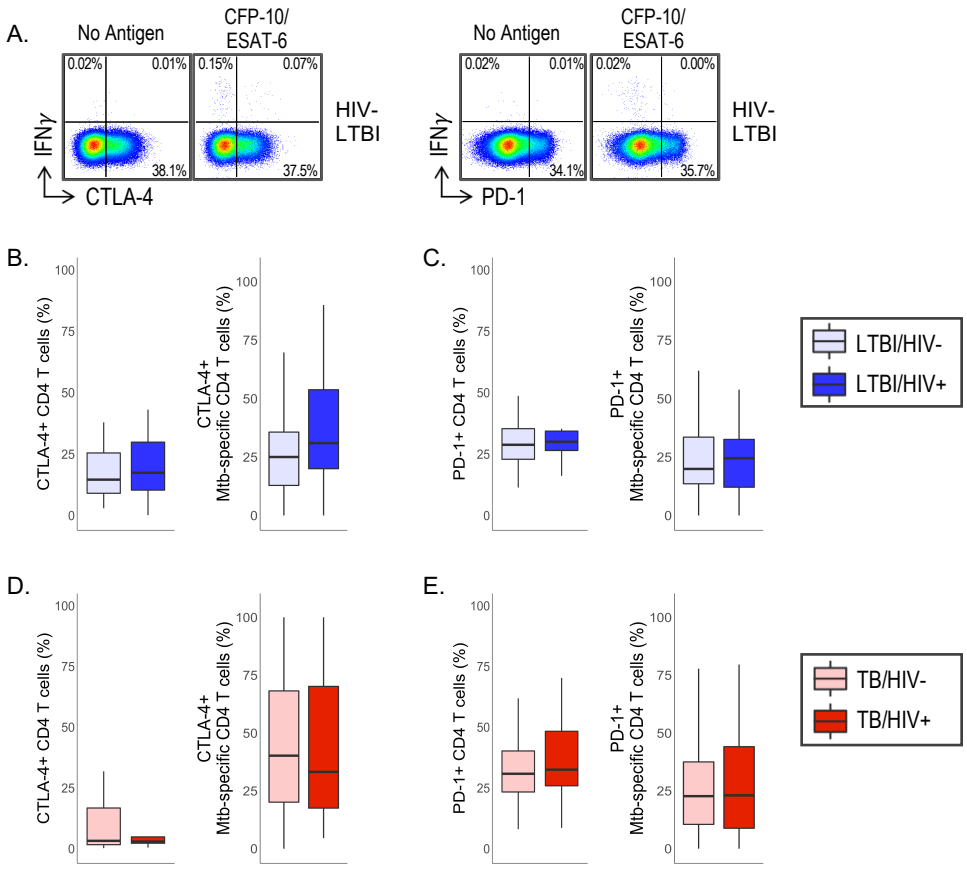

Figure S3. CTLA-4 and PD-1 expression on *Mtb*-specific CD4 T cells in latent and active TB.

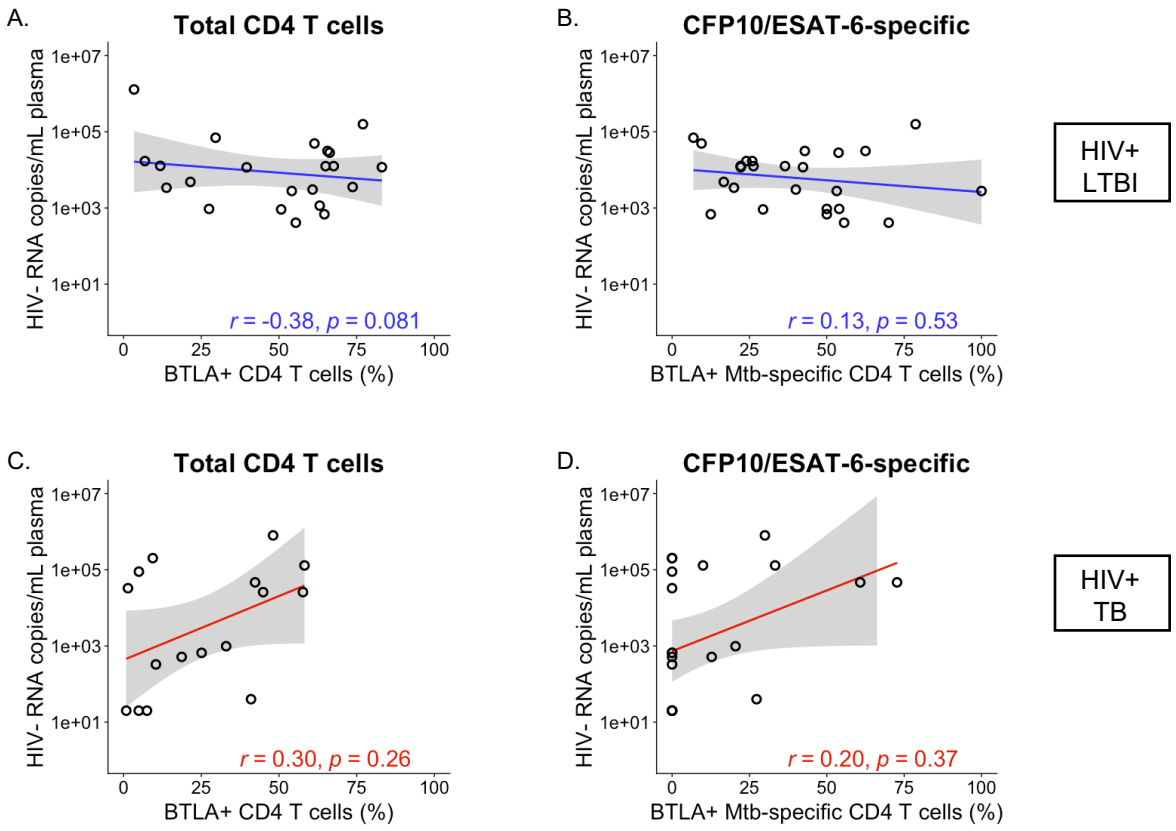

Figure S4. BTLA expression does not correlate with HIV viral load in HIV-infected individuals with LTBI and active TB.

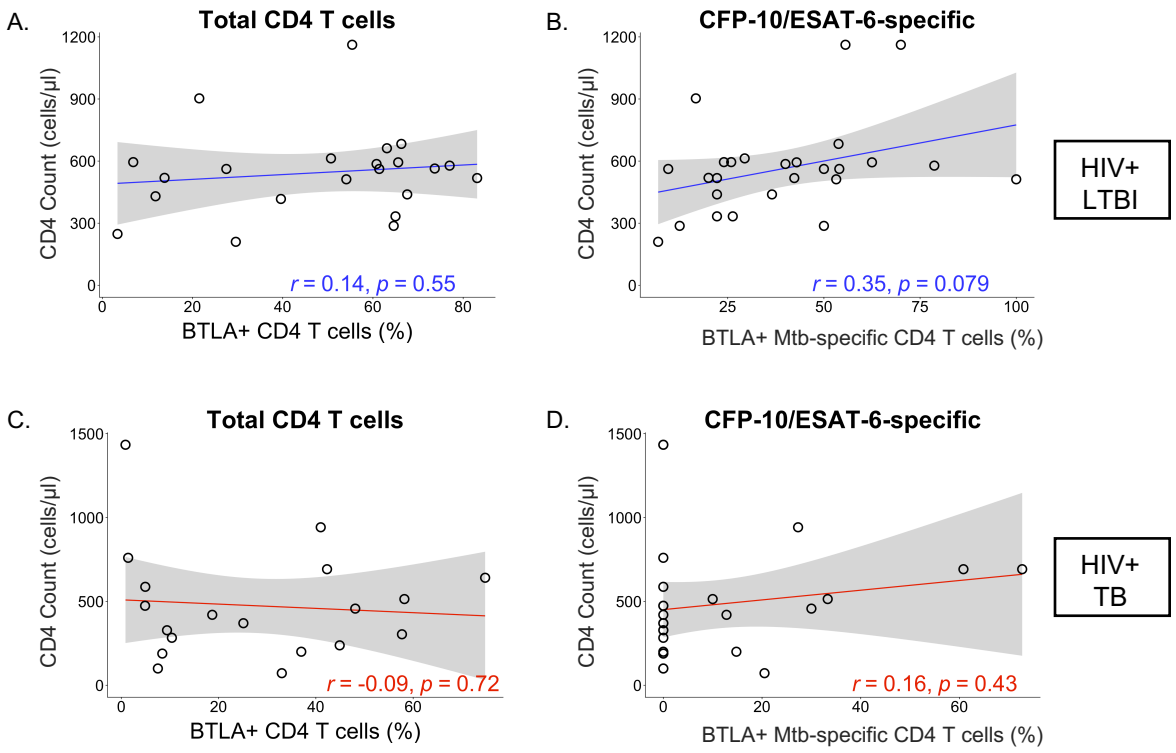

Figure S5. BTLA expression does not correlate with absolute CD4 T cell count in HIV-infected individuals with LTBI and active TB.

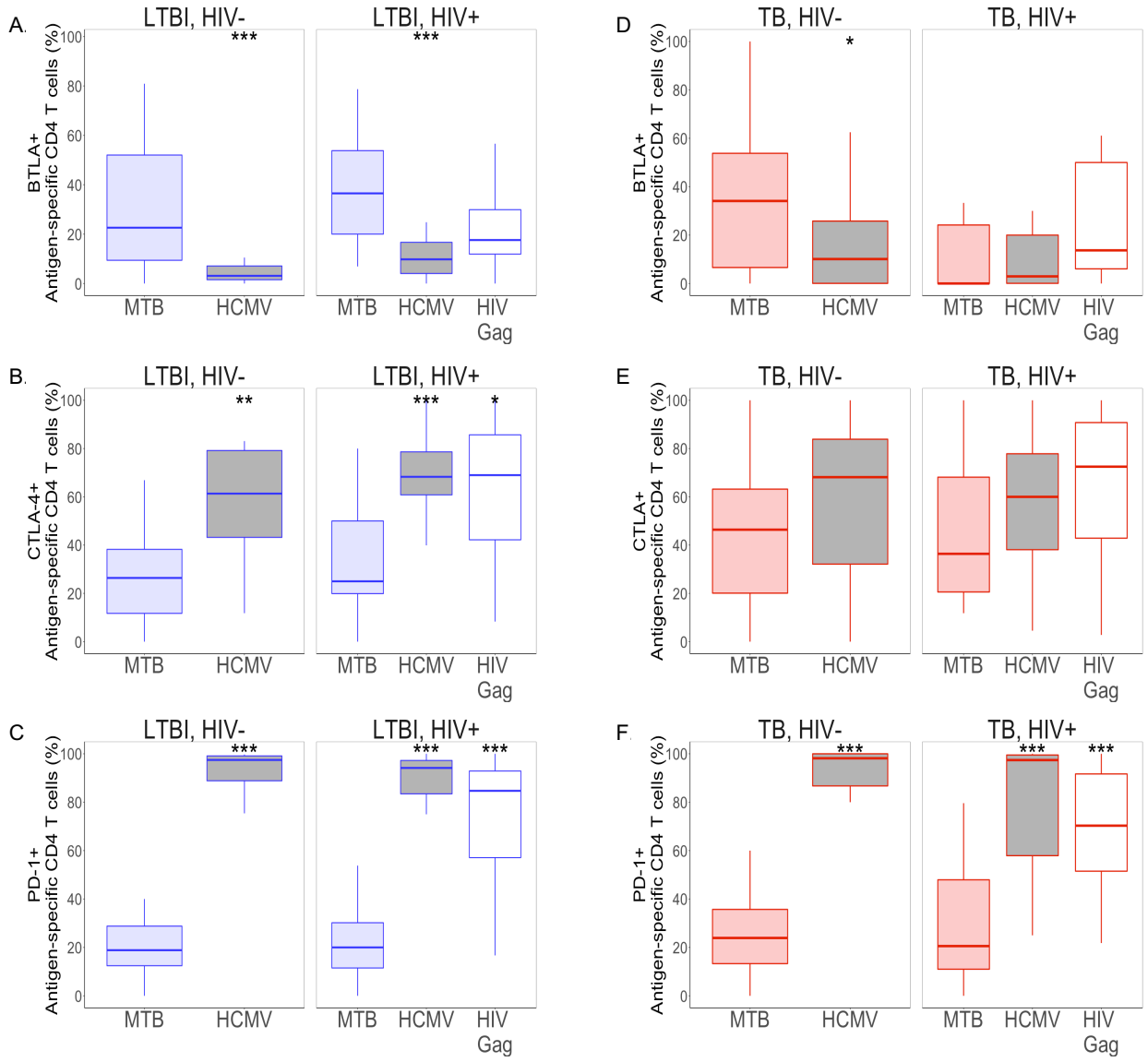

Figure S6. *Mtb*-specific CD4 T cells express higher levels of BTLA and lower levels of CTLA-4 and PD-1, compared with HCMV- and HIV-specific CD4 T cells.

CHAPTER III

Activation-induced marker expression identifies *Mtb*-specific CD4 T cells with altered cytokine production in HIV-infected individuals with latent TB

Abstract

HIV infection is a significant risk factor for reactivation of latent *Mtb* infection (LTBI) and progression to active tuberculosis (TB) disease, yet the mechanisms whereby HIV impairs T cell-mediated immune control of *Mtb* infection have not been fully defined. PBMCs from HIV-uninfected and HIV-infected Kenyan adults with LTBI were stimulated with *Mtb* antigens and evaluated by flow cytometry for detection of antigen-specific CD4 T cells by upregulation of surface activation-induced markers (AIM) CD25, OX40, CD69 and CD40L. *Mtb*-specific cytokine production profiles were evaluated by Luminex. We found that *Mtb*-specific $CD25^+OX40^+$ and $CD69^+CD40L^+$ CD4 T cells were detectable in the AIM assay in HIV-uninfected and HIV-infected individuals with LTBI. Further analysis of cytokine production profiles indicated that HIV-infected individuals have dampened *Mtb*-specific IFN-γ, IL-2, IL-17AF, and IL-22 production capacity and elevated TNF-α production capacity, compared with HIV-uninfected individuals with LTBI. These data suggest that infection with HIV modifies *Mtb*-specific cytokine production profiles towards elevated TNF-α production. Moreover, they suggest that AIM assays enable identification of *Mtb*-specific CD4 T cell responses in a cytokine-independent manner in HIV-uninfected and HIV-infected individuals, thus facilitating studies to further define immune correlates of protection to *Mtb* and elucidate HIV-associated dysregulation of anti-mycobacterial immunity.

Introduction

Globally, one-fourth of the world is burdened by latent *Mycobacterium tuberculosis* infection (LTBI) and therefore at risk for developing active tuberculosis (TB) disease (82). Ten million new cases of TB disease and 1.6 million deaths were reported in 2017 (3). TB incidence has significantly increased with the human immunodeficiency virus (HIV) pandemic, with approximately 900,000 of new reported TB cases in people living with HIV annually (3). HIV infection greatly increases the risk of reactivation of LTBI and progression to active TB disease (3, 83), although the mechanisms whereby HIV impairs successful immune control of *Mtb* infection have not been fully elucidated.

HIV infection results in CD4 T cell depletion and increasing evidence indicates that *Mtb*-specific CD4 T cells are either be preferentially depleted (75-77, 86) and/or have impaired functional capacity in HIV-infected individuals. Decreased frequencies of *Mtb*-specific CD4 T cells producing Th1, Th2, and Th17 cytokines have been described in HIV-infected individuals, compared with those not infected with HIV (75, 84, 86, 87, 130). *Mtb*-specific CD4 T cells in HIV-infected individuals also have a heightened level of activation (75, 131) and profoundly impaired proliferative capacity (75, 132). Although mounting evidence indicates HIV infection impairs *Mtb*-specific T cell immunity, the precise mechanisms whereby HIV dysregulates anti-mycobacterial immunity have not been fully elucidated.

Currently available tools for *Mtb* infection include tuberculin skin test (TST) and the IFN-γ release assays (IGRA), T-SPOT.TB and Quantiferon (QFT) (133), which detect immune sensitization to *Mtb* antigens, rather than the presence of the bacteria itself.

IGRA positivity relies on detection of IFN-γ-producing T cell responses to CPF-10 and ESAT-6 antigens, yet increasing evidence highlights the importance of IFN-γ-independent immune responses contributing to the host immune response to *Mtb* exposure and infection (134). Novel approaches to identify antigen-specific T cells in a cytokine-independent manner have been developed, based on surface expression of activation-induced markers (AIM). AIM assays for co-expression of CD25/OX40 and CD69/CD40L have been described for detection for human CD4 T cells specific for several viral (EBV, CMV, dengue virus, and HIV) and bacterial antigens (Group A *Streptococcus*, pertussis and tetanus) (135-139). AIM assays measure surface expression of activation markers on live cells, without the need for fixation and permeabilization of cells for intracellular cytokine staining, thus facilitating sorting of live antigen-specific CD4 T cells for downstream applications such as transcriptional profiling. $CD25^{+}OX40^{+}$ CD4 T cells to CFP-10 and ESAT-6 peptide pools have been reported in a small study of QFT^{+} adults with LTBI in the U.S., which indicated positive CD25/OX40 AIM assay responses in all individuals with LTBI tested (135). However, AIM assays have not been evaluated for detection of *Mtb*-specific CD4 T cell responses in high TB burden settings; moreover, the effect of HIV infection on detection of *Mtb*-specific CD4 T cell responses have not been evaluated.

To further examine how CD4 T cell responses to *Mtb* are dysregulated in HIV-infected individuals with LTBI, we evaluated the performance of AIM assays for CD25/OX40 and CD69/CD40L in QFT^{-} and QFT^{+} Kenyan adults, with and without HIV infection. Moreover, since the AIM assay measure surface expression of activation markers, without the need for Golgi transport inhibitors, we also evaluated the effect of HIV on *Mtb*-specific cytokine production profiles, including Th1, Th2, Th17 and

regulatory cytokines. We found that the AIM assay identifies *Mtb*-specific CD4 T cell responses in an IFN-γ-independent manner in both HIV-uninfected and HIV-infected individuals with LTBI in a high TB burden setting. Moreover, we determined that HIV infection is characterized by diminished *Mtb*-specific IFN-γ, IL-2, IL-17AF, and IL-22 production, and elevated TNF-α production, compared with HIV-uninfected individuals.

Materials and Methods

Study participants and sample collection: Peripheral blood samples were collected from individuals ≥18 years of age at the Kenya Medical Research Institute (KEMRI) Clinical Research Center in Kisumu, Kenya. Study participants included HIV-uninfected and HIV-infected adults with a normal chest x-ray and no symptoms of active TB disease and no history of diagnosis or treatment for active TB. *Mtb* infection status was evaluated by QuantiFERON®-TB Gold In-Tube (QFT; Qiagen). Individuals with a positive QFT result (TB Antigen-Nil ≥0.35 IU/ml) were defined as having latent *Mtb* infection (LTBI). Individuals with a TB Antigen-Nil response <0.35 IU/ml were defined as QFT-negative (QFT^-). Serologic testing for HIV antibodies was done for all individuals using the Diagnostic Kit for HIV (1+2) Antibody (Colloidal Gold) V2 (*KHB*® Shanghai Kehua Bio-engineering Co., Ltd). Plasma HIV viral load and CD4 T cell counts were measured for HIV-infected individuals. All HIV-infected participants were antiretroviral therapy-naïve at the time of analysis and had absolute CD4 T cell counts >200 cells/μl. All subjects provided written informed consent for participation in the study, which was approved by the Kenya Medical Research Institute Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

PBMC isolation and antigen stimulation: Peripheral blood was collected in sodium heparin BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes (BD Biosciences, San Jose, CA). Within 4 hours of blood collection, PBMC were isolated via density gradient centrifugation and then cryopreserved and stored in LN_2 until use. Cryopreserved PBMC 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 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 4 hours at 37°C and 5% CO_2. After resting, pure functional grade anti-CD40 antibody (0.5 μg/ml; Miltenyi Biotec) was added and cells were stimulated with the following antigens: pooled, overlapping 15-mer peptides corresponding to the sequences of CFP-10 and ESAT-6 (1 μg/ml), and human CMV (HCMV) pp65 (1 μg/ml). *Mtb* H37Rv whole cell lysate (*Mtb* lysate) was obtained from BEI Resources, NIAID, NIH (NR-14822). HCMV pp65 peptide pool was obtained from the National Institutes of Health AIDS Reagent Program, Division of AIDS, NIAID, NIH (100-102). PBMC were stimulated with staphylococcal enterotoxin B (SEB; 1 μg/ml; Toxin Technology, Inc.) as a positive control. PBMC incubated in R10 media with no antigen were used as a negative control. PMBC were incubated at 37°C for 16 hours, after which supernatants were harvested and stored for cytokine quantification (described below) and cells were analyzed by flow cytometry (described below).

