SIV increases susceptibility to tuberculosis by manipulating *M. tuberculosis*-specific immunological responses

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Submitted to the Graduate Faculty of

Molecular Virology and Microbiology in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

School of Medicine

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February 16th, 2012

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ABSTRACT

The emergence of human immunodeficiency virus-1 (HIV) exacerbated the already enormous number of cases of tuberculosis (TB) worldwide because HIV dramatically increases the susceptibility of humans to TB. The cause of this increased susceptibility to TB in HIV-infected individuals is not fully understood. In this thesis, I developed an SIV/TB cynomolgus macaque model and multiple *in vitro* and *ex vivo* assays to examine how HIV induces reactivation of latent TB and how HIV reduces various *M. tuberculosis*-specific immunological responses. In the animal model, I provide evidence that initial T cell depletion after SIV inoculation in monkeys with latent TB perturbs the control of the infection, and directly corresponds to the reactivation of TB. There was also a quantifiable decrease in the total number of T cells within granulomatous lung tissue in co-infected reactivated animals compared to animals with active TB without SIV. A significant decrease in IFN-γ releasing T cells in lung draining lymph nodes of co-infected monkeys compared to monkeys with active disease also was observed. In *in vitro* and *ex vivo* experiments we determined that the addition of exogenous SIVmac251 to cells from monkeys infected with *M. tuberculosis* (without SIV) leads to a specific decrease in TNF and

IFN-γ production in *M. tuberculosis*-specific T cells between 12 and 24hrs after incubation. This decrease in T cell cytokine production was caused by SIV-induced disruption in antigen presenting cells. Specifically, the decrease in TNF production by *M. tuberculosis*-specific T cells was caused, at least in part, by SIV-induced IL-5 production by monocytes, which demonstrates a novel role for IL-5. The wide range of experiments implemented in this thesis provides novel evidence for the increased susceptibility of TB in HIV-infected individuals.

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1.0 ACKNOWLEDGEMENTS

I'd like to first thank JoAnne for all of her support throughout all of these incredibly difficult graduate school years. JoAnne has an unbelievable ability to push me to be the best scientist I can be. I couldn't have done this without you! THANK YOU!

I'd also like to thank everyone in lab for being so supportive as well. I loved grad school and I have you to thank for that. Thanks for making lab so much fun! I am also grateful for all the help you all provided (at one time or another) in teaching me new techniques, re-teaching me those techniques when I forgot, helping out with experiments when stuff was totally crazy, helping me write, and always being there to talk to about ANYTHING (even though HR would have frowned upon those conversations). Also, thanks for dealing with me saying everything is SUPER AWESOME even when some things were just plain AWESOME. THANK YOU!

I really want to thank the 'un-sung' heroes of our lab. These are the people that work/worked at RIDC and the RBL over my tenure. Every single data point I'm presenting in this thesis (and my papers) is all here because of you people. Your work makes our science possible and I can't thank you enough for that. THANK YOU!

I'm in grad school because of my *whole damn family*. I have 2 learning disabilities (a learning disorder and a reading disorder), which has always made school really difficult for me. My family's unwavering support throughout my entire life has showed me how to overcome these difficulties without being a jaded-jerk. Their positive reinforcement has made me the

person I am today. Thanks for teaching me to, “Suck the marrow out of life!” My family is the sh*t! THANK YOU!

I’d also like to thank my wife. Robin, you are my You Are. You support me more than anyone else (that includes A LOT of people). You’re moving to South Africa with me so I can pursue my dream to continue my HIV/TB work! That’s pretty good! I will work my whole life to make it up to you. I love you, Cuun! Y, V Y. THANK YOU! ☺

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2.0 TUBERCULOSIS INTRODUCTOIN

2.1 THE HISTORY OF TUBERCULOSIS

Some of the earliest cases of presumed human TB date back thousands of years. Greek physician Hippocrates (460-370 BC) describes kyphosis, most likely caused by Pott's disease (*M. tuberculosis* infecting the spine) as the result of a disease that can occur above or below the diaphragm and is associated with solid tubercles in the lungs and abscesses in the lumbar region [1]. Hippocrates also gave patients poor prognoses if the spinal curvature was above the diaphragm in a child or young adult. Hippocrates was not the first to describe symptoms of TB. Multiple descriptions in ancient and historical texts appear to mention TB calling it phthisis, scrofula, King's Evil, lupus vulgaris, consumption, etc [2, 3]. As paleopathology and paleohistology, the study of ancient diseases, became more prevalent, the natural history of *M. tuberculosis* was revealed.

Mycobacterial tuberculosis complex (MtbC) amplified DNA was discovered in an extinct fossilized long-horned bison that died more than 17,000 years ago [4] and the first known case of human TB caused by MtbC occurred in ancient Egypt between 2050-1650 BC [5]. TB has also been identified in calcified and non-calcified tissues within mummified remains that include granulomas in lung and other organs [6]. These studies have confirmed that TB has evolved with

humans over thousands of years. This co-evolution with humans has allowed *M. tuberculosis* to become a resilient pathogen.

The 19th century was a remarkable time for scientists struggling to identify the cause of tuberculosis. Louis Pasteur and Robert Koch were fascinating infectious disease researchers in a bitter rivalry to identify the causative agent of TB. In 1882 Robert Koch identified the bacillus using his own scientific method called Koch's postulates [7]. Koch's work on *M. tuberculosis* continued as he identified 'giant cells' that contain and frequently 'degrade' the bacillus [8]. In 1901, Koch was one of the first to ask whether 1) people were susceptible to bovine tuberculosis and 2) bovine tuberculosis could be a potential vaccine for TB [9].

In 1908 Albert Calmette and Camille Guérin began passaging virulent *M. bovis* in an alkaline glycerin-ox bile-potato media until they developed an attenuated strain that could be used as a vaccine for TB. This attenuated form of *M. bovis* became known as Bacillus Calmette-Guerin (BCG) and was first given to children orally 48hrs after birth in the 1920s [10]. Despite the centuries of TB research, and the widespread use of the BCG vaccine in children in most countries for nearly 100 years, TB still remains a global pandemic (Figure 1).

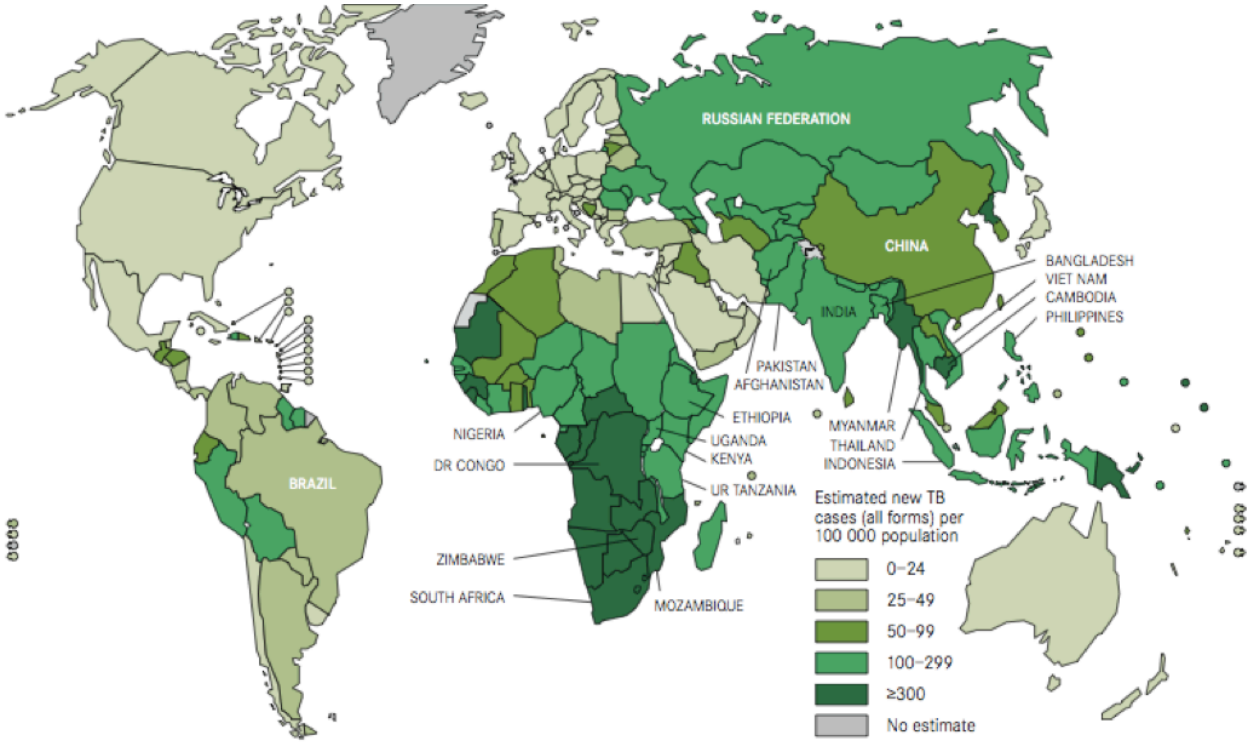

Figure 1. *TB incidence rates per country in 2010.* Heat map depicts incident cases of TB per 100,000 individuals per country. Figure was reprinted with permission from WHO 2011 Global Tuberculosis Control Report (11).