Antibodies and activation-induced marker (AIM) expression by flow cytometry: Stimulated cells were washed with PBS and stained for 20 minutes at room temperature with Zombie NIR™ Fixable Viability Dye (BioLegend). Cells were surface stained for 30 minutes at room temperature with anti-CD3 Brilliant Violet 711 (UCHT1; BD Horizon), anti-CD4 Brilliant Violet 570 (RPA-T4; BioLegend), anti-CD8 PerCP-Cy5.5 (SK1; BioLegend), anti-CD25 PE-Cy7 (MA251; BD Pharmingen), anti-OX40 (CD134) PE (ACT35; BD Pharmingen), anti-CD69 PE/Dazzle 594 (FN50; BioLegend), and anti-

CD40L (CD154) Brilliant Violet 421 (24-31; BioLegend). Cells were washed with PBS containing 1% FCS and then resuspended in PBS with 2% paraformaldehyde prior to acquisition on a BD LSRII flow cytometer.

Cytokine quantification: The following cytokines were measured in PBMC supernatants using a ProcartaPlex immunoassay (Invitrogen), according to manufacturer's instructions: IFN-γ, IL-2, IL-4, IL-10, IL-17AF, IL-21, IL-22, and TNFα. Immunoassay plates were read using a Luminex MAGPIX® system with xPONENT® software (Version 4.2) and analyzed using MILLIPLEX® Analyst 5.1 Software. The concentrations of each cytokine were expressed as pg/ml and calculated according to a standard curve.

Flow cytometry and data analysis: Cells were acquired on a BD LSRII flow cytometer with BD FACSDiva software (v8.0) and analyzed using FlowJo software (v9.9.6). Compensation was calculated using single-stained anti-mouse Ig,κ beads (BD Biosciences). Doublet cell populations were excluded by plotting forward scatter area versus forward scatter height. Viable lymphocytes were defined as Zombie NIR-low cells. Combinations of cells expressing AIM markers were determined using Boolean gating in FlowJo.

Data analysis and statistics: Functionality scores of CD4 T cells expressing AIM markers (CD25, OX40, CD69 and CD40L) were determined via the Bayesian mathematical model, COMPASS (Combinatorial Polyfunctionality Analysis of Single Cells) (140). GraphPad Prism (version 8.1.2) and R programming software (104) was

used to perform statistical analyses of frequencies and phenotype of antigen-specific CD4 T cells. Correlogram and principal component analysis (PCA) plots were generated in R. Frequencies of antigen-specific CD4 T cells expressing AIM markers are shown after subtraction of background AIM marker expression in the negative control condition. Cytokine data from antigen-stimulated cells are shown after subtraction of background cytokine production in the negative control condition. The non-parametric Mann-Whitney *U* test was used to compare AIM marker and cytokine production between groups. Correlations were conducted using Spearman's rank-order correlation. *P* values of less than 0.05 were considered to be statistically significant.

Results

Study participants.

Blood samples were collected from 38 HIV-uninfected (n=19 QFT^-, n=19 LTBI) and 37 HIV-infected (n=20 QFT^- and n=17 LTBI) adults enrolled in Kisumu, Kenya (Table 1). There were no significant differences in the absolute CD4 T cell counts or HIV plasma viral loads between HIV-infected individuals in the QFT^- and LTBI groups. The median QFT response (TB Antigen) was lower in HIV-infected individuals with LTBI, compared with HIV-uninfected individuals with LTBI (Table 1 and Supplementary Figure 1).

Upregulation of AIM markers identifies *Mtb*-specific CD4 T cells in HIV-infected and HIV-uninfected individuals.

While HIV-infected individuals with LTBI had lower IFN-γ responses to the QFT TB Antigen than HIV-uninfected individuals with LTBI, there was no difference between the two LTBI groups in their IFN-γ responses to PHA in the QFT Mitogen tube (Supplementary Figure 1). These data suggest that HIV-infected individuals either have decreased frequencies and/or diminished IFN-γ production capacity of *Mtb*-specific T cells, compared with HIV-uninfected individuals with LTBI. To evaluate the frequency of *Mtb*-specific CD4 T cells in HIV-infected individuals in a cytokine-independent manner, we next evaluated whether upregulation of AIM markers can be used to identify *Mtb*-specific CD4 T cells in both HIV-uninfected and HIV-infected individuals. PBMCs were stimulated for 16 hours with *Mtb* antigens (CFP-10/ESAT-6 peptide pool

and *Mtb* lysate) and SEB as a positive control, followed by flow cytometric analysis of antigen-induced co-expression of CD25/OX40 or CD69/CD40L (Figure 1A).

Consistent with QFT responses, the frequencies of $CD25^+OX40^+$ and $CD69^+CD40L^+$ CD4 T cells to CFP-10/ESAT-6 peptides were significantly higher in individuals with LTBI, compared with QFT^- individuals (Figure 1B). Moreover, there was a significant positive correlation between QFT responses and the percentage of CFP-10/ESAT-6-induced $CD25^+OX40^+$ and $CD69^+CD40L^+$ CD4 T cells (data not shown). By contrast with QFT responses, the frequencies of CFP-10/ESAT-6-specific CD4 T cells co-expressing AIM markers were not significantly different between HIV-infected and HIV-uninfected individuals with LTBI, thus providing further evidence that the AIM assay facilitates detection of *Mtb*-specific CD4 T cells that may otherwise be missed by evaluation of IFN-γ production alone. Moreover, the frequencies of CD4 T cells expressing AIM markers were similar across the 4 participant groups following stimulation with HCMV pp65 peptide pool (Supplementary Figure 2A), thus providing further evidence that the observed differences in CFP-10/ESAT-6-induced AIM marker expression are reflective of *Mtb* infection status.

To evaluate broader *Mtb*-specific AIM marker expression beyond the immunodominant CFP-10 and ESAT-6 antigens, we measured AIM marker expression following stimulation with *Mtb* lysate. Consistent with the greater number of potential T cell epitopes in the lysate, frequencies of CD4 T cells expressing AIM markers to *Mtb* lysate stimulation were higher than to the CFP-10/ESAT-6 peptide pool (Figure 1B, C). However, by contrast with CFP-10/ESAT-6 peptide pool, the frequency of *Mtb* lysate-induced $CD25^+OX40^+$ CD4 T cells was similar across the 4 study groups (Figure 1C), whereas $CD69^+CD40L^+$ CD4 T cells were significantly higher in LTBI, compared with

QFT^- individuals. These data indicate that, when stimulating with complex antigens such as *Mtb* lysate, evaluation of CD69/CD40L co-expression may detect a greater frequency of *Mtb*-specific CD4 T cell responses than CD25/OX40 in the AIM assay. In all 4 participant groups, stimulation with SEB induced robust expression of each of the AIM markers (Figure 1D), thus suggesting that CD4 T cell AIM marker expression is not inherently impaired in the setting of either LTBI and/or HIV infection.

We also determined whether simultaneous expression of all 4 AIM markers on antigen-specific CD4 T cells would facilitate greater differentiation of the 4 participant groups than analysis of two AIM markers. For each antigen stimulation tested, frequencies of CD4 T cells simultaneously expressing all 4 AIM markers were lower than the frequencies of either $CD25^+OX40^+$ or $CD69^+CD40L^+$ CD4 T cells, and did not enable greater discrimination of the 4 study groups than we observed by analysis of either $CD25^+OX40^+$ or $CD69^+CD40L^+$ CD4 T cells (Figure 1B-D).

To more comprehensively evaluate the impact of HIV co-infection on detection of *Mtb*-specific CD4 T cell responses in the AIM assay, we utilized COMPASS, a Bayesian hierarchical mixture model (140), to incorporate all possible combinations of antigen-induced CD25, OX40, CD69 and CD40L expression and generate an overall functionality score for each participant that summarizes CD4 T cell AIM marker expression profiles (Figure 2). While *Mtb* lysate and SEB induced higher CD4 T cell AIM marker functionality scores than CFP-10/ESAT-6 peptide pool and HCMV pp65 peptide pool (Supplementary Figure 2B), there were no significant differences overall in CD4 T cell AIM marker functionality scores following antigen stimulation of PBMCs from HIV-uninfected versus HIV-infected individuals with LTBI (Figure 2A – C and Supplementary Figure 2B). Lastly, we conducted a principal component analysis (PCA)

including each subset of antigen-specific CD4 T cells expressing all possible combinations of CD25, OX40, CD69 and CD40L. By PCA, HIV-uninfected and HIV-infected individuals with LTBI could not be clearly differentiated by CD4 T cell AIM marker expression, regardless of antigen stimulation (Figure 2A – C and Supplementary Figure 2B). Taken together, these data indicate that AIM assays for CD25/OX40 and CD69/CD40L co-expression detect *Mtb*-specific CD4 T cells in a cytokine-independent manner, and that co-expression of AIM markers is not significantly compromised in our HIV-infected cohorts.

Decreased frequencies of Th1 and Th17 cytokines and increased frequencies of TNF-α produced by *Mtb*-specific CD4 T cells in HIV-infected individuals.

Having determined that *Mtb*-specific CD4 T cells in HIV-infected and uninfected individuals are detectable by the AIM assay, we next utilized the PBMC supernatants from the AIM assay to further probe the effect of HIV infection on *Mtb*-specific cytokine production profiles. Using a Luminex assay, we measured production of Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4), Th17 (IL-17AF, IL-21, IL-22) and regulatory (IL-10) cytokines in AIM assay supernatants from HIV-uninfected and HIV-infected QFT^{-} and LTBI individuals. IL-4 and IL-21 were detectable in each participant group following stimulation with SEB, although there were no differences in SEB-stimulated IL-4 or IL-21 production capacity according to *Mtb* or HIV infection status (data not shown). Moreover, these two cytokines were not detectable above background in any participant group following stimulation with *Mtb* antigens (data not shown) and were therefore not included in our downstream analyses.

Since definition of our QFT$^-$ and LTBI groups was based on CFP-10/ESAT-6-specific IFN-γ production in the QFT assay, we first evaluated whether there was evidence of CFP-10/ESAT-6-specific cytokine production other than IFN-γ in QFT$^-$ individuals. We did not find evidence of robust cytokine production to any of the 8 cytokines measured following stimulation with CFP-10/ESAT-6 peptide pool in QFT$^-$ individuals (Supplementary Figure 3A and data not shown). As anticipated in individuals with LTBI, IFN-γ was detected at the highest concentrations following stimulation with CFP-10/ESAT-6 peptide pool, compared with the other cytokines measured (Figure 3A). However, CFP-10/ESAT-6-specific IL-2 production was significantly lower in HIV-infected individuals with LTBI, compared with HIV-uninfected individuals with LTBI (Figure 3A). By contrast, there was no evidence of diminished IL-2 production capacity to HCMV pp65 peptide pool stimulation in HIV-infected individuals (Supplementary Figure 3D).

When stimulated with a broader panel of antigens in *Mtb* lysate, PBMCs from HIV-infected individuals with LTBI also exhibited significantly lower production of IFN-γ, IL-17AF, and IL-22, compared with HIV-uninfected individuals with LTBI (Figure 3B). However, IFN-γ, IL-17AF, and IL-22 production following stimulation with SEB and HCMV pp65 peptide pool was similar between HIV-uninfected and HIV-infected individuals (Figure 3C and Supplementary Figure 3D), thus indicating production of these cytokines is not inherently impaired in HIV-infected individuals with LTBI, but may be diminished specifically in the immune response to *Mtb*. By contrast, TNF-α production to *Mtb* lysate was significantly increased in HIV-infected individuals, compared with HIV-uninfected individuals with LTBI (Figure 3B). The finding of dampened IFN-γ and IL-22 production and increased TNF-α production to *Mtb* lysate

in HIV-infected individuals with LTBI was also observed in HIV-infected QFT^- individuals, compared with HIV-uninfected QFT^- individuals (Supplementary Figure 3B). Furthermore, TNF-α production to SEB was also significantly higher in HIV-infected individuals, compared with HIV-uninfected individuals (Figure 3C), thus suggesting a propensity for elevated pro-inflammatory TNF-α production in HIV-infected individuals. We found no evidence for differential IL-10 production capacity following stimulation with any antigens in HIV-infected and HIV-uninfected individuals with LTBI (Figure 3A-C). Taken together, these data indicate that HIV infection modifies *Mtb*-specific cytokine production profiles towards enhanced TNF-α production and dampened IFN-γ, IL-2, IL-17AF, and IL-22 production.

HIV infection alters the relationship between *Mtb*-specific CD4 T cell AIM marker expression and cytokine production in individuals with LTBI

Using the AIM assay, we measured both antigen-induced co-expression of CD25/OX40 and CD69/CD40L on CD4 T cells and cytokine secretion in supernatants. To further evaluate how HIV infection may influence the relationship between antigen-specific CD4 T cell AIM marker expression and cytokine production in individuals with LTBI, we generated a correlation matrix of CD4 T cell AIM marker expression and cytokine levels for each antigen (Figure 4). As expected, there was a significant positive correlation between the frequency of $CD25^+OX40^+$ CD4 T cells and $CD69^+CD40L^+$ T cells for each antigen in HIV-infected and HIV-uninfected individuals with LTBI (Figure 4A-C).

In both HIV-uninfected and HIV-infected individuals with LTBI, the frequency of CFP-10/ESAT-6-induced $CD69^+CD40L^+$ CD4 T cells correlated more strongly with cytokine production than $CD25^+OX40^+$ CD4 T cells (Figure 4A). Moreover, there was a

significant positive correlation between the frequency of $CD69^+CD40L^+$ CD4 T cells and production of IFN-γ, TNF-γ, and IL-2, in both groups. Interestingly, while IL-22 production also correlated strongly with AIM markers in HIV-uninfected individuals, there was no correlation with IL-22 production and AIM markers in HIV-infected individuals with LTBI (Figure 4A).

While stimulation of PBMCs with CFP-10/ESAT-6 peptides has been shown to stimulate predominately CD4 T cell responses in individuals with LTBI (99, 141), stimulation with *Mtb* lysate will activate a broader range of lymphocytes in PBMCs, including innate immune cells and non-classical T cells. Despite detection of high levels of TNF-α and IL-10 in the *Mtb* lysate-stimulated AIM assay supernatants, there was no significant correlation between CD4 T cell AIM marker expression and either TNF-α and IL-10 production (Figure 4B), indicating these cytokines may be produced largely by cells other than CD4 T cells. CD4 T cell AIM marker expression to *Mtb* lysate correlated positively with IL-17AF and IL-22 in both HIV-uninfected and HIV-infected individuals. However, there was also a significant positive correlation between CD4 T cell AIM marker expression and IFN-γ and IL-2 production in HIV-infected individuals, which was not observed in HIV-uninfected individuals (Figure 4B).

Although stimulation of PBMCs with SEB induced robust AIM marker expression on CD4 T cells (Figure 1D) and cytokine production (Figure 3C), there were surprisingly little relationship between the frequency of AIM^+ CD4 T cells and cytokine production levels in SEB-stimulated cells from either HIV-uninfected or HIV-infected individuals (Figure 4C). A positive correlation between the frequency of $CD25^+OX40^+$ CD4 T cells and IL-10 production was observed in HIV-uninfected individuals, but not HIV-infected

individuals. There were otherwise no significant correlations between CD4 T cell AIM marker expression and cytokine production in either HIV-infected or HIV-uninfected individuals with LTBI (Figure 4C). Taken together, these data provide further evidence that HIV infection perturbs the relationship between *Mtb*-specific CD4 T cell AIM marker expression and cytokine production profiles in individuals with LTBI.

HIV infection modifies *Mtb*-specific cytokine production profiles in individuals with LTBI

We next used correlation matrix and PCA approaches to further dissect the impact of HIV infection on *Mtb*-specific cytokine production profiles in individuals with LTBI. CFP-10/ESAT-6-specific IL-2 production was significantly reduced in HIV-infected individuals (Figure 3A). Correlation matrices revealed that there was a significant positive correlation between IL-2 and IL-22 production in HIV-uninfected individuals, which is absent in HIV-infected individuals with LTBI (Figure 4A). PCA of CFP-10/ESAT-6-specific cytokine production indicated that 98% of the variance in cytokine production to this peptide pool could be explained by PC1. IFN-γ contributed the greatest variation in cytokine responses in HIV-uninfected individuals, whereas IL-2 and IFN-γ contributed nearly equivalent levels of variation in cytokine profiles of HIV-infected individuals with LTBI (Figure 5A). These data indicate that loss of IL-2 production capacity to CFP-10 and ESAT-6 is associated with HIV infection and that IL-2 production capacity is a significant contributor to variation in cytokine production profiles of HIV-infected individuals with LTBI.

When evaluating cytokine production profiles to a broader array of antigens in *Mtb* lysate, HIV-infected individuals produced significantly greater levels of TNF-α than HIV-uninfected individuals (Figure 3B). Although there was a significant correlation between TNF-α and IL-10 production to *Mtb* lysate in HIV-uninfected individuals, there was no correlation between TNF-α and IL-10 production in HIV-infected individuals (Figure 4B). Moreover, PCA indicated that over 93% of the variation in cytokine production profiles to *Mtb* lysate could be explained by PC1. Further analysis of PC1 indicated that TNF-α was by far the dominant contributor to variation in cytokine production capacity to *Mtb* lysate in HIV-uninfected and HIV-infected individuals with LTBI (Figure 5B).

Robust cytokine production to SEB was observed in both HIV-uninfected and HIV-infected individuals with LTBI (Figure 3C), and SEB cytokine production profiles did not clearly distinguish HIV-uninfected and HIV-infected groups by PCA (Figure 5C). While over 90% of the variation in *Mtb*-specific cytokine production profiles could be explained by PC1, only 58% of the variation in cytokine production to SEB could be explained by PC1. IL-17AF contributed the greatest variation in SEB cytokine profiles in HIV-uninfected individuals in PC1, whereas IFN-γ contributed the greatest level of variation in HIV-infected individuals.

Discussion

Although *Mtb* infection is commonly detected using IGRAs, there is mounting evidence that T cell responses to *Mtb* are highly heterogenous and include important roles for IFN-γ-independent T cells (134, 142, 143). We used a cytokine-independent AIM assay to identify *Mtb*-specific CD4 T cell responses in both HIV-uninfected and HIV-infected individuals with LTBI in a high TB burden setting. Moreover, we determined that HIV infection is characterized by diminished *Mtb*-specific IFN-γ, IL-2, IL-17AF, and IL-22 production, and elevated TNF-α production, compared with HIV-uninfected individuals. These data indicate that AIM assays have potential to identify novel populations of *Mtb*-specific CD4 T cells that may otherwise not be detected by IGRAs or traditional intracellular cytokine staining assays, and hold promise for facilitating future single-cell transcriptomic studies aimed at further defining T cell correlates of successful immune control of *Mtb* infection.

While MHC Class II tetramers enable identification of antigen-specific CD4 T cells regardless of their functional capacity, the use of tetramers is limited to detection of T cells for which the specific epitope and HLA restriction have been defined. Conventional methods for detection of antigen-specific CD4 T cells, such as flow cytometry, ELISA and ELISPOT, are inherently biased due to their dependence on cytokine production for detection of antigen-specific CD4 T cells. More recently, live-cell, cytokine-independent methods have been developed to detect Ag-specific CD4 T cells via AIM assays for CD25/OX40 and CD69/CD40L co-expression (135, 137, 144). AIM assays for CFP-10/ESAT-6-induced CD25/OX40 expression have been reported in a small number of HIV-uninfected adults with LTBI in the U.S. (135), and we now

extend these studies to a TB-endemic setting and determined that CD25/OX40 and CD69/CD40L AIM assays can be used to identify CFP-10/ESAT-6-specific CD4 T cells in HIV-uninfected and HIV-infected Kenyan adults with LTBI. While AIM assays with CFP-10/ESAT-6 peptide pool clearly differentiated QFT^- and QFT^+ individuals, AIM assays with *Mtb* lysate enable detection of a broader mycobacteria-specific CD4 T cell response in our cohorts in Kenya, a high TB burden setting where infant BCG vaccination is routine and there is high exposure to non-tuberculous mycobacteria (145). In our cohorts, AIM assays for CD69/OX40L facilitated greater distinction of QFT^- and QFT^+ individuals than CD25/OX40. Although we did not find evidence that HIV infection compromises detection of *Mtb*-specific CD4 T cells in the AIM assay, it is important to bear in mind that the participants in our cohort had relatively high CD4 T cell counts (median >500 cells/μl). It is possible that performance of the AIM assay will be more compromised in individuals with advanced HIV infection and more severe immunosuppression.

An advantage of the AIM assay is the lack of a requirement for Golgi transport inhibitors during the antigen stimulation period, thus enabling analysis of live cells and analysis of soluble molecules secreted by antigen-specific cells. The functional capacity of antigen-specific CD4 and CD8 T cells has been linked with their ability to produce multiple cytokines, with HIV disease progression and viral load associated with loss of polyfunctional T cell responses (146, 147). Using a multi-plex bead array, we analyzed a panel of 8 cytokines secreted in response to antigen stimulation in the AIM assay. We evaluated cytokine responses to CFP-10/ESAT-6 peptide pools in both QFT^- and QFT^+ individuals and did not find evidence of significant levels of cytokine production in QFT^-

individuals, regardless of HIV infection status. Importantly, we found that IL-2 production capacity to CFP-10/ESAT-6 is reduced in HIV-infected individuals with LTBI. These data are consistent with previous reports of decreased *Mtb*-specific IL-2 production capacity in HIV-infected individuals (51, 85, 87). The inverse relationship between *Mtb*-specific IL-2 production and HIV viral load suggests progressive impairment of *Mtb*-specific CD4 T cell immunity in chronic untreated HIV infection, which may be prevented or restored upon initiation of anti-retroviral therapy. Future prospective studies of *Mtb*-specific IL-2 production capacity in HIV-infected individuals with LTBI are warranted to determine if loss of IL-2 production predicts progression to active TB disease.

In addition to CFP-10/ESAT-6-specific CD4 T cell responses, we also stimulated cells with *Mtb* whole cell lysate, thus facilitating a more comprehensive analysis of cytokine production profiles to *Mtb*, beyond which is detectable to two immunodominant antigens. By stimulating with *Mtb* lysate, we identified additional perturbations in anti-mycobacterial cytokine profiles in HIV-infected individuals with LTBI, including diminished IFN-γ, IL-17AF, and IL-22 production and elevated TNF-α production. These data are consistent with several previous studies indicating decreased frequencies of *Mtb*-specific IFN-γ-producing CD4 T cells in HIV-infected individuals (75, 84, 86). There is increasing evidence from animal models and human studies for an important role of IL-17 and IL-22 in the immune response to *Mtb*. IL-17$^+$ CD4 T cells in the lung correlated with protection against *Mtb* infection in BCG-vaccinated rhesus macaques (65) and IL-22 was identified as a surrogate of protection against bovine TB in BCG-vaccinated cattle (148). In mice, IL-17$^+$ CD4 T cells in the lung recruit IFN-γ$^+$ CD4 T cells to restrict bacterial growth (149). In humans, patients with active

pulmonary TB disease have decreased frequencies of mycobacteria-specific IL-17$^+$ and IL-22$^+$ CD4 T cells, compared with healthy adults (150). Moreover, our findings of reduced IL-17AF to *Mtb* lysate in HIV-infected individuals with LTBI are consistent with two recent reports of significantly lower IL-17 production capacity in HIV-infected individuals with LTBI, compared with HIV-uninfected individuals with LTBI (87, 130). The importance of Th17 cells in immune control of *Mtb* infection is further supported by longitudinal studies of South African adolescents with LTBI, which indicate early suppression of Th17 responses in those who progressed to TB disease, compared with adolescents who did not develop TB disease (151). Th17 cells are preferentially depleted in HIV-infected individuals (152, 153) and loss of IL-17 production capacity in individuals co-infected with HIV and *Mtb* may contribute to lack of successful immune control of *Mtb* infection and subsequent progression to active TB disease.

By contrast with diminished IL-2, IFN-γ, IL-17AF, and IL-22 production, the only cytokine we found to be significantly increased in HIV-infected individuals was the pro-inflammatory cytokine TNF-α. Our findings of significantly increased TNF-α production to *Mtb* lysate in HIV-infected individuals, regardless of QFT status (Supplementary Figure 3B), as well as increased TNF-α production to SEB, are consistent with a state of persistent immune activation that has been well-described in chronic HIV infection (154). High plasma levels of TNF-α in HIV-infected individuals are associated with higher viral load set points and more rapid CD4 T cell depletion (155). Importantly, PCA of our data indicated that TNF-α was the single greatest contributor to variation in the cytokine production profiles to *Mtb* lysate in individuals with LTBI. Moreover, patients with active TB disease have significantly increased frequencies of TNF-α$^+$ *Mtb* -specific

CD4 T cells, compared with individuals with LTBI (99, 156), thus suggesting a TNF-α-dominated immune response to *Mtb* may be indicative of lack of immune control of the infection.

To our knowledge, our study is the first to report utilizing AIM assays for CD25/OX40 and CD40L/CD69 expression to identify antigen-specific CD4 T cells in QFT^- and QFT^+ individuals in a high TB burden setting, with and without HIV infection. Our results indicate that AIM assays hold great potential to identify novel populations of *Mtb*-specific CD4 T cells that may otherwise not be detected by IGRAs or traditional intracellular cytokine staining assays. While we identified perturbations in *Mtb*-specific cytokine production in HIV-infected individuals with LTBI, future studies are necessary to more comprehensively define signatures of *Mtb*-specific CD4 T cells associated with and/or predict specific outcomes following *Mtb* infection. In conclusion, the AIM assay is thus a promising approach for sorting *Mtb*-specific CD4 T cell populations for downstream applications such as single-cell RNA-sequencing, TCR sequencing, and T cell cloning, which will greatly facilitate further definition of T cell immune correlates that mediate durable control of *Mtb* infection and prevent progression to TB disease.

Acknowledgements

We thank many additional members of the Kenya Medical Research Institute (KEMRI)/Centers for Disease Control and Prevention (CDC) team who helped with enrollment and evaluation of participants, and the participants themselves.

Tables

Table 1. Characteristics of study participants

	HIV– QFT–	HIV+ QFT–	HIV– LTBI	HIV+ LTBI
n	19	20	19	17
Age, y[a] (IQR)	23 (19–28)	29 (25–36)[c]	30 (20–54)	32 (24–37)
Sex (% Male)	21	45	26	59
QFT, IFN-γ IU/ml[b] (IQR)	0 (0 – 0.07)	0 (0 – 0.08)	10 (1.91 – 10)	1.73 (0.94 – 5.41)[d]
CD4 Count, Cells/μl[b] (IQR)	N/A	515 (320 – 758)	N/A	590 (432 – 838)
HIV Viral Load, Copies RNA/ml plasma[b] (IQR)	N/A	85,488 (13,441 – 537,421)	N/A	17,550 (3,873 – 141,188)

[a] value denotes median age in years
[b] values denote median
[c] $p < 0.05$, compared with HIV– QFT–
[d] $p < 0.01$, compared with HIV– LTBI

Figure Legends

Figure 1. Differential upregulation of AIM markers on *Mtb*-specific CD4 T cells from QFT⁻ and LTBI individuals. PBMCs from HIV⁻ QFT⁻ (n=19), HIV⁺ QFT⁻ (n = 20), HIV⁻ LTBI (n = 19) and HIV⁺ LTBI (n = 17) individuals were incubated for 16 hours in media alone (negative control) or stimulated with SEB (positive control) and *Mtb* antigens (CFP-10/ESAT-6 peptide pool and *Mtb* lysate). Surface expression of the AIM markers CD25, OX40, CD40L, and CD69 on CD4 T cells was measured by flow cytometry. (**A**) Representative flow cytometry data indicating antigen-induced expression of $CD25^+OX40^+$ and $CD69^+CD40L^+$ CD4 T cells from an individual in each of the 4 groups. Plots are shown gated on live $CD3^+CD4^+$ T cells. Composite data are shown of CD4 T cell expression for the indicated AIM markers following stimulation with CFP-10/ESAT-6 peptide pool (**B**), *Mtb* lysate (**C**) and SEB (**D**). Data are shown after subtraction of background AIM marker expression on CD4 T cells incubated in media alone. Boxes represent the median and interquartile ranges; whiskers represent the 5^{th} and 95^{th} percentiles. Differences in the frequencies of CD4 T cells expressing AIM markers between QFT⁻ and LTBI groups, and between HIV⁻ and HIV⁺ groups were assessed using the Mann-Whitney *U* test.

Figure 2. Antigen-specific CD4 T cell AIM assay functionality scores are similar between HIV-infected and HIV-uninfected individuals with LTBI. Combinations of AIM markers (CD25, OX40, CD69, and CD40L) expressed on CD4 T cells from HIV-uninfected and from HIV-infected individuals with LTBI were analyzed by flow cytometry as described in Figure 1. Boolean analysis was used to define subsets

of CD4 T cells expressing all possible combinations of the 4 AIM markers. COMPASS was used to summarize the antigen-specific AIM marker upregulation profile of each group, generating an AIM assay 'functionality score'. Boxplots of functionality scores (*left panel*) for CD4 T cells stratified by HIV infection status are shown for CFP-10/ESAT-6 peptide pool (**A**), *Mtb* lysate (**B**), and SEB (**C**). Principal component analysis (PCA) plots of characterized trends exhibited by the expression profiles of AIM markers are shown in the right panel for each antigen. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the functionality scores of CD4 T cells expressing AIM markers between HIV-uninfected and HIV-infected individuals were assessed using the Mann-Whitney *U* test.

Figure 3: HIV co-infection modifies *Mtb*-specific cytokine production profiles in individuals with LTBI. PBMCs from HIV$^-$ (n=19) and HIV$^+$ (n=17) individuals with LTBI were stimulated with antigens as described in Figure 1. After 16 hours, cell supernatants were harvested, and cytokine levels quantified by Luminex bead assay. Concentrations of IFN-γ, IL-2, TNF-α, IL17AF, IL-22, and IL-10 are shown following stimulation of PBMCs with (**A**) CFP-10/ESAT-6, (**B**) *Mtb* whole cell lysate, and (**C**) SEB. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in cytokine concentration between HIV$^-$ and HIV$^+$ individuals were assessed using the Mann-Whitney *U* test.

Figure 4. Co-infection with HIV skews the relationship between antigen-specific CD4 T cell AIM marker expression and cytokine production profiles in individuals with LTBI. Correlogram analysis between antigen-specific cytokine

profiles and AIM marker expression on CD4 T cells from HIV-uninfected individuals (n=19) and HIV-infected individuals (n = 17) with LTBI. Correlograms are shown following stimulation of PBMC with (**A**) CFP-10/ESAT-6 peptide pool, (**B**) *Mtb* whole cell lysate, and (**C**) SEB. Correlations were calculated by Spearman's rank-order correlation. The size and color intensity of each circle is proportional to the strength and direction (blue: positive; red: negative) of each correlation coefficient. White asterisks indicate significant *P* values as follow: ****P*<0.001; ** *P*<0.01; * *P*<0.05. Correlograms for each antigen are ordered by the angular order of the eigenvectors (AOE) of HIV-uninfected individuals with LTBI; correlogram data from HIV-infected individuals are ordered according to the AOE of HIV-uninfected individuals.

Figure 5. Comparative analysis of the effect of HIV infection on cytokine profiles in LTBI. Cytokine production profiles from HIV^- individuals (n=19) and from HIV^+ individuals (n=17) with LTBI were analyzed as described in Figure 3. PCA plots are shown (*left panels*) of characterized trends exhibited by the cytokine expression profiles following stimulation of PBMCs with (**A**) CFP-10/ESAT-6 peptide pool, (**B**) *Mtb* whole cell lysate, and (**C**) SEB. Variables contributing to PC1 are shown from HIV^- and HIV^+ individuals (middle and right panels, respectively).

Figures

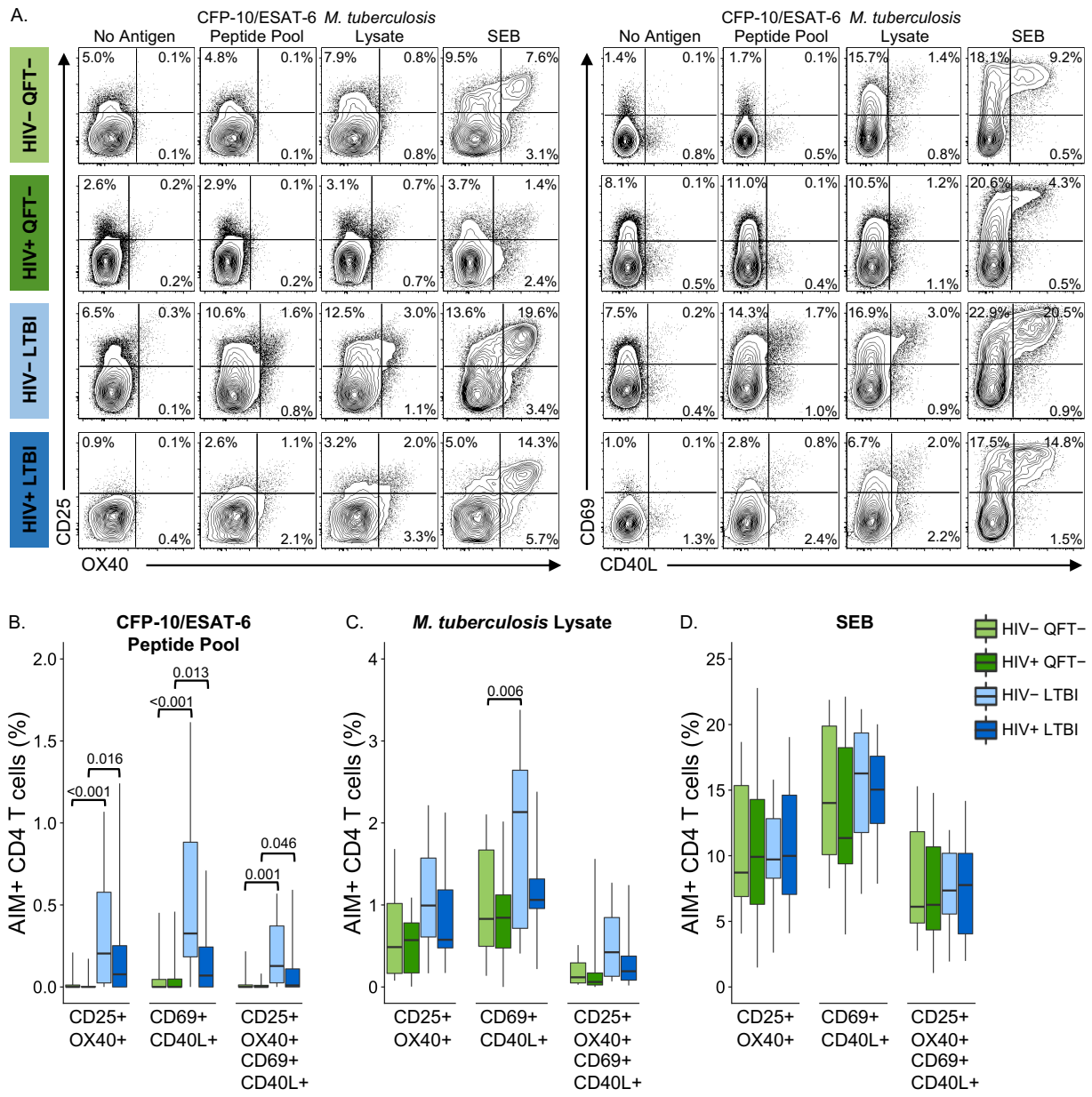

Figure 1. Differential upregulation of AIM markers on *Mtb*-specific CD4 T cells from QFT⁻ and LTBI individuals.

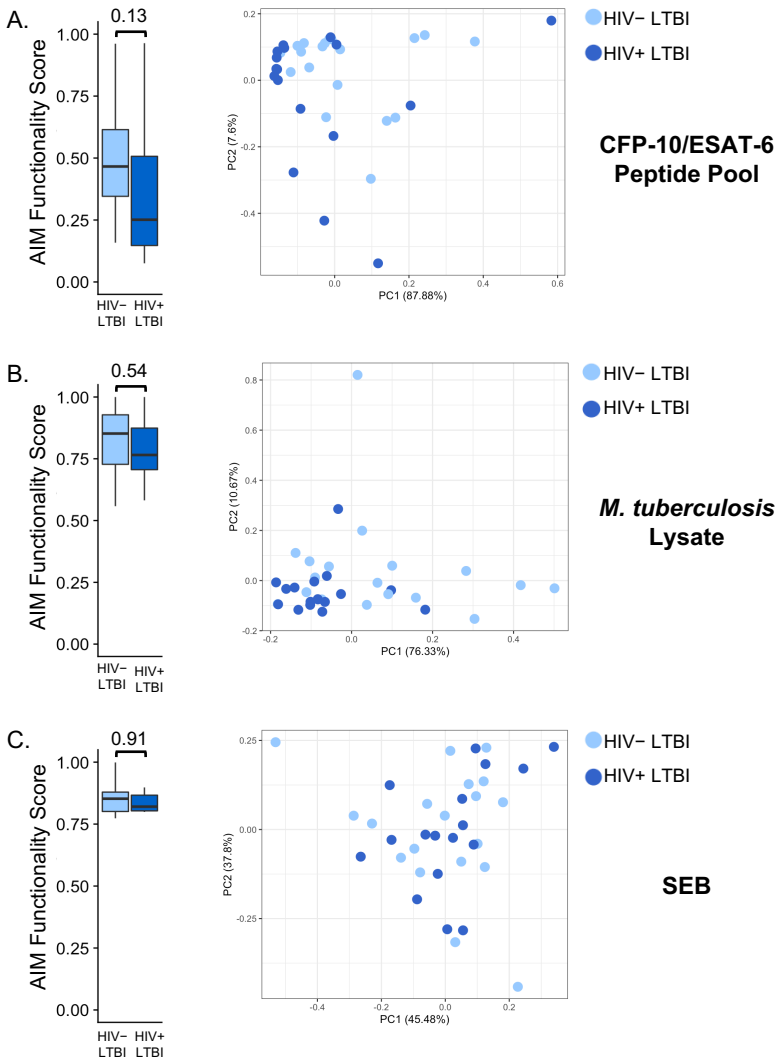

Figure 2. Antigen-specific CD4 T cell AIM assay functionality scores are similar between HIV-infected and HIV-uninfected individuals with LTBI.