2.2 TB EPIDEMIOLOGY

Roughly one third of the world's population is infected with *M. tuberculosis* [11], although only a fraction of those present with active disease. In 2010, there were 8.8 million incident cases of active TB and 1.1 million deaths from TB among HIV-uninfected people and 350,000 deaths in HIV-infected individuals [11] (Figure 2). Although the total number of TB cases has been gradually decreasing since 2006 and TB incidence rates have been falling globally since 2002, it remains a major cause of death worldwide. The highest incident rates of TB are in sub-Saharan Africa, which is due in part to the high prevalence of HIV.

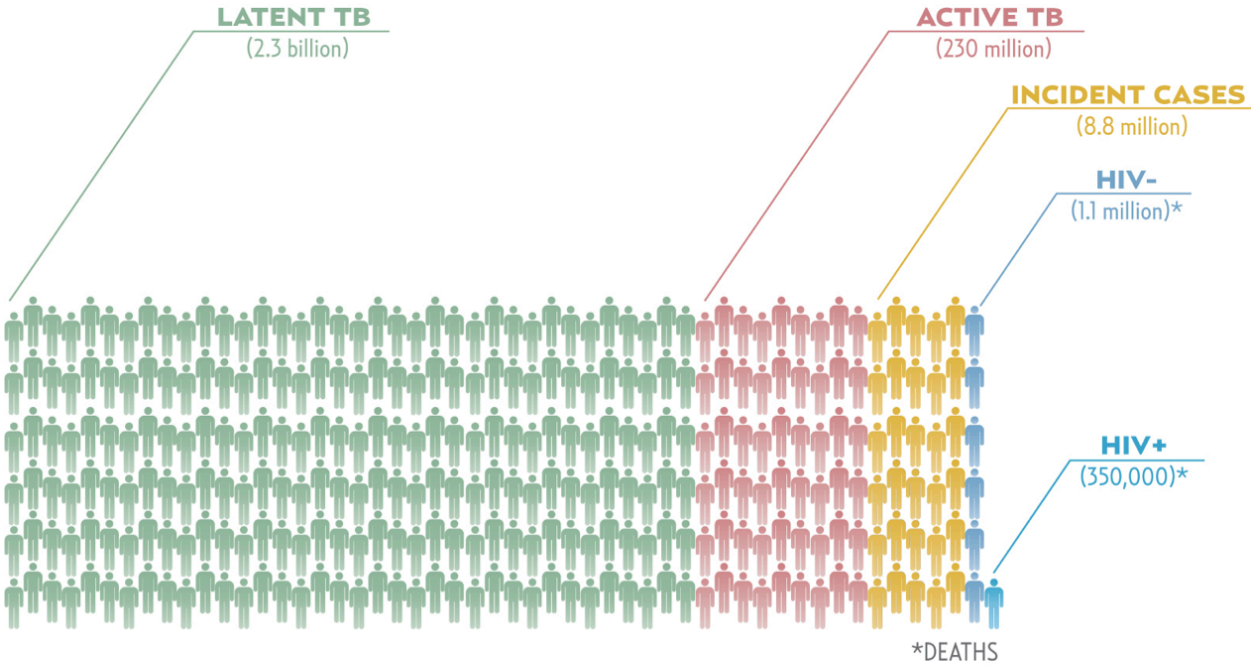

Figure 2. *2010 TB rates and death estimates.* Prevalence of individuals with latent and active TB are presented with new cases of active TB (Incident). The number of HIV-uninfected (HIV-) and HIV-infected (HIV+) individuals that died of TB in 2010 are represented. Estimates based on World Health Organization 2010 TB Control Report (11). Proportions are not to scale.

2.3 TB PATHOLOGY

M. tuberculosis is transmitted via aerosolized droplets from an individual with active TB. The bacillus infects via the respiratory tract, encountering alveolar macrophages in the airways, and transiting to the lung parenchyma, where innate and adaptive immune responses cooperate to signal that a bacterial infection is taking place. This leads to macrophage production of chemokines and proinflammatory cytokines that attract other leukocytes such as dendritic cells to the site of infection [12]. Dendritic cells phagocytose *M. tuberculosis* (either in the airways or in the parenchyma) and migrate to lung-draining lymph nodes to prime T cells. Primed T cells migrate to the infection site to form the granuloma in an attempt to wall off the infected macrophages. An asymptomatic individual with contained *M. tuberculosis* is considered to have latent TB. In contrast, active disease, in which bacterial replication and dissemination is not controlled by the granuloma, leads to the symptomatic and clinical presentation of TB and without effective treatment can be fatal [13]. The death rate of untreated TB is approximately 70% (range: 55-75%) in smear positive and 20% (10-30%) in smear negative HIV-uninfected patients (reviewed in [14]). The death rate in HIV-infected individuals with untreated TB is approximately 81% (70-99%) in patients with positive smears and 76% (63-90%) in individuals with smear negative sputum (reviewed in [14]).

The granuloma is an organized cellular structure composed of macrophages, lymphocytes, dendritic cells, neutrophils, and sometimes fibroblasts, often with a necrotic center. This structure serves to contain the bacilli, and acts as an immune microenvironment for cellular interactions that limit *M. tuberculosis* replication. However, simple formation of a granuloma is not sufficient for control of infection, as persons with active TB have multiple granulomas in the lungs and thoracic lymph nodes, and possibly extrapulmonary tissues. Instead, the granuloma

must have optimal immunologic function to contain or eliminate the bacilli. As a highly evolved pathogen, *M. tuberculosis* has devised strategies for persisting within the granuloma, and avoiding elimination by the host response. In latent infection, the host and bacillus co-exist, with the granuloma serving as the site of bacterial persistence and host resistance. Disruption of the structure or function of the granuloma is likely to lead to reactivation of latent *M. tuberculosis* infection, dissemination, and active disease.

2.4 TB INFECTION OUTCOME

The risk of becoming infected with *M. tuberculosis* upon exposure is about 25% [15], which suggests that the innate response to the bacillus is sufficient to prevent infection in the majority of immune-competent individuals. About 90-95% of the infected individuals develop asymptomatic latent TB, while the remaining develop active TB [11]. Latently infected individuals are estimated to have a 10% lifetime risk of reactivation. Co-infection with HIV dramatically increases the risk of reactivation to 10% per year [16], which will be discussed in chapter 3.

M. tuberculosis can be detected with a tuberculin skin test (TST), in which purified protein derivative (PPD) from *M. tuberculosis* is injected into the top layer of skin [17], or the newer blood tests (e.g. Interferon gamma release assays, IGRA). An individual infected with *M. tuberculosis* or vaccinated with BCG has a delayed type hypersensitivity reaction to PPD that is detected 48 to 72 hours after injection. A positive response to PPD is defined as >10 mm induration (hard swelling) at the site of injection in an immune competent individual. Individuals that are immunocompromised are considered positive with an induration of 5mm. The induration

at the injection site is caused by the recruitment of adaptive immune cells specific for mycobacteria. Epidemiologic data supports that a tuberculin test is positive in BCG-vaccinated persons for only ~5 years post-vaccine [18, 19], as the responses wane to BCG. To differentiate BCG vaccination from *M. tuberculosis* infection, the IGRA can be used, which relies on IFN-γ release to *M. tuberculosis*-specific proteins, ESAT-6 and CFP-10.