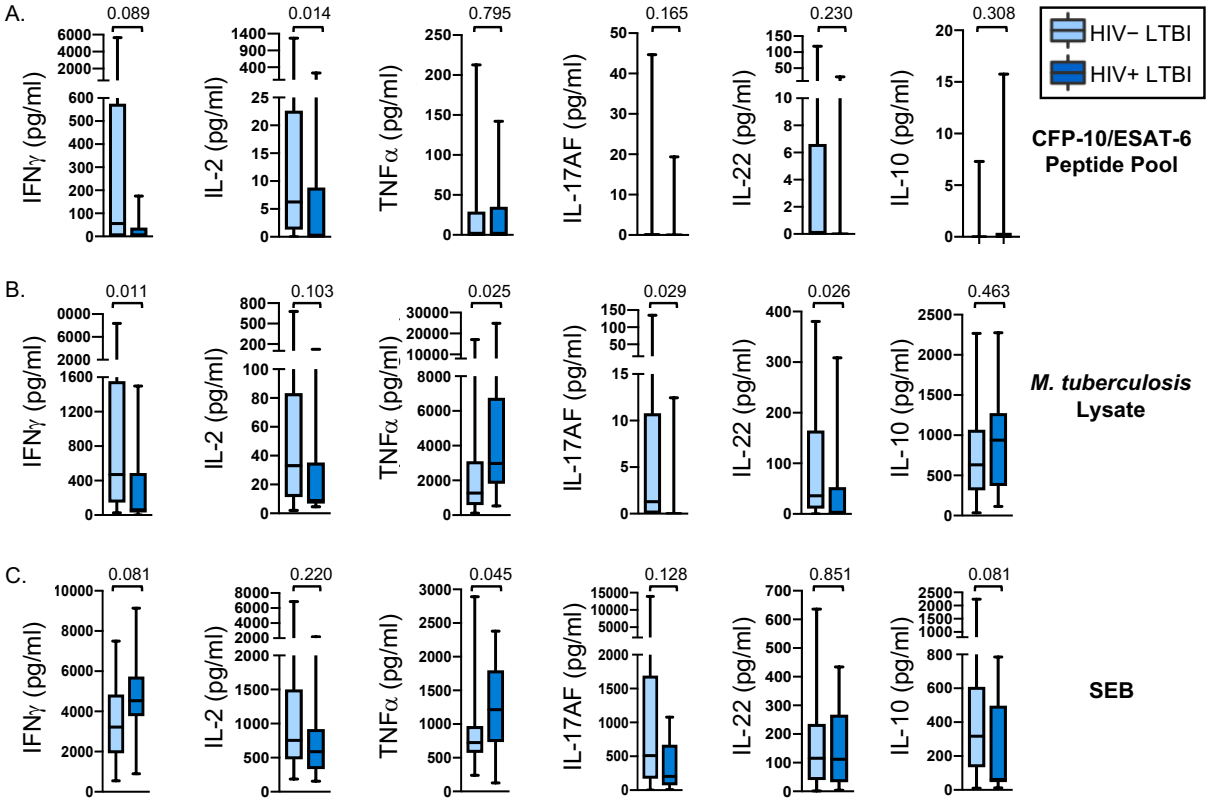

Figure 3: HIV co-infection modifies *Mtb*-specific cytokine production profiles in individuals with LTBI.

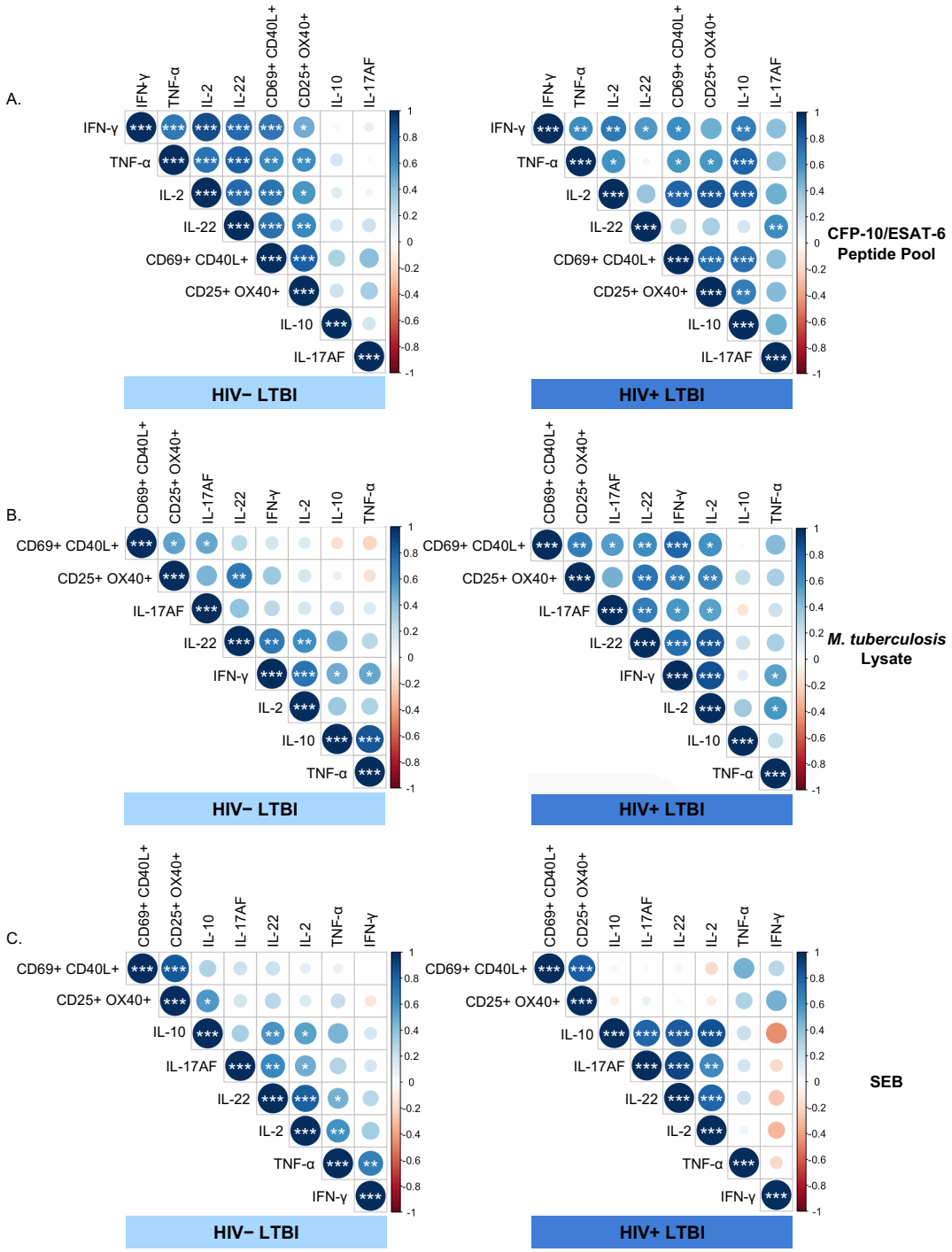

Figure 4. Co-infection with HIV skews the relationship between antigen-specific CD4 T cell AIM marker expression and cytokine production profiles in individuals with LTBI.

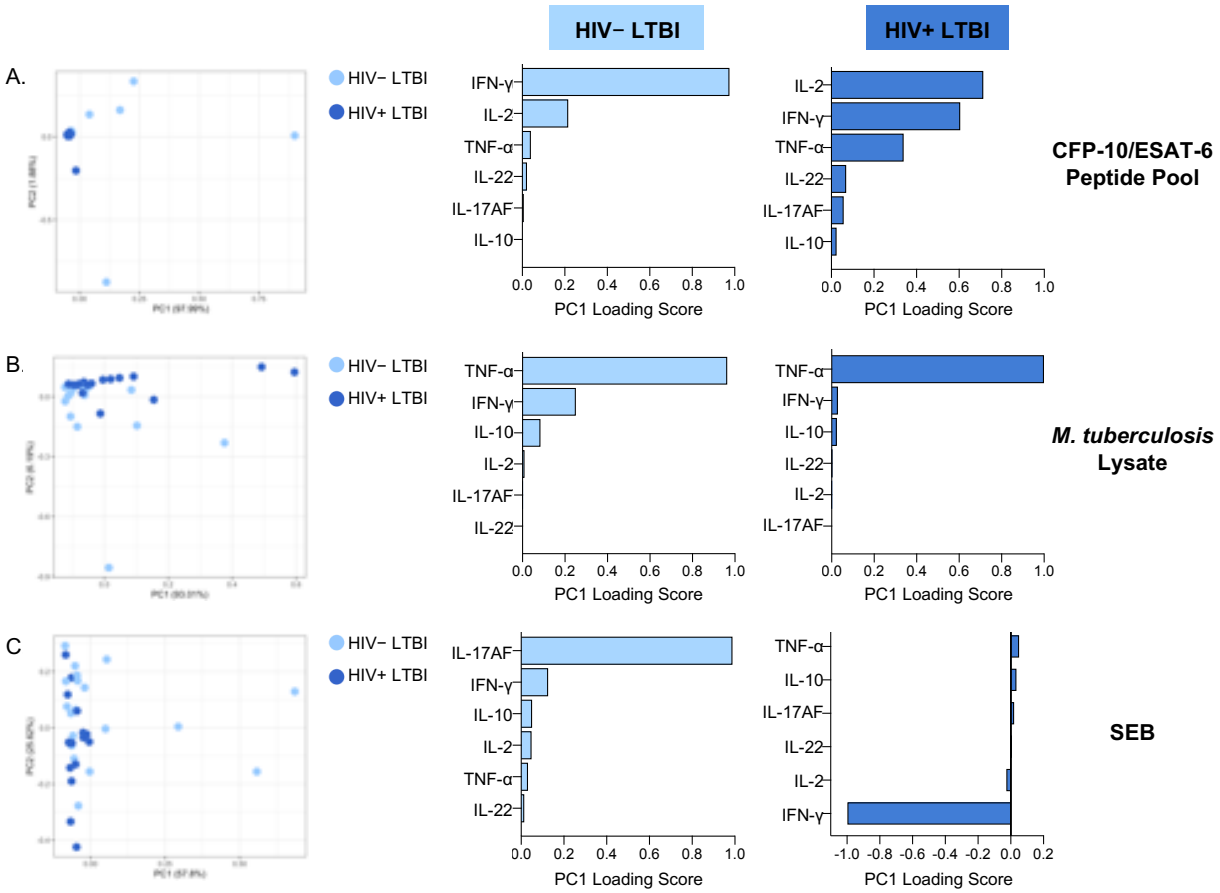

Figure 5. Comparative analysis of the effect of HIV infection on cytokine profiles in LTBI.

Supplementary Figure Legends

Supplementary Figure 1. HIV infection is associated with a lower QFT TB Antigen IFN-γ response, compared with HIV-uninfected individuals with LTBI. All study participants were tested by QFT. Composite data are shown of the IFN-γ response in the TB Antigen (left panel) and Mitogen (right panel) QFT tubes in the 4 study groups. All responses are shown after subtraction of background IFN-γ production in the QFT Nil tube. IFN-γ responses >10 IU/ml were recorded as 10 IU/ml. Horizontal lines represent the median. Differences in IFN-γ levels between HIV-uninfected and HIV-infected individuals and between LTBI and QFT^- individuals were assessed using a Mann-Whitney *U* test.

Supplementary Figure 2. HCMV-specific CD4 T cell responses in the AIM assay are not significantly impacted by *Mtb* and HIV infection status. PBMCs from HIV^- QFT^- (n=19), HIV^+ QFT^- (n = 20), HIV^- LTBI (n = 19) and HIV^+ LTBI (n = 17) individuals were incubated for 16 hours in media alone (negative control) or stimulated with HCMV pp65 peptide pool. Surface expression of the AIM markers CD25, OX40, CD40L, and CD69 on CD4 T cells was measured by flow cytometry. (**A**) Composite data of CD4 T cell expression of the indicated AIM markers following stimulation with HCMV pp65 peptide pool. Data are shown after subtraction of background AIM marker expression on CD4 T cells incubated in media alone. (**B**) HCMV pp65-specific AIM assay functionality scores generated by COMPASS (left panel). A PCA plot incorporating all possible combinations of the 4 AIM markers is

shown in the right panel. For panels A and B, boxes represent the median an interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences between groups were assessed using a Mann-Whitney *U* test. No significant differences were found between groups for HCMV pp65-specific CD4 T cell responses measured in the AIM assay.

Supplementary Figure 3. HIV infection alters antigen-specific cytokine production profiles in both QFT$^-$ and LTBI individuals. PBMC from HIV$^-$ QFT$^-$ (n=19), HIV$^+$ QFT$^-$ (n=20), HIV$^-$ LTBI (n=19), and HIV$^+$ LTBI (n=17) individuals were stimulated for 16 hours with the indicated Ags. Supernatants were collected and used to measure cytokine concentrations by Luminex. IFN-γ, IL-2, TNF-α, IL-17AF, IL-22, and IL-10 concentrations are shown following stimulation of PBMCs with (**A**) CFP-10/ESAT-6 peptide pool, (**B**) *Mtb* H37Rv whole cell lysate, (**C**) SEB, and (**D**) HCMV pp65 peptide pool. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in cytokine concentrations between HIV$^-$ and HIV$^+$ groups were assessed using a Mann-Whitney *U* test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Supplementary Figures

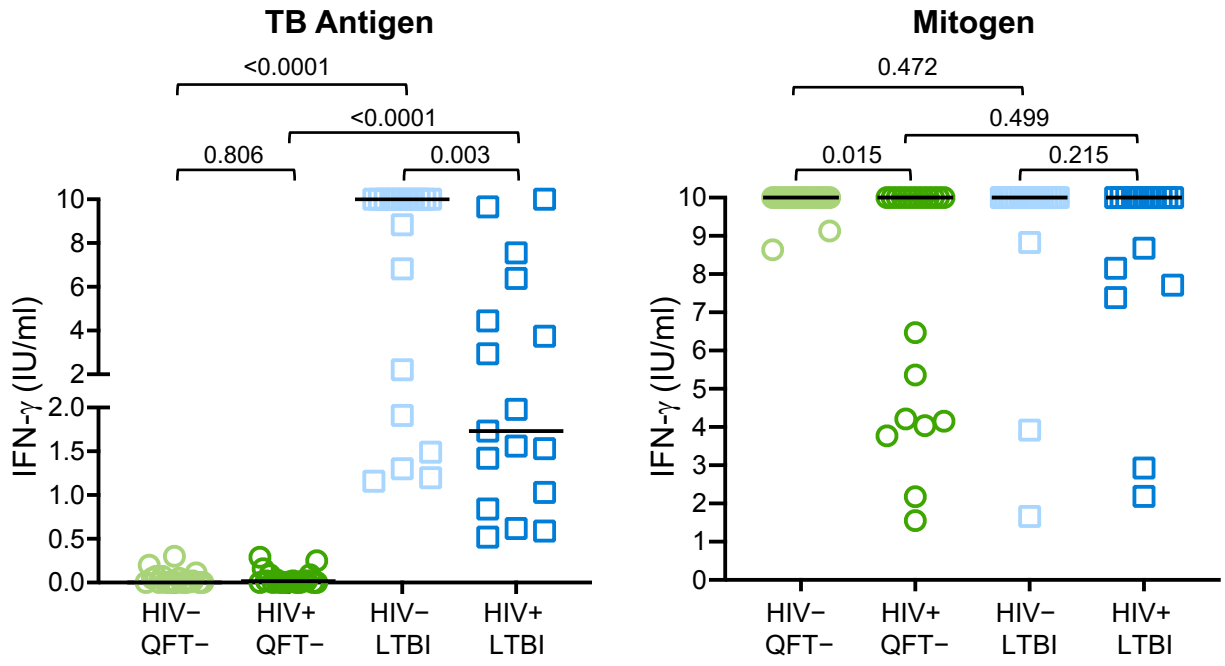

Figure S1. HIV infection is associated with a lower QFT TB Antigen IFN-γ response, compared with HIV-uninfected individuals with LTBI.

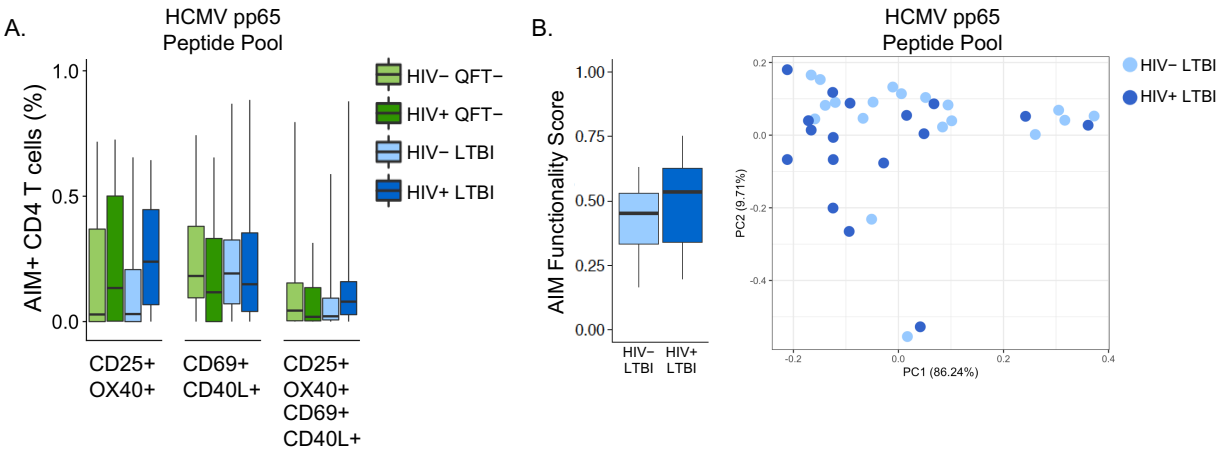

Figure S2. HCMV-specific CD4 T cell responses in the AIM assay are not significantly impacted by *Mtb* and HIV infection status.