An asymptomatic individual with a positive skin test is considered to have latent TB. In contrast, active disease, in which bacterial replication and dissemination is not controlled by the granuloma; leads to the symptomatic and clinical presentation of TB and without effective treatment may be fatal [13]. Although granulomas within individuals with latent TB can sometimes be visualized on X-rays, it is difficult for radiologists to differentiate active and latent lesions. Active TB presents with a variety of symptoms, including cough, night sweats, loss of appetite, and weight loss. In many parts of the world, TB is diagnosed based on symptoms and the observation of acid-fast bacilli in sputum smears upon microscopy. Chest radiograph findings of granulomas or infiltrates can also be useful in the diagnosis [11]. *M. tuberculosis* can be cultured from sputum, gastric aspirates and bronchial alveolar fluid in individuals with active TB, and culture remains the "gold standard" for diagnosis of active TB. However, culture is not possible in all parts of the world, and a substantial fraction of those with active TB are smear or culture negative. Thus diagnosis of TB can be very challenging, especially in children, and sometimes only improvement of symptoms upon initiation of anti-tuberculous chemotherapy confirms the diagnosis of TB.

2.5 TB TREATMENT-VACCINES

In an ideal world there would be an effective vaccine for preventing TB. BCG is the most widely used vaccine ever--it has been administered to more than 4 billion individuals worldwide over the last 90+ years (reviewed in [20]). In clinical trials, the effectiveness of BCG in preventing TB ranges significantly from 0% to 85% (reviewed in [18, 20]). There are several hypotheses for the widely disparate results of these trials, including different strains and preparations of BCG vaccine used, the influence of environmental mycobacteria or helminthes, and nutritional and genetic variations among populations. It does appear that BCG can prevent the incidence of disseminated TB in children (reviewed in [18, 20]), however it fails to prevent infection or disease in most adult populations. In addition, individuals that receive a BCG vaccination as an infant without a boost in adolescence lose their ability to respond to TST [19], which suggests that the vaccine does a poor job at inducing adaptive immunity. However, boosting with later BCG administration does not improve protection [19]. Even more telling, the vast majority of TB cases and deaths occur in countries where most newborns are vaccinated with BCG, supporting that this vaccine is ineffective. There is an urgent need for a better vaccine to prevent infection and the need for improved drugs to rapidly cure TB.

Currently there are 3 objectives for development of new vaccines: 1) delay disease progression, 2) completely eradicate the infection from the host and 3) prevent infection [11]. There are 12 potential TB vaccines in clinical trials consisting of recombinant BCG vaccines, viral-vectored booster vaccines and fusion protein in adjuvant as booster vaccines (reviewed in [21]). The current vaccines are only in Phase I or II clinical trials, so any information about efficacy is years off. To make matters worse, HIV has been shown to decrease vaccine effectiveness, which is a problem because areas with the highest HIV rates have the highest TB

rates [11, 22]. The ineffectiveness of BCG and the uncertainty of TB vaccines in the pipeline necessitate the need for more effective drug treatments.

2.6 TB TREATMENT-DRUGS

The current treatment for TB is based on drugs that have been around for more than 50 years. Individuals that have newly detected drug-susceptible TB are put on a 6-month 4-drug regimen that include rifampin (targets DNA-primed RNA polymerase), isoniazid (targets enoyl-ACP reductase and mycolic acid elongation), pyrazinamide (targets fatty acid biosynthesis/membrane, depolarization/ribosomal protein S1 (RpsA), protein translation and the ribosome-sparing process of trans-translation) and ethambutol (targets cell wall arabinan deposition) given for 2 months, followed by rifampin and isoniazid for 4 months [23, 24]. Treatment for latent infection (to prevent reactivation) includes isoniazid monotherapy for 6-9 months, or a rifampin regimen for 4 months [23, 25]. New data suggests that 3 months of rifapentine and isoniazid is also effective against latent infection [26]. HIV complicates drug treatment because protease inhibitors interact with rifampin and reduce their effectiveness and compliance [23]. The emergence of multidrug resistance (MDR) *M. tuberculosis* strains has required the use of second line, less effective drugs such as kanamycin, streptomycin and fluoroquinolones [23, 24]. The long duration of treatment, the need for directly observed therapy to improve compliance high prevalence of side effects with TB drugs, HIV-infection and the development of MDR TB add to the challenges of treating TB.

2.7 TB IMMUNOLOGY

M. tuberculosis becomes phagocytosed by alveolar macrophages when it enters the airways. If the macrophages are able to kill *M. tuberculosis* immediately, then an infection will not occur. However, if the macrophages cannot prevent infection, the bacilli are transported to the lymph nodes, where a cell mediated adaptive immune response is primed. Lymphocytes and monocytes migrate to the site of infection, usually within 2 to 6 weeks post-infection. This influx leads to the formation of a granuloma. The bacilli within the granuloma may remain there for the lifetime of the host, they may disseminate within the lungs or into other tissues to form new granulomas or they may be released into the airways following granuloma cavitation, which results in the possibility of infecting other individuals.

Granulomas play an essential role in containing the mycobacteria. The architecture and composition of a granuloma is incredibly diverse (reviewed in [27, 28]). Granulomas can consist of any combination of macrophages, dendritic cells, neutrophils, B cells, T cells and fibroblasts. Human granulomas can be necrotic, solid cellular, fibrotic, suppurative and mineralized or a combination of each with well-defined or unusual characteristics. Granuloma diversity does not just occur among people, it also occurs within the same host! It is likely that each cell type has a role to play in containing *M. tuberculosis* infection.

2.7.1 Macrophages

The phagocytosis of *M. tuberculosis* by alveolar macrophages is probably the first event that takes place when *M. tuberculosis* enters the airways. *M. tuberculosis* binds to complement receptors, mannose receptors (MR), toll like receptors (TLRs) and Fcγ-receptors to gain access to

macrophages [29]. Depending on the route of entry and the cytokine milieu, the infection can induce either pro-inflammatory or anti-inflammatory responses. Th1 cytokines (IFN-γ and TNF) can induce classically activated macrophages (CAM) while Th2 cytokines (IL-4, IL-13 and IL-10) can induce alternatively activated macrophages (AAM) [30].

CAMs up-regulate MHC Class I and Class II molecules and produce proinflammatory cytokines such as IL-1, IL-6, TNF and IL-12 and are capable of killing phagocytosed mycobacteria [28, 30]. CAMs are associated with inducible nitric oxide synthase production. AAMs up-regulate MHC Class II molecules along with various receptors such as mannose receptor and are primarily involved in humoral immunity and allergic or anti-parasitic responses [30]. Since the airway is considered by some to be an anti-inflammatory environment, due to the constant exposure to pathogens and the need to prevent excessive inflammation, it is possible that alveolar macrophages are inherently alternatively activated [31], which may decrease their ability to kill *M. tuberculosis* upon infection. Although AAMs may provide a foothold for a new infection of *M. tuberculosis,* they are probably necessary to prevent excessive pathology associated with TB. This means there must be a balance between pro- and anti-inflammatory responses within a granuloma for it to function properly.

2.7.1.1 Killing *M. tuberculosis*

When *M. tuberculosis* enters an activated macrophage it becomes subject to replication inhibition and killing by intra-lysosomal acidic hydrolases, reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) upon phago-lysosomal fusion and enzyme-mediated degradation (reviewed in [32]). TNF and IFN-γ are needed to activate macrophages [33, 34], at

least in murine models, and are likely important in activation of human macrophages. *M. tuberculosis* has developed multiple strategies to thrive in this environment. *M. tuberculosis* has the ability to prevent phagolysosomal fusion [35] and maturation [36], neutralize reactive ROIs and RNIs [37], and prevent vacuole acidification [38].