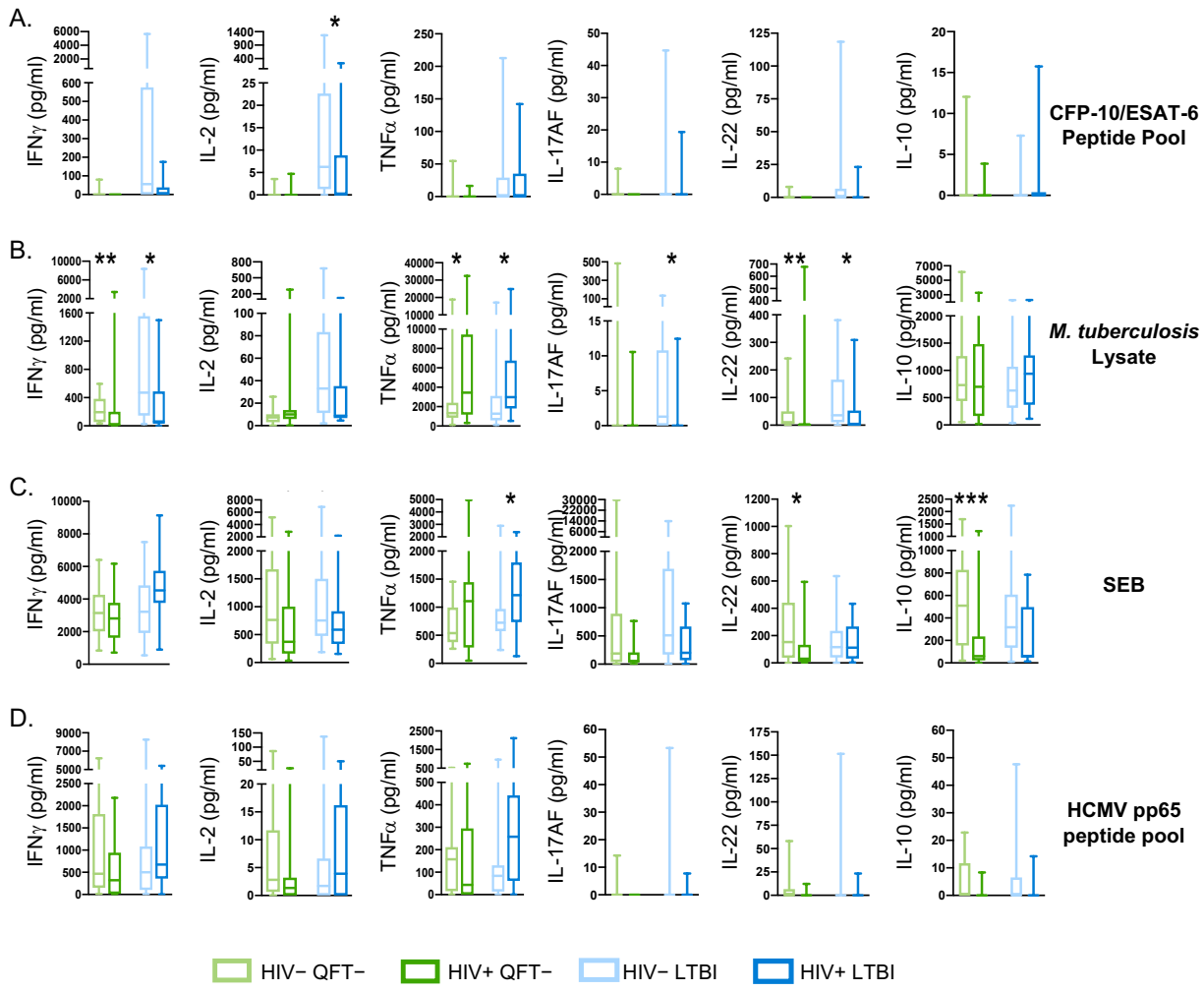

Figure S3. HIV infection alters antigen-specific cytokine production profiles in both QFT⁻ and LTBI individuals.

CHAPTER IV

DISCUSSION

Summary of Findings

This dissertation describes four main findings:

1. Ig superfamily inhibitory receptors BTLA, CTLA-4, and PD-1 are not upregulated on *Mtb*-specific CD4 T cells in peripheral blood of HIV-infected individuals, compared with HIV-uninfected individuals, in the context of either LTBI or pulmonary TB disease.
2. BTLA is markedly downregulated on *Mtb*-specific CD4 T cells in HIV-infected individuals with pulmonary TB disease, compared with HIV-uninfected individuals with pulmonary TB disease.
3. CD25/OX40 and CD69/CD40L AIM assays can be used to identify *Mtb*-specific CD4 T cells in HIV-uninfected and HIV-infected Kenyan adults with LTBI.
4. HIV infection is characterized by diminished *Mtb*-specific IFN-γ, IL-2, IL-17AF, and IL-22 production, and elevated TNF-α production, compared with HIV-uninfected individuals.

Conclusions

Mycobacterium tuberculosis (*Mtb*) infection is the leading cause of death due to a single infectious agent and has remained one of the top 10 causes of death worldwide for decades (3). *Mtb* is the infectious agent that causes tuberculosis (TB) disease, results in 10 million new cases of TB disease and approximately 1.6 million deaths (3). Furthermore, though the immune correlates of protection against TB have not been defined, co-infection with human immunodeficiency virus (HIV) is the single greatest risk factor for reactivation from latent *Mtb* infection (LTBI) to active TB disease (3, 83). In addition, co-infection with *Mtb* and HIV has proven difficult in the context of prevention, diagnosis, and treatment due to immune suppression and depletion of CD4 T cells (49). The studies described in this dissertation were conducted to evaluate the effect of HIV on the immunological response within individuals with LTBI and pulmonary TB disease via analysis of analysis of antigen-specific CD4 T cell phenotypic and functional profiles.

In Chapter 2, the effect of HIV on the concurrent expression of the Ig superfamily T cell inhibitory receptors BTLA, CTLA-4, and PD-1 on total and antigen-specific CD4 T cells from individuals with LTBI and active TB disease was described. We demonstrated that active TB disease in both HIV-uninfected and HIV-infected individuals is associated with reduced proportions of total CD4 T cells co-expressing all three inhibitory receptors, compared with LTBI. In addition, we found no evidence of increased expression of BTLA, CTLA-4, or PD-1, either alone or in combination, on *Mtb*-specific CD4 T cells in HIV-infected individuals, compared with HIV-uninfected individuals. Furthermore, expression of BTLA was significantly lower on *Mtb*-specific

CD4 T cells from HIV-infected individuals with active TB, compared with the other three participant groups. Lastly, by directly comparing inhibitory receptor phenotypes of *Mtb*-, HCMV-, and HIV-specific CD4 T cells circulating concurrently within the same individual, we provide evidence for the first time that, by contrast with virus-specific CD4 T cells, *Mtb*-specific CD4 do not co-express inhibitory receptors at high levels, regardless of HIV infection status.

In Chapter 3, the effect of HIV on the concurrent expression of the activation induced markers (AIM) CD25/OX40 and CD40L/CD69 on total and antigen-specific CD4 T cells from QFT^- and LTBI individuals, as well as cytokine production profiles, was described. We demonstrated that CD25/OX40 and CD69/CD40L AIM assays can be used to identify CFP-10/ESAT-6-specific CD4 T cells in HIV-uninfected and HIV-infected Kenyan adults with LTBI. We also show that AIM assays for CD69/OX40L provided greater differentiation between QFT^- and QFT^+ individuals than CD25/OX40. Furthermore, we evaluated *Mtb*-specific cytokine production profiles in both HIV-uninfected and HIV-infected individuals with LTBI in a high TB burden setting and showed that HIV infection is characterized by diminished *Mtb*-specific IFN-γ, IL-2, IL-17AF, and IL-22 production, and elevated TNF-α production, compared with HIV-uninfected individuals.

Future Implications

Nearly a quarter of the global population is infected with *Mycobacterium tuberculosis* (*Mtb*), with 10 million people developing active tuberculosis (TB) annually. The World Health Organization's 'End TB Strategy' aims to end the TB pandemic by drastically decreasing TB incidence, morbidly and death by 2030. To achieve this goal, TB incidence rates need to decrease from an average 1.4% per year to 4-5% per year(6). In order to do so, TB transmission, pathogenesis, vaccines and treatment need to be addressed. In addition to defining immune correlates of *Mtb* infection, co-infection with HIV has long been recognized as a significant risk factor for progression to TB disease, yet the mechanisms whereby HIV impairs T cell-mediated control of *Mtb* infection remain poorly defined. Thus, there is an urgent need to further define elements of protective immunity to *Mtb* infection and determine precisely how co-infection with HIV impairs anti-mycobacterial immunity.

In summary, this dissertation contributes novel evidence that the majority of *Mtb*-specific CD4 T cells do not co-express multiple inhibitory receptors, regardless of HIV infection status. Our data suggest that immune checkpoint blockade, which is currently in use for cancer therapy and proposed for treatment of certain chronic viral infections, may not be an effective approach for boosting T cell-mediated immune control of *Mtb* infection. Furthermore, this dissertation highlights the previously unrecognized role of BTLA expression on *Mtb*-specific CD4 T cells as a potential biomarker of *Mtb* infection status, particularly in people living with HIV, the population at greatest risk for development of active TB disease. In addition, this dissertation demonstrates for the first time that AIM assays have potential to identify novel

populations of *Mtb*-specific CD4 T cells in HIV-infected and uninfected individuals in a high TB burden setting that may otherwise not be detected by IGRAs or traditional intracellular cytokine staining assays. An additional important consideration in evaluating the effect of HIV co-infection on *Mtb*-specific T cell immunity is HIV disease state. While the HIV-infected participants in our cohorts had relatively preserved CD4 T cell counts, it is possible that more substantial differences in inhibitory receptor expression, AIM marker expression, or cytokine production profiles of *Mtb*-specific CD4 T cells would be apparent in HIV-infected individuals with CD4 T cell counts <200 cells/μl and more advanced HIV disease.

Moreover, the finding presented in this dissertation describe multiple phenotypic and functional markers that facilitate characterization of antigen-specific CD4 T cells within individuals across a spectrum of *Mtb* infection, with and without HIV. Further studies are warranted to investigate additional markers, including but not limited to activation markers, inhibitory and costimulatory markers, as well as cytokine production by *Mtb*-specific CD4 T cells in order to better understand mechanisms and develop more efficacious therapeutics and vaccines to combat the reactivation of LTBI to active TB disease in individuals with *Mtb*/HIV co-infection. Further, in order to more comprehensively define CD4 T cell responses in latent and active TB and further define how co-infection with HIV impairs protective T cell immunity to *Mtb* infection, detailed studies such as transcriptional and epigenetic profiling of *Mtb*-specific CD4 T cells will be necessary to comprehensively define *Mtb*-specific CD4 T cell signatures associated with successful control of *Mtb* infection. Our AIM assay can be utilized in future studies to sort *Mtb*-specific CD4 T cells for single-cell RNA-sequencing, TCR sequencing, and T cell cloning to define T cell immune correlates that mediate durable control of *Mtb*

infection and prevent progression to TB disease. In addition, although our studies were conducted using samples from cohorts enrolled in a cross-sectional manner, our results provide rationale for conducting prospective, longitudinal studies of individuals with LTBI and household contacts of TB index cases to define signatures of *Mtb*-specific CD4 T cells that can identify individuals at greatest risk for subsequent progression to TB disease. Moreover, longitudinal studies of *Mtb*-specific CD4 T cell responses in individuals with newly acquired HIV infection will be necessary to fully elucidate mechanisms of HIV-associated dysregulation of anti-mycobacterial immunity that occur very early in HIV infection. It remains to be seen whether early treatment of HIV with antiretroviral therapy can preserve *Mtb*-specific CD4 T cell responses and reduce the incidence of active TB disease in people living with HIV. Taken together, this knowledge of immune correlates of *Mtb* infection can then be employed to develop novel prophylactic and therapeutic vaccines and immunotherapies to significantly contribute to decreasing TB incidence as proposed by the 'End TB Strategy'.

CHAPTER V

REFERENCES

1. Dye, C., S. Scheele, P. Dolin, V. Pathania, and M. C. Raviglione. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* 282: 677-686.
2. Cambau, E., and M. Drancourt. 2014. Steps towards the discovery of *Mycobacterium tuberculosis* by Robert Koch, 1882. *Clin Microbiol Infect* 20: 196-201.
3. WHO Publication. Global Tuberculosis Report 2018. Available from http://www.who.int/tb/publications/global_report/en/
4. Navin, T. R., S. J. McNabb, and J. T. Crawford. 2002. The continued threat of tuberculosis. *Emerg Infect Dis* 8: 1187.
5. Grange, J. M., and M. D. Yates. 1993. Re-emergence of tuberculosis. *BMJ* 306: 931-932.
6. Hervas-Stubbs, S., L. Majlessi, M. Simsova, J. Morova, M. J. Rojas, C. Nouze, P. Brodin, P. Sebo, and C. Leclerc. 2006. High frequency of CD4+ T cells specific for the TB10.4 protein correlates with protection against *Mycobacterium tuberculosis* infection. *Infect Immun* 74: 3396-3407.
7. Brandt, L., M. Elhay, I. Rosenkrands, E. B. Lindblad, and P. Andersen. 2000. ESAT-6 subunit vaccination against *Mycobacterium tuberculosis*. *Infect Immun* 68: 791-795.
8. Pawlowski, A., M. Jansson, M. Skold, M. E. Rottenberg, and G. Kallenius. 2012. Tuberculosis and HIV co-infection. *PLoS Pathog* 8: e1002464.
9. Hopewell, P. C. 2000. Translating discoveries into control of tuberculosis in HIV-infected persons. *Int J Tuberc Lung Dis* 4: S82-87.

10. Horsburgh, C. R., Jr. 2004. Priorities for the treatment of latent tuberculosis infection in the United States. *N Engl J Med* 350: 2060-2067.

11. Lawn, S. D., A. D. Harries, B. G. Williams, R. E. Chaisson, E. Losina, K. M. De Cock, and R. Wood. 2011. Antiretroviral therapy and the control of HIV-associated tuberculosis. Will ART do it? *Int J Tuberc Lung Dis* 15: 571-581.

12. Ulrichs, T., M. E. Munk, H. Mollenkopf, S. Behr-Perst, R. Colangeli, M. L. Gennaro, and S. H. Kaufmann. 1998. Differential T cell responses to *Mycobacterium tuberculosis* ESAT6 in tuberculosis patients and healthy donors. *Eur J Immunol* 28: 3949-3958.

13. Sakula, A. 1983. Robert koch: centenary of the discovery of the tubercle bacillus, 1882. *Can Vet J* 24: 127-131.

14. Sakula, A. 1983. Robert Koch (1843-1910): Founder of the Science of Bacteriology and Discoverer of the Tubercle Bacillus : A Study of his Life and Work. *Can Vet J* 24: 124-127.

15. Ross, L. N., and J. F. Woodward. 2016. Koch's postulates: An interventionist perspective. *Stud Hist Philos Biol Biomed Sci* 59: 35-46.

16. Delogu, G., M. Sali, and G. Fadda. 2013. The biology of *Mycobacterium tuberculosis* infection. *Mediterr J Hematol Infect Dis* 5: e2013070.

17. Simeone, R., A. Bobard, J. Lippmann, W. Bitter, L. Majlessi, R. Brosch, and J. Enninga. 2012. Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. *PLoS Pathog* 8: e1002507.

18. van der Wel, N., D. Hava, D. Houben, D. Fluitsma, M. van Zon, J. Pierson, M. Brenner, and P. J. Peters. 2007. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 129: 1287-1298.

19. Fennelly, K. P., J. W. Martyny, K. E. Fulton, I. M. Orme, D. M. Cave, and L. B. Heifets. 2004. Cough-generated aerosols of *Mycobacterium tuberculosis*: a new method to study infectiousness. *Am J Respir Crit Care Med* 169: 604-609.

20. Russell, D. G., C. E. Barry, 3rd, and J. L. Flynn. 2010. Tuberculosis: what we don't know can, and does, hurt us. *Science* 328: 852-856.

21. Kaufmann, S. H. 2006. Tuberculosis: back on the immunologists' agenda. *Immunity* 24: 351-357.

22. Pai, M., L. W. Riley, and J. M. Colford, Jr. 2004. Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. *Lancet Infect Dis* 4: 761-776.

23. Desikan, P. 2013. Sputum smear microscopy in tuberculosis: is it still relevant? *Indian J Med Res* 137: 442-444.

24. Matee, M., L. Mtei, T. Lounasvaara, W. Wieland-Alter, R. Waddell, J. Lyimo, M. Bakari, K. Pallangyo, and C. F. von Reyn. 2008. Sputum microscopy for the diagnosis of HIV-associated pulmonary tuberculosis in Tanzania. *BMC public health* 8: 68.

25. Boehme, C. C., P. Nabeta, D. Hillemann, M. P. Nicol, S. Shenai, F. Krapp, J. Allen, R. Tahirli, R. Blakemore, R. Rustomjee, A. Milovic, M. Jones, S. M. O'Brien, D. H. Persing, S. Ruesch-Gerdes, E. Gotuzzo, C. Rodrigues, D. Alland, and M. D. Perkins. 2010. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med* 363: 1005-1015.

26. Dorman, S. E., S. G. Schumacher, D. Alland, P. Nabeta, D. T. Armstrong, B. King, S. L. Hall, S. Chakravorty, D. M. Cirillo, N. Tukvadze, N. Bablishvili, W. Stevens, L. Scott, C. Rodrigues, M. I. Kazi, M. Joloba, L. Nakiyingi, M. P. Nicol, Y.

Ghebrekristos, I. Anyango, W. Murithi, R. Dietze, R. Lyrio Peres, A. Skrahina, V. Auchynka, K. K. Chopra, M. Hanif, X. Liu, X. Yuan, C. C. Boehme, J. J. Ellner, C. M. Denkinger, and t. study. 2018. Xpert MTB/RIF Ultra for detection of *Mycobacterium tuberculosis* and rifampicin resistance: a prospective multicentre diagnostic accuracy study. *Lancet Infect Dis* 18: 76-84.

27. Ramakrishnan, L. 2012. Revisiting the role of the granuloma in tuberculosis. *Nat Rev Immunol* 12: 352-366.

28. Pai, M., M. A. Behr, D. Dowdy, K. Dheda, M. Divangahi, C. C. Boehme, A. Ginsberg, S. Swaminathan, M. Spigelman, H. Getahun, D. Menzies, and M. Raviglione. 2016. Tuberculosis. *Nat Rev Dis Primers* 2: 16076.

29. Barry, C. E., 3rd, H. I. Boshoff, V. Dartois, T. Dick, S. Ehrt, J. Flynn, D. Schnappinger, R. J. Wilkinson, and D. Young. 2009. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* 7: 845-855.

30. Furin, J., H. Cox, and M. Pai. 2019. Tuberculosis. *Lancet* 393: 1642-1656.

31. Achkar, J. M., and E. R. Jenny-Avital. 2011. Incipient and subclinical tuberculosis: defining early disease states in the context of host immune response. *J Infect Dis* 204 Suppl 4: S1179-1186.

32. Shah, N. S., P. Kim, B. D. Kana, and R. Rustomjee. 2017. Getting to Zero New Tuberculosis Infections: Insights From the National Institutes of Health/US Centers for Disease Control and Prevention/Bill & Melinda Gates Foundation Workshop on Research Needs for Halting Tuberculosis Transmission. *J Infect Dis* 216: S627-S628.

33. Sharma, S. K., and A. Mohan. 2017. Miliary Tuberculosis. *Microbiol Spectr* 5.

34. Davis, A. G., U. K. Rohlwink, A. Proust, A. A. Figaji, and R. J. Wilkinson. 2019. The pathogenesis of tuberculous meningitis. *J Leukoc Biol* 105: 267-280.

35. Behr, M. A., P. H. Edelstein, and L. Ramakrishnan. 2018. Revisiting the timetable of tuberculosis. *BMJ* 362: k2738.

36. Ganguly, N., I. Siddiqui, and P. Sharma. 2008. Role of M. tuberculosis RD-1 region encoded secretory proteins in protective response and virulence. *Tuberculosis (Edinb)* 88: 510-517.

37. Fine, P. E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 346: 1339-1345.

38. Trunz, B. B., P. Fine, and C. Dye. 2006. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet* 367: 1173-1180.

39. McShane, H. 2019. Insights and challenges in tuberculosis vaccine development. *Lancet Respir Med*.

40. Harris, R. C., T. Sumner, G. M. Knight, and R. G. White. 2016. Systematic review of mathematical models exploring the epidemiological impact of future TB vaccines. *Hum Vaccin Immunother* 12: 2813-2832.

41. WHO. 2018. Latent TB Infection : Updated and consolidated guidelines for programmatic management.

42. LoBue, P. A., and J. H. Mermin. 2017. Latent tuberculosis infection: the final frontier of tuberculosis elimination in the USA. *Lancet Infect Dis* 17: e327-e333.

43. Bahuguna, A., and D. S. Rawat. 2019. An overview of new antitubercular drugs, drug candidates, and their targets. *Med Res Rev*.

44. Davenne, T., and H. McShane. 2016. Why don't we have an effective tuberculosis vaccine yet? *Expert Rev Vaccines* 15: 1009-1013.