2.7.1.2 Signaling T cells

Not only can *M. tuberculosis* prevent its degradation within an infected macrophage it can also reduce the ability of macrophages to signal T cells. *M. tuberculosis* can prevent MHC-II and MHC-I antigen processing (reviewed in [39]). Mycobacterial 19-kDa lipoprotein can reduce MHC-II expression on infected macrophages through a TLR-2 dependent mechanism [40]. *M. tuberculosis* can also reduce cathepsin S activity which is essential in processing MHC class-II-associated variant chain, a necessary step in peptide loading [41]. The decreased degradation along with the reduction in antigen processing and MHC-II expression allow *M. tuberculosis* bacilli to evade CD4 T cell surveillance.

Peptides presented by MHC-I molecules on macrophages and dendritic cells are targets for CD8 T cell recognition, and subsequent degradation of the cell. Since MHC-I molecules generally load peptides in the cytosol and *M. tuberculosis* typically remains within phagosomes non-traditional loading of MHC-I molecules with *M. tuberculosis*-peptides needs to occur. Secreted membrane proteins ESAT-6 and CFP-10 may cause the phagosomal membrane to become leaky [42], which would allow bacterial peptides to enter the cytosol and be processed by the proteasome for MHC-I processing. It is also possible that the bacteria are transported to the cytosol from the phagolysosome through a dislocon [43]. Other possible mechanisms for cross presentation by MHC-I molecules are the vacuolar cross presentation model [44] and the "detour pathway" [45]. The vacuolar cross presentation model suggests that peptides derived

from bacterial proteins that are generated within the phagolysosomal lumen, load directly on MHC-I molecules when they are recycled through the endocytic system and the detour pathway suggests that uninfected antigen presenting cells take up apoptotic bodies containing *M. tuberculosis* or its peptides from an infected phagocyte.

2.7.2 Dendritic Cells

Dendritic cells are professional antigen-presenting cells that have the ability to prime naïve T cells [46]. Dendritic cells are capable of expressing high levels of co-stimulatory molecules while releasing IL-12 to induce priming [47]. After the initial infection, *M. tuberculosis* is engulfed by resident dendritic cells and migrate to the draining lymph node to prime lymphocytes [48-51]. Priming initiates when specific naïve CD4 or CD8 T cells bind to either MHC-II or to MHC-I molecules, respectively, presenting a specific epitope, Dendritic cells can also induce T cells to produce IL-2 along with expressing costimulatory molecules that help T cells grow and differentiate [46, 50]. Dendritic cells are necessary in developing an adaptive immune response to *M. tuberculosis*; depleting them in mice during acute TB results in higher bacterial burden and delays CD4 T cell priming [52].

Since infected dendritic cells have the ability to migrate, they may also play a role in the dissemination of *M. tuberculosis* [51]. Dendritic cells also support *M. tuberculosis* growth [47]. While macrophages have the ability to reduce mycobacterial burden, dendritic cells do not, which indicates they may also serve as a reservoir for *M. tuberculosis* [50]. Dendritic cells are necessary for priming T cells to *M. tuberculosis,* however they can be exploited by *M. tuberculosis* to increase both dissemination and growth.

2.7.3 T Lymphocytes

Host defense against *M. tuberculosis* is dependent on both innate and adaptive cellular responses. CD4 and CD8 T cells act as the adaptive arm of the immune system and are essential in controlling TB [53-57]. T cells are primary produces of cytokines needed to activate macrophages and also directly kill *M. tuberculosis* within infected macrophage through cytolytic activity. T cells are also a major component of granulomas and are essential in preventing the dissemination of *M. tuberculosis* [28, 58, 59].

2.7.3.1 CD4 T cells

The primary role of CD4 T cells is to orchestrate the immune system by producing cytokines and chemokines. CD4 T cells are essential for containment of *M. tuberculosis* and long-term survival of mice during TB, which was demonstrated by a significant decrease in survival time and an increase in bacterial burden in *M. tuberculosis* infected MHC-II-/- and CD4-/- mice [56] and CD4 T cell depleted mice [60]. In humans, the importance of CD4 T cells was "confirmed" by HIV-infected individual's increased susceptibility to TB [16, 61], albeit this is an oversimplification of the incredibly complex interaction between HIV and *M. tuberculosis,* which is discussed below.

CD4 T Cells- Th1 Responses

The orchestration of the immune system by CD4 T cells during TB revolves around the production of Th1 cytokines, which include TNF and IFN-γ which activate macrophages [33, 34], and the production of IL-2 that is needed for T cell proliferation [62]. IL-12 is produced by

dendritic cells and plays a central role in inducing Th1 immunity during TB [63]. Humans with IL-12Rβ1 deficiency cannot respond to IL-12 and are more susceptible to TB [64].

IFN-γ is a pro-inflammatory cytokine that is produced by both CD4 and CD8 T cells (reviewed in [65]). As mentioned above, IFN-γ activates macrophages, which facilitates killing of intracellular pathogens through the induction of reactive oxygen species, nitrogen intermediates and increased phagosome-lysosome fusion [66]. During the acute phase of infection, CD4 T cells are the main producers of IFN-γ [56]. IFN-γ production is delayed in the absence of CD4 T cells during acute TB in mice but recovers after 3 weeks [56]. In the absence of IFN-γ, mice infected with *M. tuberculosis* have dramatically reduced survival and an increase in bacterial burden [67]. These mice also have a reduced ability to produce reactive nitrogen intermediates [67]. These studies suggest that IFN-γ and CD4 T cell production of IFN-γ are important in controlling *M. tuberculosis*.

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that is not only produced by CD4 T cells but also macrophages, dendritic cells, natural killer cells and CD8 T cells (reviewed in [65]). TNF has both transmembrane and soluble cytokine forms that are involved in lymphocyte activation, production of cytokines and chemokines, angiogenesis, and upregulation of cell adhesion molecule expression [68, 69]. CD4 T cells can also produce TNF which can act synergistically with IFN-γ to activate infected macrophages [70]. The neutralization of TNF exacerbates TB in mice [71], macaques with acute and latent TB [72] and humans [73].

Cytokine production by CD4 T cells is a dynamic process that occurs along a spectrum. CD4 T cells can produce only one cytokine or they can be multifunctional, producing more than four cytokines at a time [74, 75]. Multifunctional CD4 T cell responses wax and wane throughout TB [76], but there is some evidence that multifunctional activity (IFN-γ, TNF, IL-2)

increased post-TB treatment in infected individuals [74]. However, it is difficult to differentiate between cause and effect when it comes to an increase in these cells because it is possible that the temporary increase in antigen load associated with treatment may result in more priming of T cells. There is also evidence that multifunctional T cells are seen in the setting of increased disease [77, 78], likely reflecting increased antigen availability from replicating bacilli.

CD4 T Cells- Th2 Responses

Th2 responses are essential mediators of asthma and are an effective response against extracellular pathogens [79]. Th2 CD4 T cells can produce IL-10, IL-4, IL-5 and IL-13. As mentioned above, Th2 cytokines are capable of inducing AAMs [30], which fail to kill *M. tuberculosis* [80]. Increases in Th2 responses have been associated with increased pathology in individuals with TB [77, 81-83]. For example, a positive correlation was made between IL-4 production and humans with increased cavitary pulmonary lesions [83]. IL-10 produced by CD4 T cells can directly antagonize CD4 T cell responses by inhibiting macrophage function of cells infected with mycobacteria [84] and decreasing IL-12 production [85].

Th2-mediated dampening of immune responses is not necessarily a detrimental occurrence within TB. If pro-inflammatory responses remained unchecked, granulomas would induce more tissue damage, which could lead to a functional disruption [28]. Th2 responses may function to balance the proinflammatory response with an anti-inflammatory response within the granuloma.

2.7.3.2 CD8 T Cells

M. tuberculosis-specific CD8 T cells are very similar to their *M. tuberculosis*-specific CD4 T cell counterparts. CD8 T cells can also produce Th1 cytokines [86, 87] and can be cytolytic to *M. tuberculosis* infected macrophages [57]. CD8 T cells contain specific cytolytic activity that involves the degranulation of a cytolytic granule that release perforin, granzyme B and granulysin into an infected cell presenting a specific antigen on MHC-I [88]. Perforin inserts into the membrane of the target cell, and upon dimerization, it creates a pore that allows granzyme B and granulysin to enter. Granzyme B plays a role in apoptosis and granulysin has anti-microbial activity.