45. Chaplin, D. D. 2010. Overview of the immune response. *J Allergy Clin Immunol* 125: S3-23.

46. Mayer-Barber, K. D., and D. L. Barber. 2015. Innate and Adaptive Cellular Immune Responses to *Mycobacterium tuberculosis* Infection. *Cold Spring Harb Perspect Med* 5.

47. Sanchez, D., M. Rojas, I. Hernandez, D. Radzioch, L. F. Garcia, and L. F. Barrera. 2010. Role of TLR2- and TLR4-mediated signaling in *Mycobacterium tuberculosis*-induced macrophage death. *Cell Immunol* 260: 128-136.

48. Chensue, S. W., J. H. Ruth, K. Warmington, P. Lincoln, and S. L. Kunkel. 1995. In vivo regulation of macrophage IL-12 production during type 1 and type 2 cytokine-mediated granuloma formation. *J Immunol* 155: 3546-3551.

49. Ernst, J. D. 2012. The immunological life cycle of tuberculosis. *Nat Rev Immunol* 12: 581-591.

50. Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* 178: 2243-2247.

51. Day, C. L., N. Mkhwanazi, S. Reddy, Z. Mncube, M. van der Stok, P. Klenerman, and B. D. Walker. 2008. Detection of polyfunctional *Mycobacterium tuberculosis*-specific T cells and association with viral load in HIV-1-infected persons. *J Infect Dis* 197: 990-999.

52. Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* 178: 2249-2254.

53. Jouanguy, E., F. Altare, S. Lamhamedi, P. Revy, J. F. Emile, M. Newport, M. Levin, S. Blanche, E. Seboun, A. Fischer, and J. L. Casanova. 1996. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. *N Engl J Med* 335: 1956-1961.

54. Jouanguy, E., S. Lamhamedi-Cherradi, D. Lammas, S. E. Dorman, M. C. Fondaneche, S. Dupuis, R. Doffinger, F. Altare, J. Girdlestone, J. F. Emile, H. Ducoulombier, D. Edgar, J. Clarke, V. A. Oxelius, M. Brai, V. Novelli, K. Heyne, A. Fischer, S. M. Holland, D. S. Kumararatne, R. D. Schreiber, and J. L. Casanova. 1999. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. *Nat Genet* 21: 370-378.

55. Keane, J., S. Gershon, R. P. Wise, E. Mirabile-Levens, J. Kasznica, W. D. Schwieterman, J. N. Siegel, and M. M. Braun. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 345: 1098-1104.

56. Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol* 162: 5407-5416.

57. Mogues, T., M. E. Goodrich, L. Ryan, R. LaCourse, and R. J. North. 2001. The relative importance of T cell subsets in immunity and immunopathology of airborne *Mycobacterium tuberculosis* infection in mice. *J Exp Med* 193: 271-280.

58. Serbina, N. V., V. Lazarevic, and J. L. Flynn. 2001. CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during *Mycobacterium tuberculosis* infection. *J Immunol* 167: 6991-7000.

59. Lin, P. L., T. Rutledge, A. M. Green, M. Bigbee, C. Fuhrman, E. Klein, and J. L. Flynn. 2012. CD4 T Cell Depletion Exacerbates Acute *Mycobacterium tuberculosis* While Reactivation of Latent Infection Is Dependent on Severity of Tissue Depletion in Cynomolgus Macaques. *AIDS Res Hum Retroviruses* 28: 1693-1702.

60. Yao, S., D. Huang, C. Y. Chen, L. Halliday, R. C. Wang, and Z. W. Chen. 2014. CD4+ T cells contain early extrapulmonary tuberculosis (TB) dissemination and rapid TB progression and sustain multieffector functions of CD8+ T and CD3- lymphocytes: mechanisms of CD4+ T cell immunity. *J Immunol* 192: 2120-2132.

61. Andersson, J., A. Samarina, J. Fink, S. Rahman, and S. Grundstrom. 2007. Impaired expression of perforin and granulysin in CD8+ T cells at the site of infection in human chronic pulmonary tuberculosis. *Infect Immun* 75: 5210-5222.

62. Bruns, H., C. Meinken, P. Schauenberg, G. Harter, P. Kern, R. L. Modlin, C. Antoni, and S. Stenger. 2009. Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against *Mycobacterium tuberculosis* in humans. *J Clin Invest* 119: 1167-1177.

63. Smith, S. M., M. R. Klein, A. S. Malin, J. Sillah, K. Huygen, P. Andersen, K. P. McAdam, and H. M. Dockrell. 2000. Human CD8(+) T cells specific for *Mycobacterium tuberculosis* secreted antigens in tuberculosis patients and healthy BCG-vaccinated controls in The Gambia. *Infect Immun* 68: 7144-7148.

64. Chen, C. Y., D. Huang, R. C. Wang, L. Shen, G. Zeng, S. Yao, Y. Shen, L. Halliday, J. Fortman, M. McAllister, J. Estep, R. Hunt, D. Vasconcelos, G. Du, S. A. Porcelli, M. H. Larsen, W. R. Jacobs, Jr., B. F. Haynes, N. L. Letvin, and Z. W. Chen. 2009. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS Pathog* 5: e1000392.

65. Dijkman, K., C. C. Sombroek, R. A. W. Vervenne, S. O. Hofman, C. Boot, E. J. Remarque, C. H. M. Kocken, T. H. M. Ottenhoff, I. Kondova, M. A. Khayum, K. G. Haanstra, M. P. M. Vierboom, and F. A. W. Verreck. 2019. Prevention of tuberculosis infection and disease by local BCG in repeatedly exposed rhesus macaques. *Nat Med* 25: 255-262.

66. Santosuosso, M., X. Zhang, S. McCormick, J. Wang, M. Hitt, and Z. Xing. 2005. Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. *J Immunol* 174: 7986-7994.

67. Sonnenberg, P., J. R. Glynn, K. Fielding, J. Murray, P. Godfrey-Faussett, and S. Shearer. 2005. How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. *J Infect Dis* 191: 150-158.

68. Dye, C., and B. G. Williams. 2019. Tuberculosis decline in populations affected by HIV: a retrospective study of 12 countries in the WHO African Region. *Bull World Health Organ* 97: 405-414.

69. Esmail, H., C. Riou, E. D. Bruyn, R. P. Lai, Y. X. R. Harley, G. Meintjes, K. A. Wilkinson, and R. J. Wilkinson. 2018. The Immune Response to *Mycobacterium tuberculosis* in HIV-1-Coinfected Persons. *Annu Rev Immunol* 36: 603-638.

70. Datiko, D. G., M. A. Yassin, L. T. Chekol, L. E. Kabeto, and B. Lindtjorn. 2008. The rate of TB-HIV co-infection depends on the prevalence of HIV infection in a community. *BMC public health* 8: 266.

71. Whalen, C., C. R. Horsburgh, Jr., D. Hom, C. Lahart, M. Simberkoff, and J. Ellner. 1997. Site of disease and opportunistic infection predict survival in HIV-associated tuberculosis. *AIDS* 11: 455-460.

72. Whalen, C. C., P. Nsubuga, A. Okwera, J. L. Johnson, D. L. Hom, N. L. Michael, R. D. Mugerwa, and J. J. Ellner. 2000. Impact of pulmonary tuberculosis on survival of HIV-infected adults: a prospective epidemiologic study in Uganda. *AIDS* 14: 1219-1228.

73. Kumawat, K., S. K. Pathak, A. L. Spetz, M. Kundu, and J. Basu. 2010. Exogenous Nef is an inhibitor of *Mycobacterium tuberculosis*-induced tumor necrosis factor-alpha production and macrophage apoptosis. *J Biol Chem* 285: 12629-12637.

74. Mwandumba, H. C., D. G. Russell, M. H. Nyirenda, J. Anderson, S. A. White, M. E. Molyneux, and S. B. Squire. 2004. *Mycobacterium tuberculosis* resides in nonacidified vacuoles in endocytically competent alveolar macrophages from patients with tuberculosis and HIV infection. *J Immunol* 172: 4592-4598.

75. Day, C. L., D. A. Abrahams, L. D. Harris, M. van Rooyen, L. Stone, M. de Kock, and W. A. Hanekom. 2017. HIV-1 Infection Is Associated with Depletion and

Functional Impairment of *Mycobacterium tuberculosis*-Specific CD4 T Cells in Individuals with Latent Tuberculosis Infection. *J Immunol* 199: 2069-2080.

76. Geldmacher, C., N. Ngwenyama, A. Schuetz, C. Petrovas, K. Reither, E. J. Heeregrave, J. P. Casazza, D. R. Ambrozak, M. Louder, W. Ampofo, G. Pollakis, B. Hill, E. Sanga, E. Saathoff, L. Maboko, M. Roederer, W. A. Paxton, M. Hoelscher, and R. A. Koup. 2010. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. *J Exp Med* 207: 2869-2881.

77. Geldmacher, C., A. Schuetz, N. Ngwenyama, J. P. Casazza, E. Sanga, E. Saathoff, C. Boehme, S. Geis, L. Maboko, M. Singh, F. Minja, A. Meyerhans, R. A. Koup, and M. Hoelscher. 2008. Early depletion of *Mycobacterium tuberculosis*-specific T helper 1 cell responses after HIV-1 infection. *J Infect Dis* 198: 1590-1598.

78. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27: 670-684.

79. Diedrich, C. R., and J. L. Flynn. 2011. HIV-1/*Mycobacterium tuberculosis* coinfection immunology: how does HIV-1 exacerbate tuberculosis? *Infect Immun* 79: 1407-1417.

80. Diedrich, C. R., J. O'Hern, M. G. Gutierrez, N. Allie, P. Papier, G. Meintjes, A. K. Coussens, H. Wainwright, and R. J. Wilkinson. 2016. Relationship Between HIV Coinfection, Interleukin 10 Production, and *Mycobacterium tuberculosis* in Human Lymph Node Granulomas. *J Infect Dis* 214: 1309-1318.

81. Diedrich, C. R., J. O'Hern, and R. J. Wilkinson. 2016. HIV-1 and the *Mycobacterium tuberculosis* granuloma: A systematic review and meta-analysis. *Tuberculosis (Edinb)* 98: 62-76.

82. Houben, R. M., and P. J. Dodd. 2016. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. *PLoS Med* 13: e1002152.

83. Lawn, S. D., and A. I. Zumla. 2011. Tuberculosis. *Lancet* 378: 57-72.

84. Bunjun, R., C. Riou, A. P. Soares, N. Thawer, T. L. Muller, A. Kiravu, Z. Ginbot, T. Oni, R. Goliath, B. Kalsdorf, F. von Groote-Bidlingmaier, W. Hanekom, G. Walzl, R. J. Wilkinson, and W. A. Burgers. 2017. Effect of HIV on the Frequency and Number of *Mycobacterium tuberculosis*-Specific CD4+ T Cells in Blood and Airways During Latent M. tuberculosis Infection. *J Infect Dis* 216: 1550-1560.

85. Pollock, K. M., D. J. Montamat-Sicotte, L. Grass, G. S. Cooke, M. S. Kapembwa, O. M. Kon, R. D. Sampson, G. P. Taylor, and A. Lalvani. 2016. PD-1 Expression and Cytokine Secretion Profiles of *Mycobacterium tuberculosis*-Specific CD4+ T-Cell Subsets; Potential Correlates of Containment in HIV-TB Co-Infection. *PLoS One* 11: e0146905.

86. Riou, C., R. Bunjun, T. L. Muller, A. Kiravu, Z. Ginbot, T. Oni, R. Goliath, R. J. Wilkinson, and W. A. Burgers. 2016. Selective reduction of IFN-gamma single positive mycobacteria-specific CD4+ T cells in HIV-1 infected individuals with latent tuberculosis infection. *Tuberculosis (Edinb)* 101: 25-30.

87. Amelio, P., D. Portevin, J. Hella, K. Reither, L. Kamwela, O. Lweno, A. Tumbo, L. Geoffrey, K. Ohmiti, S. Ding, G. Pantaleo, C. Daubenberger, and M. Perreau.

2019. HIV Infection Functionally Impairs *Mycobacterium tuberculosis*-Specific CD4 and CD8 T-Cell Responses. *J Virol* 93.

88. Attanasio, J., and E. J. Wherry. 2016. Costimulatory and Coinhibitory Receptor Pathways in Infectious Disease. *Immunity* 44: 1052-1068.

89. Doering, T. A., A. Crawford, J. M. Angelosanto, M. A. Paley, C. G. Ziegler, and E. J. Wherry. 2012. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. *Immunity* 37: 1130-1144.

90. Odorizzi, P. M., and E. J. Wherry. 2012. Inhibitory receptors on lymphocytes: insights from infections. *J Immunol* 188: 2957-2965.

91. Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali, and E. J. Wherry. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10: 29-37.

92. Crawford, A., J. M. Angelosanto, C. Kao, T. A. Doering, P. M. Odorizzi, B. E. Barnett, and E. J. Wherry. 2014. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40: 289-302.

93. Wykes, M. N., and S. R. Lewin. 2018. Immune checkpoint blockade in infectious diseases. *Nat Rev Immunol* 18: 91-104.

94. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression

on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350-354.

95. Kaufmann, D. E., D. G. Kavanagh, F. Pereyra, J. J. Zaunders, E. W. Mackey, T. Miura, S. Palmer, M. Brockman, A. Rathod, A. Piechocka-Trocha, B. Baker, B. Zhu, S. Le Gall, M. T. Waring, R. Ahern, K. Moss, A. D. Kelleher, J. M. Coffin, G. J. Freeman, E. S. Rosenberg, and B. D. Walker. 2007. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol* 8: 1246-1254.

96. Kassu, A., R. A. Marcus, M. B. D'Souza, E. A. Kelly-McKnight, L. Golden-Mason, R. Akkina, A. P. Fontenot, C. C. Wilson, and B. E. Palmer. 2010. Regulation of virus-specific CD4+ T cell function by multiple costimulatory receptors during chronic HIV infection. *J Immunol* 185: 3007-3018.

97. Teigler, J. E., G. Zelinskyy, M. A. Eller, D. L. Bolton, M. Marovich, A. D. Gordon, A. Alrubayyi, G. Alter, M. L. Robb, J. N. Martin, S. G. Deeks, N. L. Michael, U. Dittmer, and H. Streeck. 2017. Differential Inhibitory Receptor Expression on T Cells Delineates Functional Capacities in Chronic Viral Infection. *J Virol* 91: e01263-01217.

98. Porichis, F., D. S. Kwon, J. Zupkosky, D. P. Tighe, A. McMullen, M. A. Brockman, D. F. Pavlik, M. Rodriguez-Garcia, F. Pereyra, G. J. Freeman, D. G. Kavanagh, and D. E. Kaufmann. 2011. Responsiveness of HIV-specific CD4 T cells to PD-1 blockade. *Blood* 118: 965-974.

99. Day, C. L., D. A. Abrahams, L. Lerumo, E. Janse van Rensburg, L. Stone, T. O'Rie, B. Pienaar, M. de Kock, G. Kaplan, H. Mahomed, K. Dheda, and W. A. Hanekom. 2011. Functional Capacity of *Mycobacterium tuberculosis*-Specific T Cell

Responses in Humans Is Associated with Mycobacterial Load. *J Immunol* 187: 2222-2232.

100. Kern, F., T. Bunde, N. Faulhaber, F. Kiecker, E. Khatamzas, I. M. Rudawski, A. Pruss, J. W. Gratama, R. Volkmer-Engert, R. Ewert, P. Reinke, H. D. Volk, and L. J. Picker. 2002. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. *J Infect Dis* 185: 1709-1716.

101. Kern, F., N. Faulhaber, C. Frommel, E. Khatamzas, S. Prosch, C. Schonemann, I. Kretzschmar, R. Volkmer-Engert, H. D. Volk, and P. Reinke. 2000. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. *Eur J Immunol* 30: 1676-1682.

102. Maecker, H. T., H. S. Dunn, M. A. Suni, E. Khatamzas, C. J. Pitcher, T. Bunde, N. Persaud, W. Trigona, T. M. Fu, E. Sinclair, B. M. Bredt, J. M. McCune, V. C. Maino, F. Kern, and L. J. Picker. 2001. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J Immunol Methods* 255: 27-40.

103. Finak, G., A. McDavid, P. Chattopadhyay, M. Dominguez, S. De Rosa, M. Roederer, and R. Gottardo. 2014. Mixture models for single-cell assays with applications to vaccine studies. *Biostatistics* 15: 87-101.

104. R Development Core Team. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

105. Harari, A., V. Dutoit, C. Cellerai, P. A. Bart, R. A. Du Pasquier, and G. Pantaleo. 2006. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol Rev* 211: 236-254.

106. Zhang, Z., X. Xu, J. Lu, S. Zhang, L. Gu, J. Fu, L. Jin, H. Li, M. Zhao, J. Zhang, H. Wu, L. Su, Y. X. Fu, and F. S. Wang. 2011. B and T lymphocyte attenuator down-regulation by HIV-1 depends on type I interferon and contributes to T-cell hyperactivation. *J Infect Dis* 203: 1668-1678.

107. Wherry, E. J. 2011. T cell exhaustion. *Nat Immunol* 12: 492-499.

108. Lewinsohn, D. A., D. M. Lewinsohn, and T. J. Scriba. 2017. Polyfunctional CD4(+) T Cells As Targets for Tuberculosis Vaccination. *Frontiers in immunology* 8: 1262.

109. Borsuk, S., J. Newcombe, T. A. Mendum, O. A. Dellagostin, and J. McFadden. 2009. Identification of proteins from tuberculin purified protein derivative (PPD) by LC-MS/MS. *Tuberculosis (Edinb)* 89: 423-430.

110. Cho, Y. S., K. M. Dobos, J. Prenni, H. Yang, A. Hess, I. Rosenkrands, P. Andersen, S. W. Ryoo, G. H. Bai, M. J. Brennan, A. Izzo, H. Bielefeldt-Ohmann, and J. T. Belisle. 2012. Deciphering the proteome of the in vivo diagnostic reagent "purified protein derivative" from *Mycobacterium tuberculosis*. *Proteomics* 12: 979-991.

111. Pollock, K. M., H. S. Whitworth, D. J. Montamat-Sicotte, L. Grass, G. S. Cooke, M. S. Kapembwa, O. M. Kon, R. D. Sampson, G. P. Taylor, and A. Lalvani. 2013. T-cell immunophenotyping distinguishes active from latent tuberculosis. *J Infect Dis* 208: 952-968.

112. Riou, C., N. Berkowitz, R. Goliath, W. A. Burgers, and R. J. Wilkinson. 2017. Analysis of the Phenotype of *Mycobacterium tuberculosis*-Specific CD4+ T Cells to Discriminate Latent from Active Tuberculosis in HIV-Uninfected and HIV-Infected Individuals. *Frontiers in immunology* 8: 968.

113. Strickland, N., T. L. Muller, N. Berkowitz, R. Goliath, M. N. Carrington, R. J. Wilkinson, W. A. Burgers, and C. Riou. 2017. Characterization of *Mycobacterium tuberculosis*-Specific Cells Using MHC Class II Tetramers Reveals Phenotypic Differences Related to HIV Infection and Tuberculosis Disease. *J Immunol* 199: 2440-2450.

114. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15: 486-499.

115. Piasecka, B., D. Duffy, A. Urrutia, H. Quach, E. Patin, C. Posseme, J. Bergstedt, B. Charbit, V. Rouilly, C. R. MacPherson, M. Hasan, B. Albaud, D. Gentien, J. Fellay, M. L. Albert, L. Quintana-Murci, and C. Milieu Interieur. 2018. Distinctive roles of age, sex, and genetics in shaping transcriptional variation of human immune responses to microbial challenges. *Proc Natl Acad Sci U S A* 115: E488-E497.

116. Schmiedel, B. J., D. Singh, A. Madrigal, A. G. Valdovino-Gonzalez, B. M. White, J. Zapardiel-Gonzalo, B. Ha, G. Altay, J. A. Greenbaum, G. McVicker, G. Seumois, A. Rao, M. Kronenberg, B. Peters, and P. Vijayanand. 2018. Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell* 175: 1701-1715 e1716.

117. Sterling, T. R., P. A. Pham, and R. E. Chaisson. 2010. HIV infection-related tuberculosis: clinical manifestations and treatment. *Clin Infect Dis* 50 Suppl 3: S223-230.

118. Watanabe, N., M. Gavrieli, J. R. Sedy, J. Yang, F. Fallarino, S. K. Loftin, M. A. Hurchla, N. Zimmerman, J. Sim, X. Zang, T. L. Murphy, J. H. Russell, J. P. Allison, and K. M. Murphy. 2003. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 4: 670-679.

119. Gonzalez, L. C., K. M. Loyet, J. Calemine-Fenaux, V. Chauhan, B. Wranik, W. Ouyang, and D. L. Eaton. 2005. A coreceptor interaction between the CD28 and TNF receptor family members B and T lymphocyte attenuator and herpesvirus entry mediator. *Proc Natl Acad Sci U S A* 102: 1116-1121.

120. Sedy, J. R., M. Gavrieli, K. G. Potter, M. A. Hurchla, R. C. Lindsley, K. Hildner, S. Scheu, K. Pfeffer, C. F. Ware, T. L. Murphy, and K. M. Murphy. 2005. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol* 6: 90-98.

121. Han, P., O. D. Goularte, K. Rufner, B. Wilkinson, and J. Kaye. 2004. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. *J Immunol* 172: 5931-5939.

122. Murphy, T. L., and K. M. Murphy. 2010. Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM. *Annu Rev Immunol* 28: 389-411.

123. Deppong, C., T. I. Juehne, M. Hurchla, L. D. Friend, D. D. Shah, C. M. Rose, T. L. Bricker, L. P. Shornick, E. C. Crouch, T. L. Murphy, M. J. Holtzman, K. M. Murphy, and J. M. Green. 2006. Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. *J Immunol* 176: 3909-3913.

124. Hurchla, M. A., J. R. Sedy, and K. M. Murphy. 2007. Unexpected role of B and T lymphocyte attenuator in sustaining cell survival during chronic allostimulation. *J Immunol* 178: 6073-6082.

125. Flynn, R., T. Hutchinson, K. M. Murphy, C. F. Ware, M. Croft, and S. Salek-Ardakani. 2013. CD8 T cell memory to a viral pathogen requires trans cosignaling between HVEM and BTLA. *PLoS One* 8: e77991.

126. Kaufmann, D. E., and B. D. Walker. 2009. PD-1 and CTLA-4 inhibitory cosignaling pathways in HIV infection and the potential for therapeutic intervention. *J Immunol* 182: 5891-5897.

127. Wong, E. A., L. Joslyn, N. L. Grant, E. Klein, P. L. Lin, D. E. Kirschner, and J. L. Flynn. 2018. Low Levels of T Cell Exhaustion in Tuberculous Lung Granulomas. *Infect Immun* 86: e00426-00418.

128. Jayaraman, P., M. K. Jacques, C. Zhu, K. M. Steblenko, B. L. Stowell, A. Madi, A. C. Anderson, V. K. Kuchroo, and S. M. Behar. 2016. TIM3 Mediates T Cell Exhaustion during *Mycobacterium tuberculosis* Infection. *PLoS Pathog* 12: e1005490.

129. Qiu, Y., J. Chen, H. Liao, Y. Zhang, H. Wang, S. Li, Y. Luo, D. Fang, G. Li, B. Zhou, L. Shen, C. Y. Chen, D. Huang, J. Cai, K. Cao, L. Jiang, G. Zeng, and Z. W. Chen. 2012. Tim-3-expressing CD4+ and CD8+ T cells in human tuberculosis (TB) exhibit polarized effector memory phenotypes and stronger anti-TB effector functions. *PLoS Pathog* 8: e1002984.

130. Murray, L. W., I. Satti, J. Meyerowitz, M. Jones, C. B. Willberg, J. E. Ussher, D. Goedhals, J. Hurst, R. E. Phillips, H. McShane, C. V. Vuuren, and J. Frater. 2018. Human Immunodeficiency Virus Infection Impairs Th1 and Th17 *Mycobacterium tuberculosis*-Specific T-Cell Responses. *J Infect Dis* 217: 1782-1792.

131. Wilkinson, K. A., T. Oni, H. P. Gideon, R. Goliath, R. J. Wilkinson, and C. Riou. 2016. Activation Profile of *Mycobacterium tuberculosis*-Specific CD4(+) T Cells Reflects Disease Activity Irrespective of HIV Status. *Am J Respir Crit Care Med* 193: 1307-1310.

132. Zhang, M., J. Gong, D. V. Iyer, B. E. Jones, R. L. Modlin, and P. F. Barnes. 1994. T cell cytokine responses in persons with tuberculosis and human immunodeficiency virus infection. *J Clin Invest* 94: 2435-2442.

133. Walzl, G., R. McNerney, N. du Plessis, M. Bates, T. D. McHugh, N. N. Chegou, and A. Zumla. 2018. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. *Lancet Infect Dis* 18: e199-e210.

134. Lu, L. L., M. T. Smith, K. K. Q. Yu, C. Luedemann, T. J. Suscovich, P. S. Grace, A. Cain, W. H. Yu, T. R. McKitrick, D. Lauffenburger, R. D. Cummings, H. Mayanja-Kizza, T. R. Hawn, W. H. Boom, C. M. Stein, S. M. Fortune, C. Seshadri, and G. Alter. 2019. IFN-gamma-independent immune markers of *Mycobacterium tuberculosis* exposure. *Nat Med* 25: 977-987.

135. Dan, J. M., C. S. Lindestam Arlehamn, D. Weiskopf, R. da Silva Antunes, C. Havenar-Daughton, S. M. Reiss, M. Brigger, M. Bothwell, A. Sette, and S. Crotty. 2016. A Cytokine-Independent Approach To Identify Antigen-Specific Human Germinal Center T Follicular Helper Cells and Rare Antigen-Specific CD4+ T Cells in Blood. *J Immunol* 197: 983-993.

136. Porichis, F., M. G. Hart, J. Zupkosky, L. Barblu, D. S. Kwon, A. McMullen, T. Brennan, R. Ahmed, G. J. Freeman, D. G. Kavanagh, and D. E. Kaufmann. 2014. Differential impact of PD-1 and/or interleukin-10 blockade on HIV-1-specific CD4 T cell and antigen-presenting cell functions. *J Virol* 88: 2508-2518.

137. Reiss, S., A. E. Baxter, K. M. Cirelli, J. M. Dan, A. Morou, A. Daigneault, N. Brassard, G. Silvestri, J. P. Routy, C. Havenar-Daughton, S. Crotty, and D. E. Kaufmann. 2017. Comparative analysis of activation induced marker (AIM)

assays for sensitive identification of antigen-specific CD4 T cells. *PLoS One* 12: e0186998.

138. da Silva Antunes, R., S. Paul, J. Sidney, D. Weiskopf, J. M. Dan, E. Phillips, S. Mallal, S. Crotty, A. Sette, and C. S. Lindestam Arlehamn. 2017. Definition of Human Epitopes Recognized in Tetanus Toxoid and Development of an Assay Strategy to Detect Ex Vivo Tetanus CD4+ T Cell Responses. *PLoS One* 12: e0169086.

139. Frentsch, M., O. Arbach, D. Kirchhoff, B. Moewes, M. Worm, M. Rothe, A. Scheffold, and A. Thiel. 2005. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. *Nat Med* 11: 1118-1124.

140. Lin, L., G. Finak, K. Ushey, C. Seshadri, T. R. Hawn, N. Frahm, T. J. Scriba, H. Mahomed, W. Hanekom, P. A. Bart, G. Pantaleo, G. D. Tomaras, S. Rerks-Ngarm, J. Kaewkungwal, S. Nitayaphan, P. Pitisuttithum, N. L. Michael, J. H. Kim, M. L. Robb, R. J. O'Connell, N. Karasavvas, P. Gilbert, C. D. R. S, M. J. McElrath, and R. Gottardo. 2015. COMPASS identifies T-cell subsets correlated with clinical outcomes. *Nature biotechnology* 33: 610-616.

141. Penn-Nicholson, A., E. Nemes, W. A. Hanekom, M. Hatherill, and T. J. Scriba. 2015. *Mycobacterium tuberculosis*-specific CD4 T cells are the principal source of IFN-gamma in QuantiFERON assays in healthy persons. *Tuberculosis (Edinb)* 95: 350-351.

142. Gallegos, A. M., J. W. van Heijst, M. Samstein, X. Su, E. G. Pamer, and M. S. Glickman. 2011. A gamma interferon independent mechanism of CD4 T cell mediated control of *M. tuberculosis* infection in vivo. *PLoS Pathog* 7: e1002052.

143. Sakai, S., K. D. Kauffman, M. A. Sallin, A. H. Sharpe, H. A. Young, V. V. Ganusov, and D. L. Barber. 2016. CD4 T Cell-Derived IFN-gamma Plays a Minimal Role in Control of Pulmonary *Mycobacterium tuberculosis* Infection and Must Be Actively Repressed by PD-1 to Prevent Lethal Disease. *PLoS Pathog* 12: e1005667.

144. Havenar-Daughton, C., S. M. Reiss, D. G. Carnathan, J. E. Wu, K. Kendric, A. Torrents de la Pena, S. P. Kasturi, J. M. Dan, M. Bothwell, R. W. Sanders, B. Pulendran, G. Silvestri, and S. Crotty. 2016. Cytokine-Independent Detection of Antigen-Specific Germinal Center T Follicular Helper Cells in Immunized Nonhuman Primates Using a Live Cell Activation-Induced Marker Technique. *J Immunol* 197: 994-1002.

145. Okoi, C., S. T. B. Anderson, M. Antonio, S. N. Mulwa, F. Gehre, and I. M. O. Adetifa. 2017. Non-tuberculous Mycobacteria isolated from Pulmonary samples in sub-Saharan Africa - A Systematic Review and Meta Analyses. *Sci Rep* 7: 12002.

146. Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107: 4781-4789.

147. Kannanganat, S., C. Ibegbu, L. Chennareddi, H. L. Robinson, and R. R. Amara. 2007. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *J Virol* 81: 8468-8476.

148. Bhuju, S., E. Aranday-Cortes, B. Villarreal-Ramos, Z. Xing, M. Singh, and H. M. Vordermeier. 2012. Global gene transcriptome analysis in vaccinated cattle

revealed a dominant role of IL-22 for protection against bovine tuberculosis. *PLoS Pathog* 8: e1003077.

149. Khader, S. A., G. K. Bell, J. E. Pearl, J. J. Fountain, J. Rangel-Moreno, G. E. Cilley, F. Shen, S. M. Eaton, S. L. Gaffen, S. L. Swain, R. M. Locksley, L. Haynes, T. D. Randall, and A. M. Cooper. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol* 8: 369-377.

150. Scriba, T. J., B. Kalsdorf, D. A. Abrahams, F. Isaacs, J. Hofmeister, G. Black, H. Y. Hassan, R. J. Wilkinson, G. Walzl, S. J. Gelderbloem, H. Mahomed, G. D. Hussey, and W. A. Hanekom. 2008. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol* 180: 1962-1970.

151. Scriba, T. J., A. Penn-Nicholson, S. Shankar, T. Hraha, E. G. Thompson, D. Sterling, E. Nemes, F. Darboe, S. Suliman, L. M. Amon, H. Mahomed, M. Erasmus, W. Whatney, J. L. Johnson, W. H. Boom, M. Hatherill, J. Valvo, M. A. De Groote, U. A. Ochsner, A. Aderem, W. A. Hanekom, D. E. Zak, and A. C. S. c. s. t. other members of the. 2017. Sequential inflammatory processes define human progression from M. tuberculosis infection to tuberculosis disease. *PLoS Pathog* 13: e1006687.

152. Prendergast, A., J. G. Prado, Y. H. Kang, F. Chen, L. A. Riddell, G. Luzzi, P. Goulder, and P. Klenerman. 2010. HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. *AIDS* 24: 491-502.

153. El Hed, A., A. Khaitan, L. Kozhaya, N. Manel, D. Daskalakis, W. Borkowsky, F. Valentine, D. R. Littman, and D. Unutmaz. 2010. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. *J Infect Dis* 201: 843-854.

154. Paiardini, M., and M. Muller-Trutwin. 2013. HIV-associated chronic immune activation. *Immunol Rev* 254: 78-101.

155. Vaidya, S. A., C. Korner, M. N. Sirignano, M. Amero, S. Bazner, J. Rychert, T. M. Allen, E. S. Rosenberg, R. J. Bosch, and M. Altfeld. 2014. Tumor necrosis factor alpha is associated with viral control and early disease progression in patients with HIV type 1 infection. *J Infect Dis* 210: 1042-1046.

156. Harari, A., V. Rozot, F. B. Enders, M. Perreau, J. M. Stalder, L. P. Nicod, M. Cavassini, T. Calandra, C. L. Blanchet, K. Jaton, M. Faouzi, C. L. Day, W. A. Hanekom, P. A. Bart, and G. Pantaleo. 2011. Dominant TNF-alpha(+) *Mycobacterium tuberculosis*-specific CD4(+) T cell responses discriminate between latent infection and active disease. *Nat Med* 17: 372-376.

CHAPTER VI

APPENDIX

Unpublished Data on Inhibitory Receptor Phenotype of *Mtb*-specific CD8 T cells

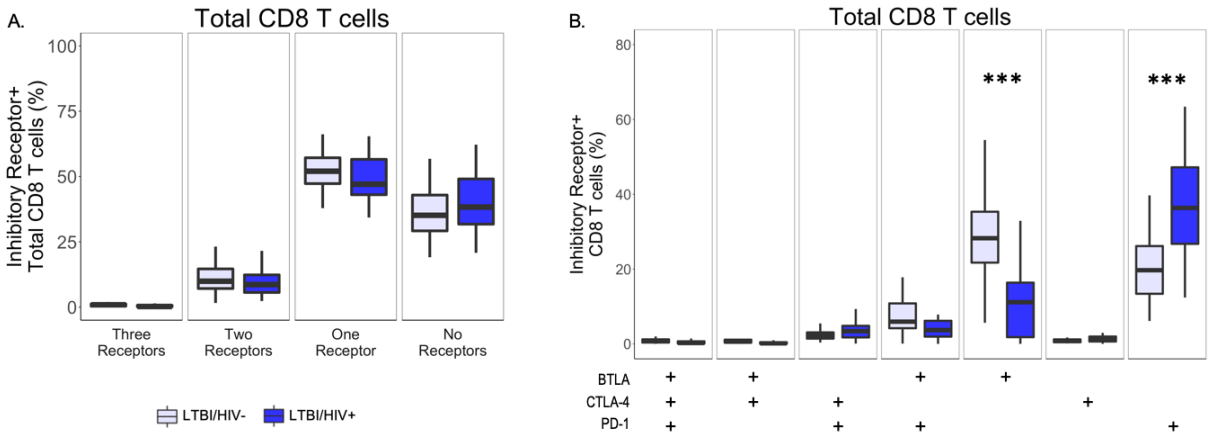

Figure 1. Similar frequencies of inhibitory receptor co-expression on total CD8 T cells in HIV-infected and HIV-uninfected individuals with LTBI.

PBMCs from HIV-uninfected and HIV-infected individuals with LTBI (n=32 and n=22, respectively) were analyzed by flow cytometry for expression of the inhibitory receptors BTLA, CTLA-4, and PD-1 on total CD8 T cells. (A) Summary data representing the proportion of total CD8 T cells expressing three, two, one, or no inhibitory receptors from HIV-uninfected and HIV-infected individuals with LTBI. (B) The frequencies of total CD8 T cells expressing each combination of BTLA, CTLA-4 and PD-1 are shown for individuals with LTBI. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the proportion of CD8 T cells expressing each inhibitory receptor population between HIV-uninfected and HIV-infected individuals with LTBI (A, B) were assessed using a Mann-Whitney *U* test. *** $p < 0.001$.

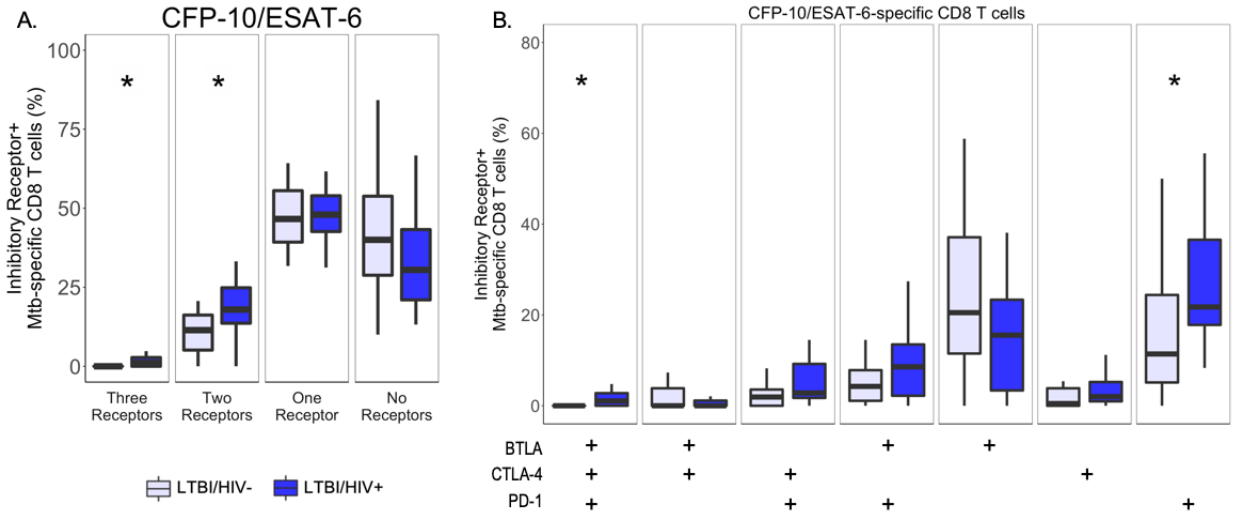

Figure 2. HIV infection is associated with upregulation of PD-1 expression on *Mtb*-specific CD8 T cells in individuals with LTBI. PBMCs from HIV-uninfected and HIV-infected individuals were stimulated with CFP-10 and ESAT-6 peptide pools and evaluated for expression of IFN-γ and TNF-α by flow cytometry. *Mtb*-specific CD8 T cells meeting the criteria for a positive response (see Chapter II, Materials and Methods) were evaluated for expression of BTLA, CTLA-4, and PD-1. (A) Composite data from individuals with LTBI indicating the proportion of CFP-10/ESAT-6-specific CD8 T cells expressing three, two, one or no inhibitory receptors. (B) Composite data from individuals with LTBI indicating the proportion of CFP-10/ESAT-6-specific CD8 T cells expressing the indicated combinations of BTLA, PD-1 and CTLA-4 (LTBI/HIV$^-$, n=27; LTBI/HIV$^+$, n=17). Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the proportions of *Mtb*-specific CD8 T cells expressing the indicated inhibitory receptors between HIV-uninfected and HIV-infected individuals were assessed using a Mann-Whitney *U* test. * $p < 0.05$.