CD8 T cells have not been as well researched in TB immunology as CD4 T cells. They appear to not be as essential for the containment of TB in mice. CD8-/- mice have increased bacterial burden during the chronic infection, with small differences in survival compared to wild type animals [89]. The depletion of CD8 T cells in a murine TB model did not lead to increased pathology [90]. However, the depletion of CD8 T cells at the beginning of infection in nonhuman primates infected with *M. tuberculosis* prevents containment of the infection, leading to higher bacterial burden and pathology ([91], Flynn and Lin unpublished data). It has been suggested that CD8 T cells preferentially recognize heavily infected cells, which is not the case with CD4 T cells [92]. CD8 T cells are also important to study because MHC-I molecules are on all cells and *M. tuberculosis* DNA can be detected on MHC-class-II negative cells [93], which would allow these cells to be susceptible to only CD8 T cells.

2.7.4 Animal Models

Animal models are essential for better understanding various human diseases. The TB field has gained an incredible amount of information from the multiple animals used to study *M. tuberculosis* including zebrafish, mice, guinea pigs, rabbits, cows, and non-human primates. Although each model has its own limitations, these models allow researchers to explore microbial and immunological changes that cannot be performed in humans.

2.7.4.1 Zebrafish (*Danio rerio*)

Zebrafish are used in multiple areas of biomedical research that range from disease modeling to drug screening (reviewed in [94]). *Mycobacterium marinum*, a close relative to the MtbC [95] is a natural pathogen to fish and amphibians [96], which makes in an ideal TB model (reviewed in [97]). Some advantages to *M. marinum* are that it grows more quickly than *M. tuberculosis* and is less virulent so it requires less stringent biosafety protocols. However, there are differences between *M. marinum* and *M. tuberculosis*, including temperature of growth, and the ability of *M. marinum* to quickly escape the phagosome and live in the cytosol of cells, which *M. tuberculosis* does not, in general, do. Zebrafish have transparent larvae, which are ideal for live imagining and examining host-pathogen interactions in real-time [98, 99]. This animal model has verified many 'firsts' in the TB world. Using fluorescent *M. marinum* this animal model has provided us with details about bacilli transfer from one granuloma to another, that "mycobacterial expansion of early granulomas is driven by the continual cycle of death of infected macrophages and their phagocytosis by multiple newly recruited macrophages" and that innate immunity was sufficient to initiate granuloma formation without adaptive immunity, which were never observed before [98, 99].

2.7.4.2 Guinea Pigs (*Cavia porcellus*)

Guinea pigs were first used by Robert Koch to develop vaccines against TB [8]. Guinea pigs are very susceptible to the disease and display a wide range of granuloma types and pathology, with some similarities to humans [100, 101]. Guinea pigs have been used to assess dissemination of *M. tuberculosis* from the site of inoculation [102, 103] and to determine virulence of human TB strains [104]. The mouse model of TB has overshadowed the guinea pig model because there are more reagents developed for mice compared to guinea pigs, which makes immunological analysis of mice easier. However, the guinea pig model is still used today to assess vaccine efficacy [105], drug efficacy [106] and pathogenesis [107].

2.7.4.3 Rabbits (*Oryctolagus cuniculus*)

Unlike most other mammals, rabbits are naturally resistant to *M. tuberculosis* and extremely susceptible to *M. bovis* [108]. Although rabbits are resistant to *M. tuberculosis,* they still develop granulomas that eventually resolve [108]. *M. bovis* infection of rabbits leads to the development of granulomas that do not contain infection, and often develop into cavities that resemble the ones found in humans [109]. Rabbits may develop a spectrum of TB, but the lack of available reagents for studying their immunology and the high cost of their housing in a BSL3 facility make this animal model less than ideal for TB.

2.7.4.4 Mice (*Mus musculus*)

Mice are by far the most studied research animal in the world [110]. Mice are ideal animals to study because of the plethora of available reagents, and the availability of genetically identical mice and hundreds of knockout and transgenic strains. They are relatively cheap and easy to breed and house, and their short life spans allow researchers to follow the entire course of

disease in a fraction of the time it would take in a human. In light of these benefits, mice are the most widely used animals in TB research.

The immunologic and bacterial response to *M. tuberculosis* in mice differs from humans. When C57/BL6 mice are exposed to ~100 CFU of aerosolized *M. tuberculosis* they develop an adaptive immune response in 2 weeks with bacterial load stabilizing in the lungs around 10^{5-6} CFU/lung from about 4 weeks to up to 1 year [53, 54, 71, 111]. This high bacterial load results in the recruitment of CD4 and CD8 T cells that are maintained there throughout the infection [53]. These T cells and macrophages make up granulomas that form in the mice, however these granulomas differ significantly than the ones that form in humans [28, 33, 37, 53, 72, 112, 113]. Murine granulomas appear to be more of a cluster of infected and uninfected macrophages, T cells and other immune cells with less organization than those found in humans and macaques. There is no caseous necrosis in standard mice, and the granulomas are not as variable as humans.

Despite the differences in immunologic response to *M. tuberculosis* by humans and mice they are great starting point for examining a specific immunologic function that has not been examined in humans. For example, studies determining that TNF neutralization in TB mice caused increased pathology [114] lead to the examination of TNF neutralization for patients with rheumatoid arthritis and other chronic inflammatory diseases (reviewed in [68]), and now the risk of TB in patients receiving anti-TNF agents is well known, with protective measures in place for these patients. However, additional research demonstrated that the mechanism by which TNF controls murine TB may be different than in human TB; in mice, TNF neutralization leads to the inability to form or maintain granulomas. In humans and non-human primates, neutralization of TNF leads to exacerbation of disease, but the overall structure of the granuloma is similar to non-

neutralized subjects [72]. Although mouse research has its drawbacks, it still has been a good predictor of TB in humans (reviewed in [115]).

2.7.4.5 Non-Human Primates (*Macaca mulatta* & *Macaca fascicularis*)

Rhesus and cynomolgus macaques are both currently being used as TB animal models [72, 91, 112, 113]. Cynomolgus macaques bronchoscopically inoculated with a low dose (~ 25CFU) of Erdman strain *M. tuberculosis* become TST positive by 4-6 weeks post-infection. Half of the animals will develop active TB and the other half will develop latent infection [113]. Monkeys with active TB present the same signs of disease as humans, which include weight loss, coughing, radiological evidence of disease, high sedimentation rates and culturable *M. tuberculosis* from either gastric aspirates or bronchial alveolar fluid [72, 112, 113, 116]. Latent TB is defined as being TST positive without any signs of disease.

These infection outcomes (active or latent) are clinically defined, using methodology similar, although more rigorous, to that used in diagnosing human TB. The clinical classifications were validated at necropsy. Monkeys classified clinically as having active TB had significantly higher gross pathology scores, overall bacterial burden, and increased dissemination of infection compared to those classified as having latent infection [113]. Natural reactivation occurs in <5% of latently infected macaques. In our lab, we have demonstrated induction of reactivation of latent infection with anti-TNF antibody and with CD4 depletion via antibody.

In addition to having similar outcomes of disease as humans, the pathology of TB in monkeys and humans is similar [116]. Macaques form well defined granulomas that represent the spectrum of granulomas seen in humans. Granulomas can be necrotic, solid cellular, mineralized, suppurative, fibrotic and cavitary, and include coalescing and satellite granulomas [72, 112, 113, 116]. These granulomas may be located in the lungs or be disseminated to the

draining lymph nodes, spine, liver, spleen, kidneys, colon and other organs. Granulomas within individual monkeys vary dramatically, which is similar to humans. Because non-human primates are more similar to humans than the other animal models, they have the opportunity to provide us with a better understanding of the disease that we cannot ethically explore in humans.

3.0 HIV INTRODUCTION

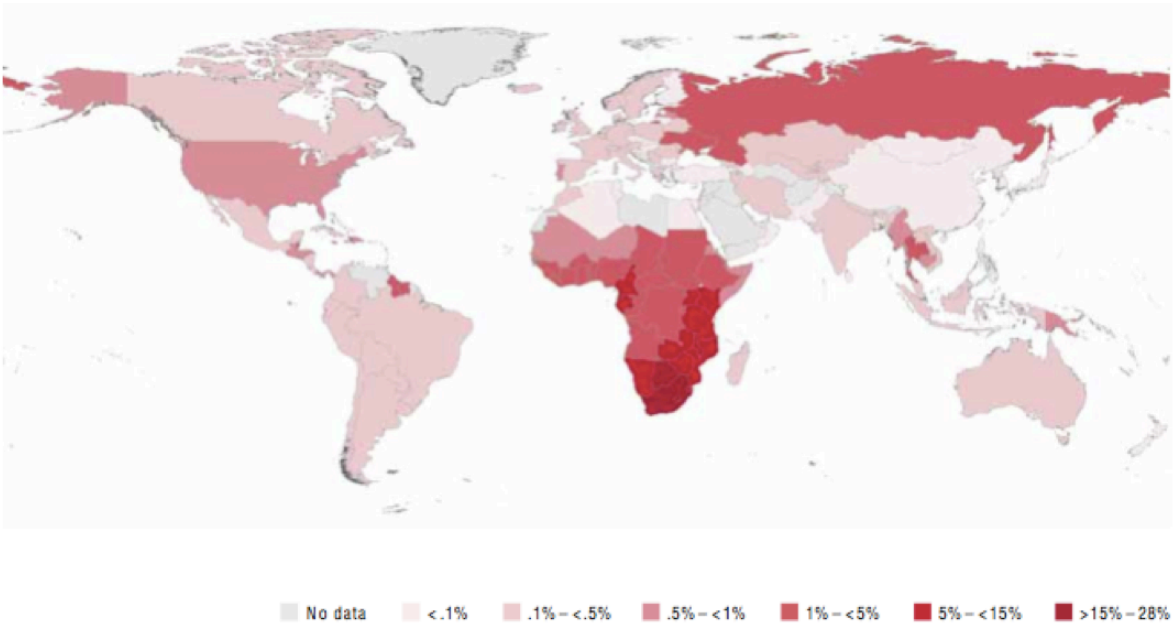

Figure 3. *Prevalence of HIV in 2009 per country.* Heat map displays the percentage of HIV-infected individuals per country. Figure was reprinted with permission from UNAIDS (22).

3.1 HIV EPIDEMIOLOGY

More than 33.3 million people are infected with HIV worldwide (Figure 3) and 2.6 million were newly infected with HIV in 2009 [22] (Figure 4). There is an income disparity in HIV incidence rates because 97% of new HIV infections in 2009 occurred in low and middle-income countries [22]. There were approximately 1.8 million deaths associated with AIDS in 2009 (Figure 4). Sub-Saharan Africa has the highest prevalence of HIV-1 infection with an estimated 22.5 million people infected [22].

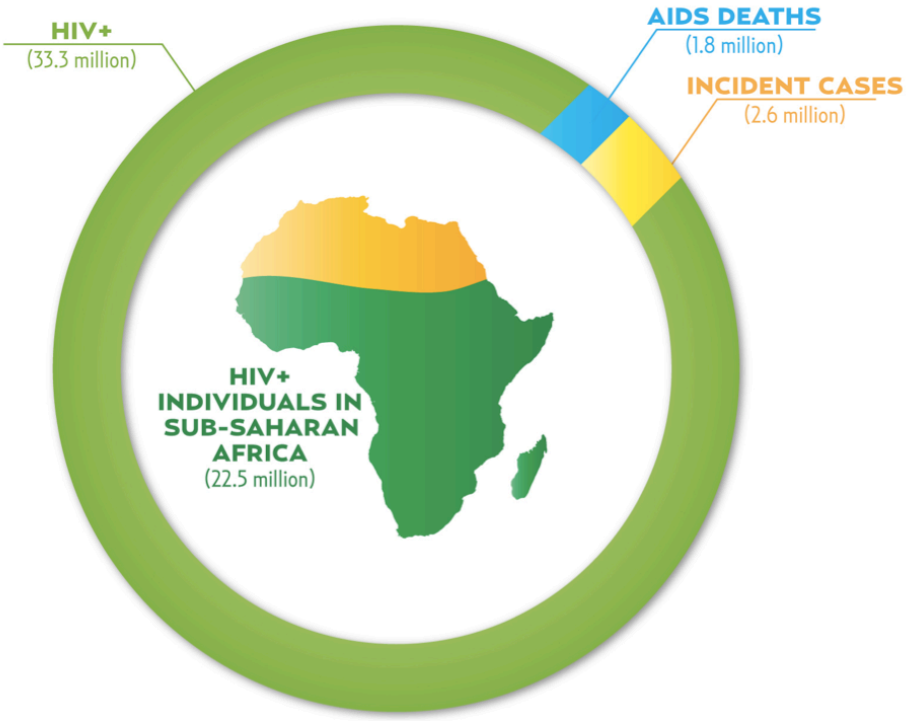

Figure 4. *HIV infection and AIDS-related death rates in 2009.* HIV prevalence, new cases of HIV (Incident) and the number of AIDS-related deaths in 2009 are represented. Total number of infected individuals in Sub-Saharan Africa are also depicted. Estimates are based on UNAIDS 2010 report (22).

3.2 HIV DIVERSITY

Based on full-length viral genome sequencing there are three HIV-1 groups known as M (main; 99% of total infections), O (outlier; <0%) and N (non M or O; 0.22%) and 8 groups of HIV-2 (Groups A-H, 0.17%) [117, 118]. Each group of HIV is divided into various clades. Group M, for example has 9 clades that are designated A to D, F to H, J, K. Each clade differs from the others in amino acid composition by at least 20% in the envelope region [119, 120]. Within each clade there are multiple subtypes of HIV that add to its diversity.

3.3 HIV HISTORY

The HIV pandemic has developed over decades. The first indication of acquired immune deficiency syndrome (AIDS) was observed in 1981 when seemingly healthy men became severely ill and developed *Pneumocystis carinii* pneumonia, mucosal candidiasis and multiple viral infections [121, 122]. These individuals were mostly homosexuals and drug users that developed these diseases in specific communities [122]. Patterns of these opportunistic infections began to dramatically rise in these cohorts and possible causes were examined. HIV was eventually isolated from an enlarged lymph node of a man with persistent generalized lymphadenopathy in 1983 [123]. From here it was determined that HIV actually infected blood lymphocytes and could be horizontally transmitted [123, 124].

HIV did not just randomly emerge in the 1980's *de novo.* Simian immunodeficiency virus (SIV) that infects non-human primates probably gave rise to HIV [125]. It is estimated that the most recent common ancestor for HIV-1 group M is 1908 [126], which suggests that HIV-1 has

been adapting to humans long before the first viral sequence of HIV was identified in an individual from a 1959 human sample [127].

3.4 THE HIV VIRION

HIV is a lentivirus with a cone shaped core composed of p24 Gag capsid (CA) protein. The virion is 100 to 120 nm in diameter. HIV has 3 structural Gag proteins: matrix, capsid and nucleocapsid. The envelope contains viral proteins (gp120 and gp41) [118]. Below the envelope, the matrix forms the inner shell, the capsid forms the conical core encapsulating the viral genomic RNA and the nucleocapsid interacts with the viral RNA inside the capsid. There are two identical RNA strands that are associated with viral RNA-dependent DNA polymerase (Pol), which is a reverse transcriptase (RT) along with protease (PR) and integrase (IN) in complex with the nucleocapsid proteins [118]. HIV contains three major enzymes that function at different times during replication. These enzymatic activities include the RNA-dependent DNA polymerase with its RNase H function that acts in the early steps of viral replication to form double stranded DNA of viral RNA [118]. IN functions inside the nucleus to incorporate viral cDNA into the host chromosome and the protease processes Gag and Gag-Pol polyproteins within the budding virion and induces maturation of the viral particle into an infectious virus [128].