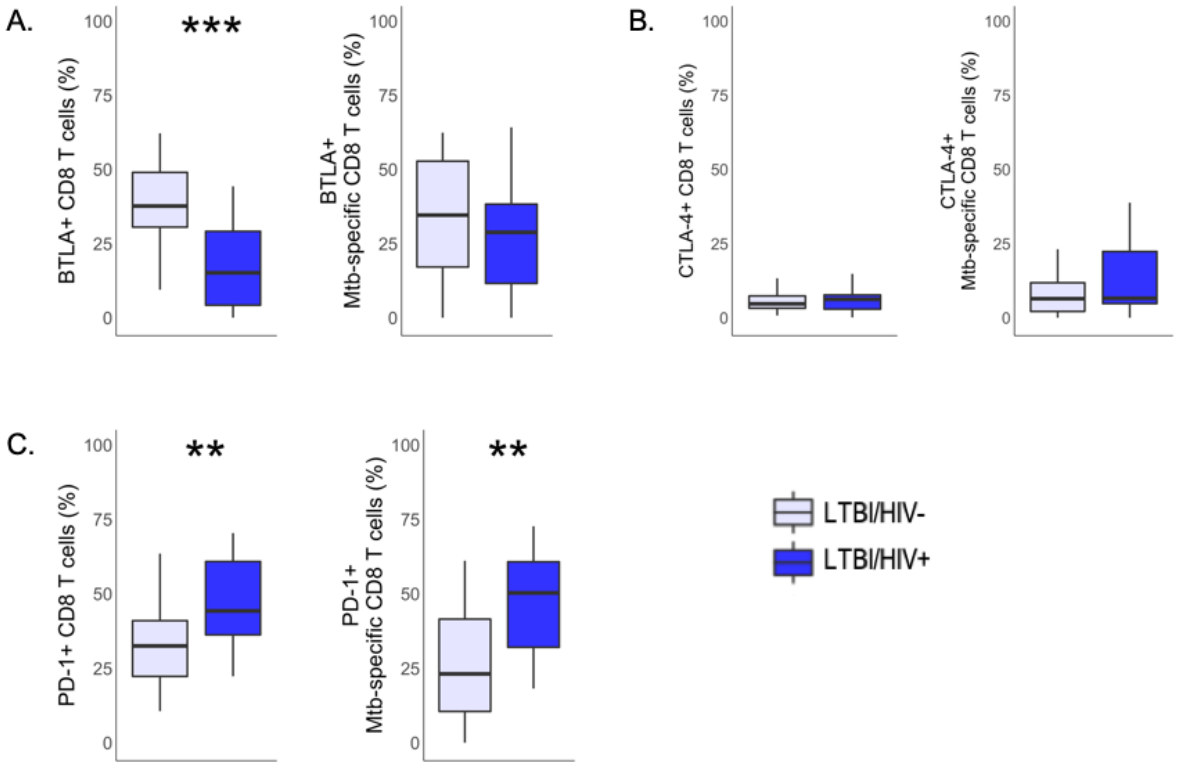

Figure 3. BTLA, CTLA-4 and PD-1 expression on total and *Mtb*-specific CD8 T cells in individuals with latent *Mtb* infection. PBMCs from HIV-uninfected and HIV-infected individuals with LTBI were either left unstimulated or stimulated with CFP-10 and ESAT-6 peptide pools. Composite data of the percentage of BTLA (A), CTLA-4 (B) and PD-1 (C) expression on total unstimulated CD8 T cells (*left panel*) and CFP-10/ESAT-6-specific CD8 T cells (*right panel*) from individuals with LTBI. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the percentages between HIV-uninfected and HIV-infected individuals were assessed using a Mann-Whitney *U* test. ** p <0.01; *** p <0.001.

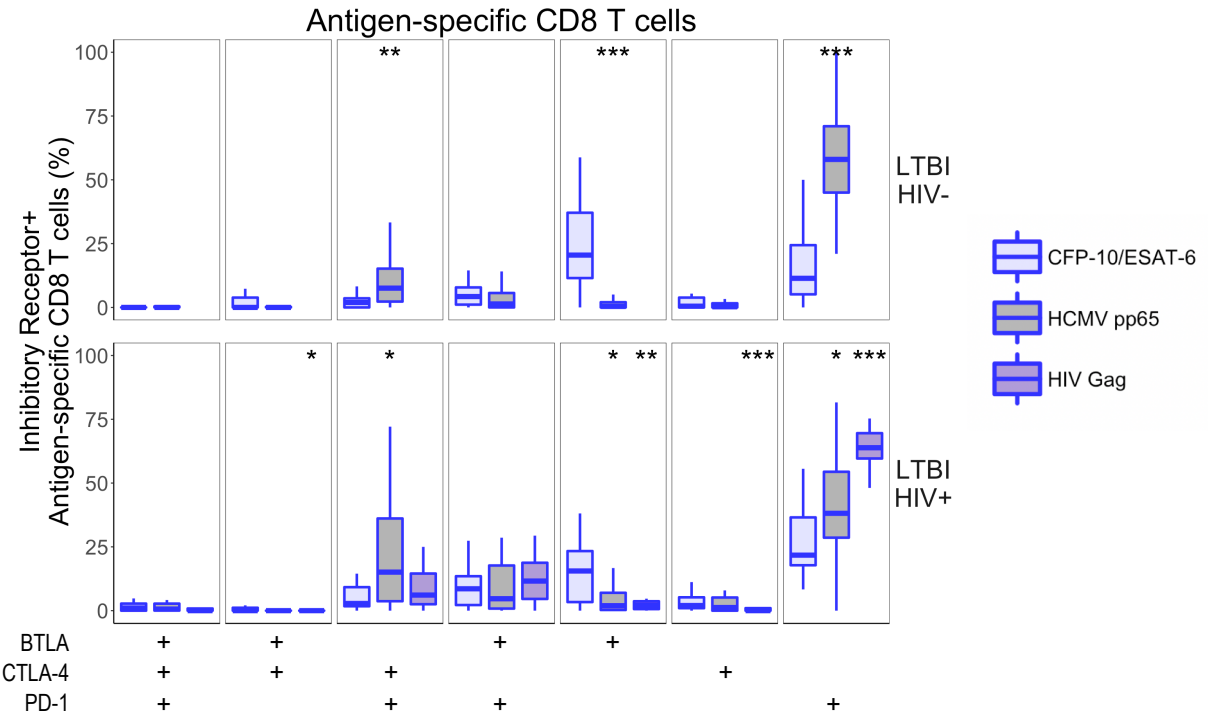

Figure 4. Differential inhibitory receptor expression profiles on *Mtb*-specific CD8 T cells, compared with HCMV- and HIV-specific CD8 T cells within the same individuals. PBMCs were stimulated with CFP-10 and ESAT-6 peptide pools, as well as HCMV pp65 peptide pool and HIV Gag peptide pool. Ag-specific CD8 T cells meeting the criteria for a positive response (see Chapter II, Materials and Methods) were evaluated for expression of BTLA, CTLA-4, and PD-1. Summary data of the proportion of Ag-specific CD8 T cells expressing the indicated subsets of inhibitory receptors in individuals with LTBI (top row, HIV-uninfected LTBI; bottom row, HIV-infected LTBI). Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the inhibitory receptor expression of *Mtb*-specific CD8 T cells was compared with paired HCMV- and HIV-specific CD8 T cells from the same individuals using a Wilcoxon matched pairs test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

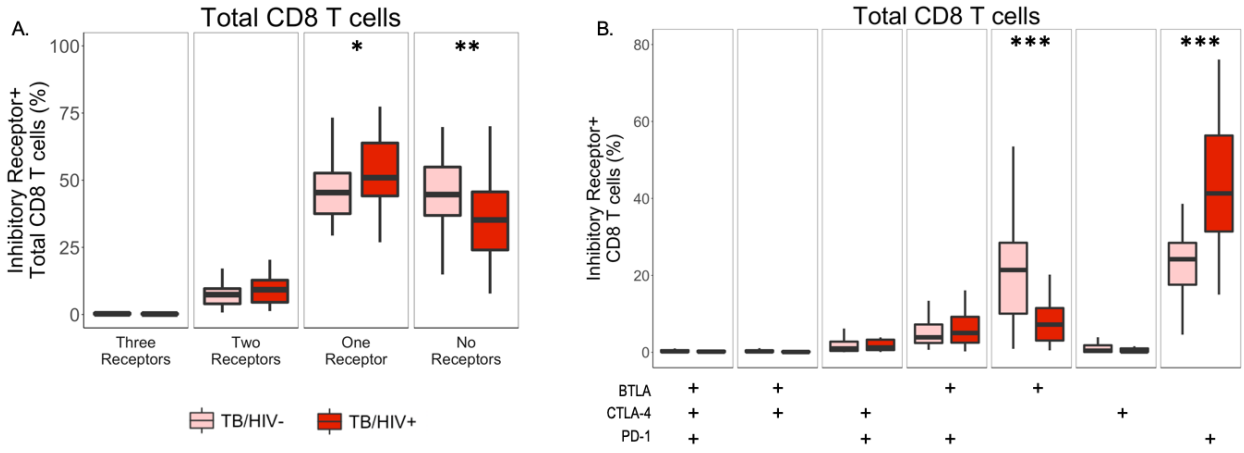

Figure 5. Similar frequencies of inhibitory receptor co-expression on total CD8 T cells in HIV-infected and HIV-uninfected individuals with active TB. PBMCs from HIV-uninfected and HIV-infected individuals with active TB (n=37 and n=19, respectively) were analyzed by flow cytometry for expression of the inhibitory receptors BTLA, CTLA-4, and PD-1 on total CD8 T cells. (A) Summary data representing the proportion of total CD8 T cells expressing three, two, one, or no inhibitory receptors from HIV-uninfected and HIV-infected individuals with active TB. (B) The frequencies of total CD8 T cells expressing each combination of BTLA, CTLA-4 and PD-1 are shown for individuals with active TB. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the proportion of CD8 T cells expressing each inhibitory receptor population between HIV-uninfected and HIV-infected individuals with active TB (A, B) were assessed using a Mann-Whitney *U* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, after applying the Bonferroni correction for multiple comparisons.

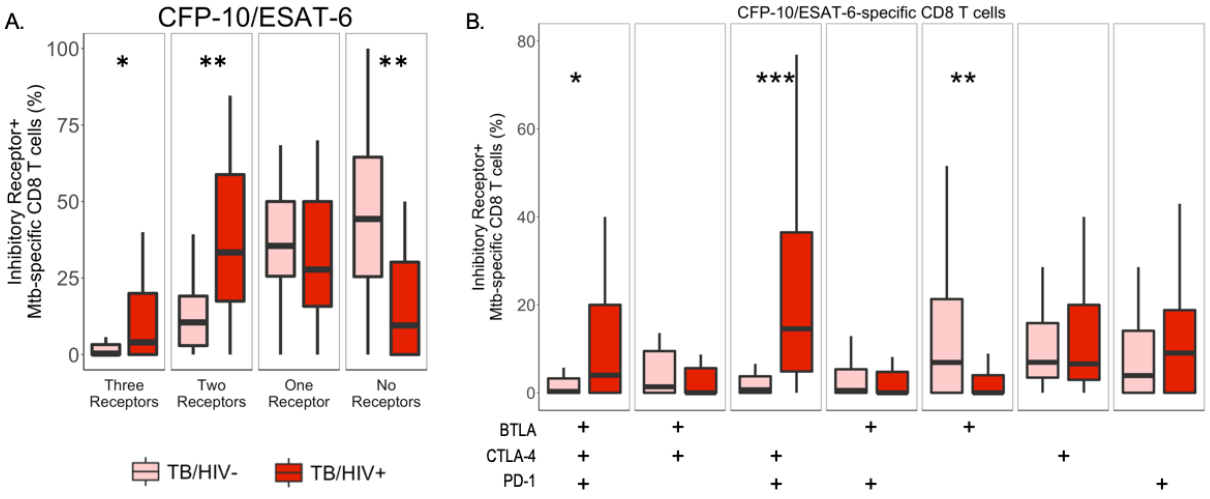

Figure 6. HIV infection is associated with upregulation of CTLA-4 and PD-1 expression and downregulation of BTLA on *Mtb*-specific CD8 T cells in individuals with active TB disease. PBMCs from HIV-uninfected and HIV-infected individuals were stimulated with CFP-10 and ESAT-6 peptide pools and evaluated for expression of IFN-γ and TNF-α by flow cytometry. *Mtb*-specific CD8 T cells meeting the criteria for a positive response (see Chapter II, Materials and Methods) were evaluated for expression of BTLA, CTLA-4, and PD-1. (A) Composite data from individuals with active TB indicating the proportion of CFP-10/ESAT-6-specific CD8 T cells expressing three, two, one or no inhibitory receptors. (B) Composite data from individuals with active TB indicating the proportion of CFP-10/ESAT-6-specific CD8 T cells expressing the indicated combinations of BTLA, PD-1 and CTLA-4 (TB/HIV$^-$, n=36; TB/HIV$^+$, n=16). Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the proportions of *Mtb*-specific CD8 T cells expressing the indicated inhibitory receptors between HIV-uninfected and HIV-infected individuals were assessed using a Mann-Whitney *U* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

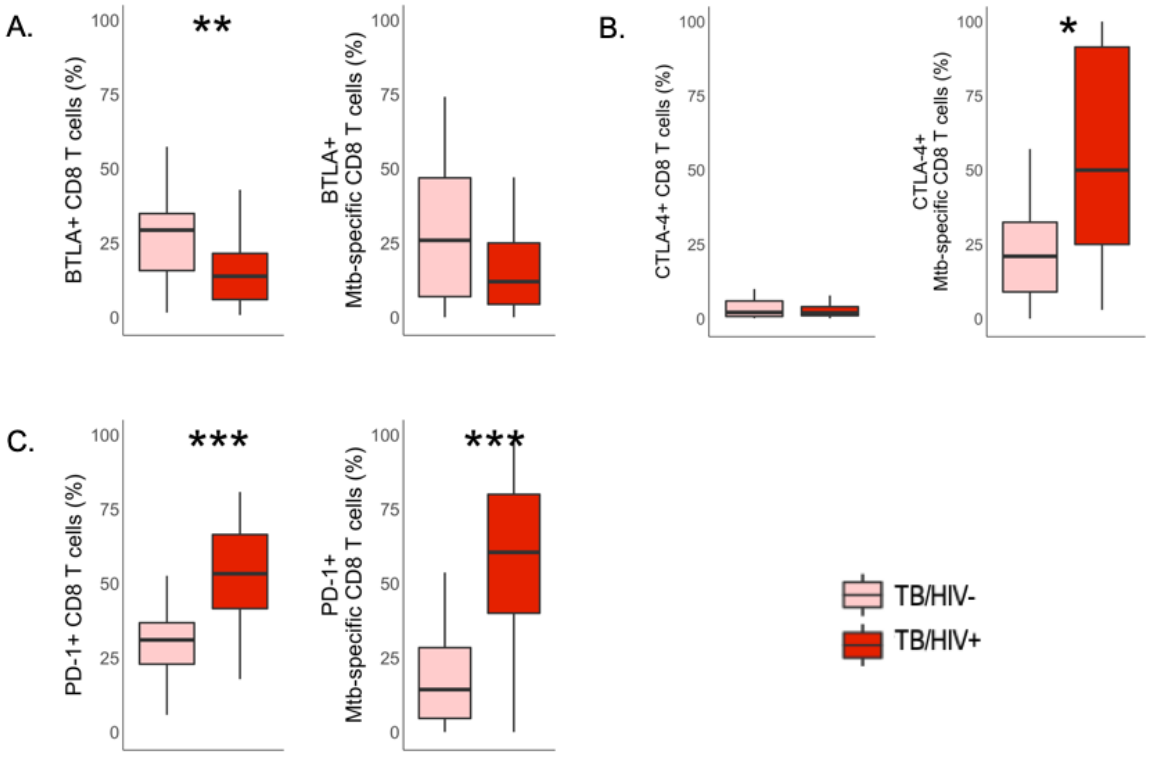

Figure 7. BTLA, CTLA-4 and PD-1 expression on total and *Mtb*-specific CD8 T cells in individuals with active TB. PBMCs from HIV-uninfected and HIV-infected individuals with active TB were either left unstimulated or stimulated with CFP-10 and ESAT-6 peptide pools. Composite data of the percentage of BTLA (A), CTLA-4 (B) and PD-1 (C) expression on total unstimulated CD8 T cells (*left panel*) and CFP-10/ESAT-6-specific CD8 T cells (*right panel*) from individuals with active TB. Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the percentages between HIV-uninfected and HIV-infected individuals were assessed using a Mann-Whitney *U* test.

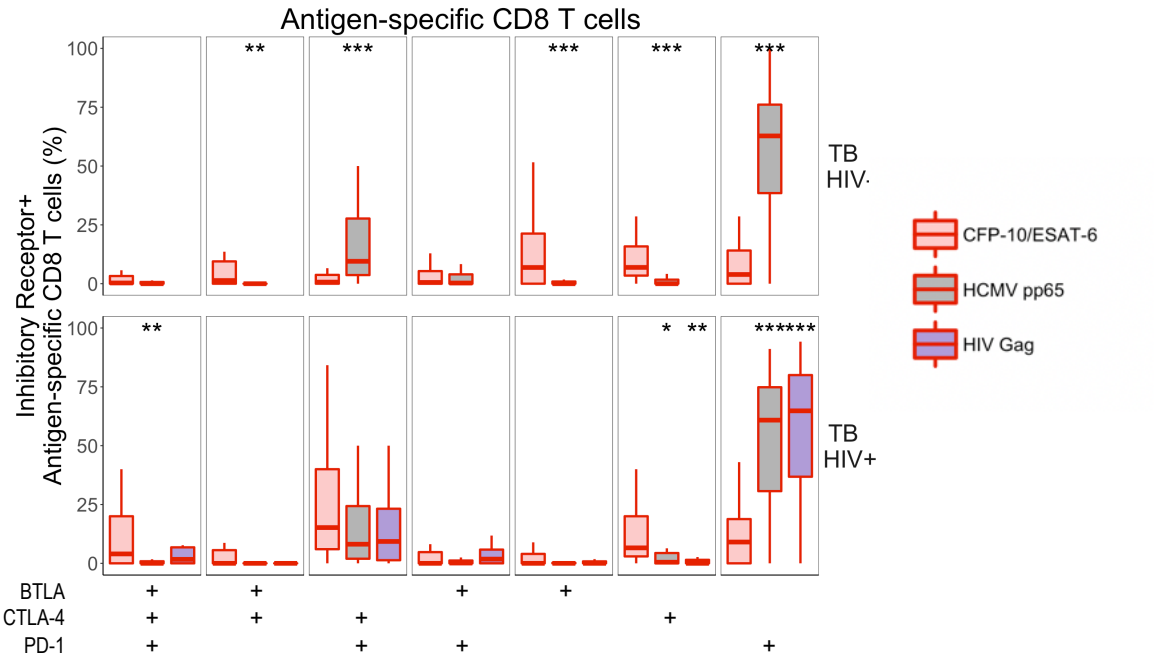

Figure 8. Differential inhibitory receptor expression profiles on *Mtb*-specific CD8 T cells, compared with HCMV- and HIV-specific CD8 T cells within the same individuals. PBMCs were stimulated with CFP-10 and ESAT-6 peptide pools, as well as HCMV pp65 peptide pool and HIV Gag peptide pool. Ag-specific CD8 T cells meeting the criteria for a positive response (see Chapter II, Materials and Methods) were evaluated for expression of BTLA, CTLA-4, and PD-1. Summary data of the proportion of Ag-specific CD8 T cells expressing the indicated subsets of inhibitory receptors in individuals with active TB (top row, HIV-uninfected TB; bottom row, HIV-infected TB). Boxes represent the median and interquartile ranges; whiskers represent the 5th and 95th percentiles. Differences in the inhibitory receptor expression of *Mtb*-specific CD8 T cells was compared with paired HCMV- and HIV-specific CD8 T cells from the same individuals using a Wilcoxon matched pairs test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.