The genomic size of HIV is 10kb. The primary transcript of HIV is a full length viral mRNA, which is translated into Pol and Gag proteins, which get cleaved by PR into RT, PR, IN and other accessory proteins such as MA, CA and NC [118]. HIV also contains env, vpr, vif, vpu, tat, rev and nef genes that are transcribed into proteins of the same names. Splicing is an

important event that is needed for the transcription of multiple subgenomic precursors. The relative amounts of spliced mRNA are determined by the *rev* gene, which is a product of multiply spliced mRNA [129]. Tat is a regulatory protein that interacts with an RNA loop structure formed in the 3' portion of the viral long terminal repeat (LTR), which increases viral replication. Rev also binds an RNA stem-loop structure called Rev-responsive element located in the viral envelope mRNA [130]. This interaction involves cellular and viral proteins, which permit unspliced mRNA to enter the cytoplasm from the nucleus to give rise to full-length viral proteins.

A variety of other viral proteins are associated with the core, such as Nef and Vif. Nef has multiple functions but is primarily involved in cellular activation and enhanced infectivity [131]. Vif is involved in increasing infectivity. Other proteins associated with the virion are Tat, Rev and Vpr. Envelope (Env) protein, precursor gp160 is cleaved by furin, an endoprotease into gp41 and gp120 [132], which make up the receptor responsible for binding host cells.

3.5 HIV REPLICATION

HIV usually infects cells through an interaction between a gp120 viral envelope protein and cell surface expressed CD4 and either CCR5 or CXCR4 [118]. Using CD4 and the various co-receptors, HIV can infect CD4 T cells, macrophages and to a lesser extent, dendritic cells. Since HIV is a retrovirus that must integrate into the DNA of the host cell, it must adapt to that particular cell.

3.5.1 Viral Replication in CD4 T cells

Viral replication is efficient and occurs rapidly in activated CD4 T cells. Viral long terminal repeats (LTR) contain binding sites for cellular factors that regulate HIV transcription and are abundant in activated T cells and macrophages [133-135]. Since host factors are not efficient enough to induce the appropriate amount of replication, viral proteins such as Tat [136], Rev [137] and Vpr [138] increase gene expression. Activated cells produce more virus than quiescent cells within plasma of HIV-infected individuals [139, 140]. This increased replication within activated lymphocytes also leads to a rapid turnover of these cells [140].

Cell activation is not required for HIV infection [141]. However, quiescent cells, in G_0 phase of replication are refractory to infection because they have the ability to block reverse transcription of HIV [142]. In order for the establishment of provirus, the cell needs to go beyond G_0 cell cycle phase. CD4 T cells may receive a subtle stimulatory signal that helps push it into an early stage of cell cycle (G_1) that may be sufficient to begin viral replication from integrated viral DNA [143]. Most cells that express viral RNA do not express cellular activation markers [144] and these cells persist after antiretroviral therapy [143]. This means that the virus may temporarily push a cell from G_0 to G_1, which will allow replication to commence while reducing cytotoxicity. HIV Nef may create conditions that allow the virus to enter nonactivated cells [145]. Nef and Tat genes may be transcribed before the provirus is established [146], which means that they may help prepare a cell for infection.

3.5.2 Viral Replication in Macrophages

HIV infects and replicates in macrophages both *in vitro* [147, 148] and *in vivo* [149]. Despite macrophages being a non-dividing cell they have been shown to contain proviral DNA [150]. HIV may establish infection in macrophages through a 'reverse transcription complex' (RTC, reviewed in [151]). When HIV enters a cell the viral core undergoes a rearrangement that becomes the RTC. The RTC contains nuclear targeting signals that guide it through the nuclear pore and help establish an active infection within the cell.

Rhesus macaques infected with highly pathogenic SIV/HIV chimeric virus SHIV(DH12R) that completely depletes CD4 T cells maintain plasma viremia though tissue macrophages [152]. The infection of macrophages by HIV is important because they may act as reservoirs in tissues (reviewed in [153]). These reservoirs prevent the eradication of virus during antiretroviral therapy ([154], reviewed in [155]) and also may help disseminate the virus to autologous CD4 T cells [156], which can lead to the preferential infection and depletion of HIV-specific CD4 T cells.

3.5.3 HIV Pathology

A person infected with HIV usually sustains a significant loss of CD4 T cells during chronic infection. Decreases in CD4 T cell counts are used as a clinical benchmark for the course of disease. Patients with <200 CD4 T cells/μl of blood are considered to have acquired immune deficiency syndrome (AIDS) [157]. CD4 T cell reduction leads to compromised cellular and humoral immune responses, which renders the patient more susceptible to opportunistic

pathogens. These opportunistic infections can eventually lead to the death of an infected individual.

HIV infection can be characterized by three specific phases: 1) Primary infection (acute) phase is associated with a massive increase in viral load that is accompanied by a decrease in peripheral and gut CD4 T cells that eventually leads to a viral set-point; 2) chronic phase which is associated with a gradual increase in viral load and an irreversible decrease in CD4 T cell counts and 3) the final phase (AIDS) which is associated with the terminal failure of the immune system (reviewed in [158, 159]) (Figure 5).

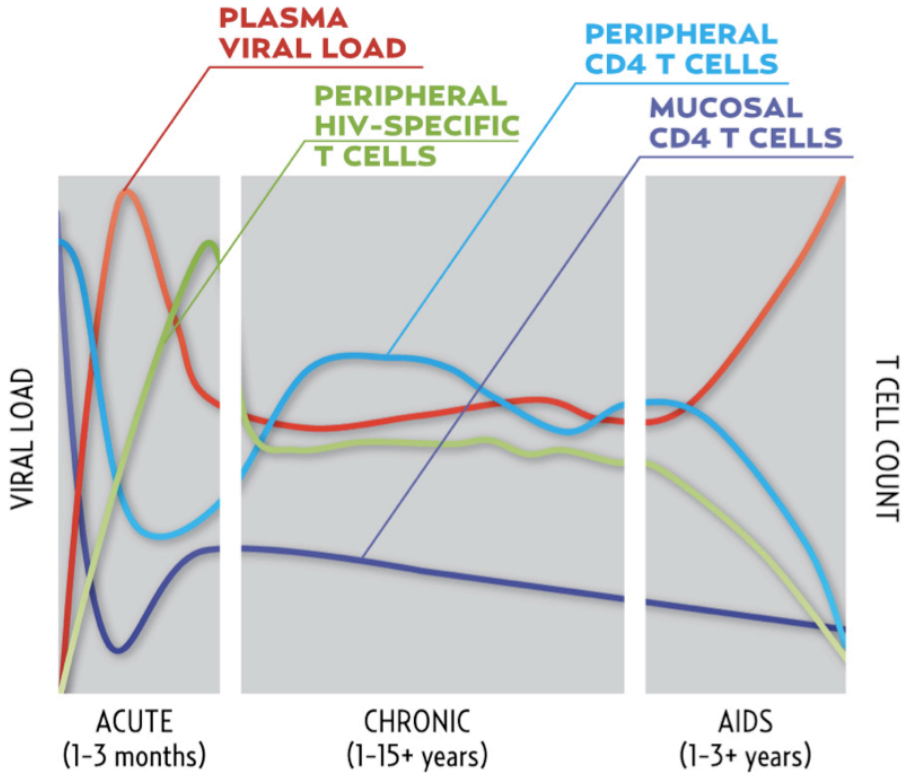

Figure 5. *Changes in viral load and T cells in an HIV-infected individual.* Relative viral loads and T cell counts in an average HIV-infected individual receiving no treatment over time.

3.5.3.1 Primary Infection Phase

Viral load observed in blood directly correlates to the risk of HIV transmission [160]. HIV is usually transmitted during sexual intercourse, through blood or vertically from mother to fetus [161]**.** During this early stage, the infected individual may become symptomatic presenting with clinical symptoms such as fever, skin rash, oral ulcers and lymphadenopathy that is associated with seroconversion [162, 163].

After HIV is transmitted through the mucosal barrier, the virus will be well established in a lymphatic tissue reservoir within the first few weeks of infection [164]. This HIV reservoir acts a principal site for viral production and persistence that leads to CD4 T cell depletion and destruction of follicles and the lymphatic tissue architecture [165]. During this period there is a profound depletion in gut-associated lymphoid tissue (GALT) that results in a reduction of CD4 T cells that remain depleted throughout the course of disease [166]. The depletion is predominately limited to CCR5+ (CD45RA-CD45RO+) memory CD4 T cells [166, 167]. This depletion does not only occur within the GALT but also in most lymph nodes and peripheral blood [166-169]. This depletion of CD4 T cells occurs independent of route of transmission. Once this infection is established, an adaptive cellular response to HIV is primed that generates a partial control of viral replication [170].

During this early stage millions of resting CD4 T cells and macrophages become infected [171] and billions of virions in immune complexes bind to follicular dendritic cells (fDC) within lymph nodes [162, 165, 172]. The resting CD4 T cells remain infected throughout the course of disease, which provide an opportunity for the virus to evade immune surveillance until replication becomes activated. Infected fDCs provide an opportunity for HIV to preferentially infect HIV-specific CD4 T cells when they begin priming [173]. These reservoirs are

maintained throughout the disease course and become a major issue during anti-retroviral therapy because drugs target integrated viral DNA. The acute phase of HIV ends when viral titers decrease and hit their target set point and there is a significant increase in peripheral HIV-specific T cells [174].

3.5.3.2 Chronic Phase

The massive depletion of CD4 T cells within GALT persists in the chronic phase [166], with an initial increase in peripheral CD4 T cell levels [169, 174]. This phase can last between 1 to 15+ years in individuals that are not being treated. During this phase there is a constant balance between CD4 T cell killing and viral production. Since 10-100 million infected activated CD4 T cells are killed each day [175], there is a constant turnover of CD4 T cells. The massive amount of CD4 T cell death along with the constant turnover of T cells can place a lot of stress on the proliferative ability of naïve T cells and disrupt lymph node architecture [176].

HIV also preferentially infects HIV-specific CD4 T cell [173]. HIV does not just kill CD4 T cells during the chronic phase of infection, but also impairs CD8 and CD4 T cell function [177-179]. The constant stimulation of HIV-specific CD8 and CD4 T cells eventually causes them to become exhausted, which means they are less responsive to antigenic stimulation and/or cellular proliferation[180-183]. This decrease in responsiveness of T cells to HIV and the extensive amounts of cell death eventually lead to a loss of viral control and the final stage of disease.

3.5.3.3 The Final Phase (AIDS)

AIDS occurs when HIV-infected individuals have <200 CD4 T cells/μl of blood. This results in a significant loss of the immune system's ability to prevent infections. As peripheral CD4 T cell

levels decrease, there is a direct correlation to the number of opportunistic pathogens that can infect the host [157]. Individuals with AIDS typically live between 1-3+ years in the United States.

3.6 HIV TREATMENT

When HIV was identified in 1983 scientists set out to develop both drugs and vaccines to cure and prevent disease, respectively. Although we cannot cure or prevent HIV, chemotherapy has dramatically decreased morbidity and mortality associated with HIV [184].

3.6.1 Drugs

The first antiretroviral therapy that was approved by the FDA was 3'-azido-3'-deoxythymidine (AZT) in 1987 [185]. AZT dramatically decreased viral load by inhibiting viral RT and HIV replication [186]. Its mechanism of action involves both the termination of viral DNA production and a competition for nucleosides used by viral polymerase. Because HIV has such a high mutation rate, AZT did not prove to be an effective mono-therapy long term [185]. This eventually led to the development of multiple drugs that work by inhibiting multiple viral proteins.

There are over 20 anti-retrovirals approved by the FDA to treat HIV [187]. These drugs include nucleoside RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PI), integrase inhibitors (II) and fusion inhibitors (FI). Each of these drugs targets a specific viral protein that is required for viral replication or fusion. The error-prone HIV RT

causes a approximately 10^{-5} to 10^{-6} mutations per base pair/cycle [188], which leads to mutations that allow HIV to bypass these inhibitors. To decrease the probability that a mutation will cause drug resistance, individuals are given a combination of anti-retrovirals (highly active anti-retroviral therapy; HAART) that include different families of drugs to prevent multiple steps in the viral replication pathway. HAART has dramatically increased the life expectancy of HIV-infected individuals.

3.6.2 Vaccines

Although HAART has dramatically increased life expectancy in HIV-infected individuals, there are severe side effects associated with the drug cocktail [184]. These side effects and the need to prevent disease have led to the search for an effective vaccine. With any vaccine, the overall aim is to reduce infection in the population. A few of the vaccine approaches have involved whole killed virus, live attenuated variants, subunits of viral proteins, viral proteins in live vectors, sequence-derived peptides, anti-HIV neutralizing antibodies, DNA gene transfer and viral like particles [118, 181, 188-191].

Some challenges face the development of an HIV vaccine: HIV integrates into cellular genome resulting in latent reservoirs; HIV can be transmitted from infected cells; cell-to-cell transfer of infection takes place; there are multiple HIV variants; the virus infects cells that are immune sanctuaries such as the brain and testes; the virus compromises immune function; and HIV has a high mutation rate [118, 181, 188-191]. These challenges have prevented the development of a successful vaccine but increasing our basic understanding of HIV biology will increase the odds of overcoming this obstacle.

3.7 IMMUNOLOGIC RESPONSE TO HIV

The immune response to HIV is dynamic and involves both adaptive and innate cellular responses. Dendritic cells and macrophages provide a bridge between the two responses by presenting viral epitopes that either prime T and B cells or activate them, respectively. HIV has developed multiple mechanisms that evade immune surveillance and leads to the destruction of both the innate and adaptive immune responses [118].

3.7.1 Dendritic Cells

Dendritic cells play an important role in HIV immunology (reviewed in [192]). Dendritic cells prime HIV-specific lymphocytes. HIV has exploited these cells by using them to infect HIV-specific CD4 T cells upon priming [173]. Dendritic cells are also exploited by HIV to traverse the epithelial barrier in the genital tract to initial a productive infection [193].

3.7.2 Monocytes and Macrophages

As discussed above, macrophages can be infected with HIV. HIV can infect both tissue macrophages and peripheral blood monocytes. Although HIV infects a small fraction of macrophages and monocytes, they contribute significantly to viral replication [152]. Infected monocytes traffic to various tissues and cause a significant amount of dissemination of HIV [194] and they act as a reservoir of HIV during antiretroviral therapy [195]. HIV not only uses macrophages and monocytes as reservoirs for viral transcription it also has the ability to disrupt

macrophage phagocytosis [196-198], intracellular killing [196] and cytokine production [199, 200].

Macrophages respond to pathogens through pathogen-associated molecular patters using pattern-recognition receptors (PRR), that include Toll-like receptors (TLR) and they can respond by releasing appropriate cytokines [88]. TLR signaling is directed through two pathways that either increase the expression of proinflammatory cytokine or type 1 IFN genes, which can be affected by HIV-1 (reviewed in [201]). HIV and SIV have been shown to decrease TLR4 and CD14 on PBMC [202, 203]. HIV also reduces downstream signaling from TLR pathways [204]. HIV can modulate NF-κB [205], IRAK-4 phosphorylation [206], MyD88 activation [207] and various kinases JNK, ERK1/2 and MAPK phosphorylation [208]. These changes in signal transduction manipulate the ability of macrophages to respond to other pathogens. For example, Tat has the ability to inhibit LPS-activation of ERK1/2 in monocyte-derived macrophages [209], which can decrease macrophage ability to recognize microorganisms.

HIV has the ability to modulate macrophage-induced cytokine production (reviewed in [30, 199]). Monocytes from healthy donors that are infected with HIV and differentiated into macrophages initially release more TNF, IL-1β, IL-6 and IL-8 in response to LPS compared to HIV uninfected macrophages [210]. This increase in cytokine production eventually decreases in both monocyte-derived macrophages and alveolar macrophages [204, 211]. HIV-infected individuals also have higher levels of anti-inflammatory cytokines such as IL-10 in their bronchial alveolar fluid than HIV-uninfected individuals [212]. However, the decrease in pro-inflammatory cytokines in HIV-infected macrophages does not always occur because LPS-induced TNF and IL-1β has also been observed in asymptomatic HIV-infected individuals [213].

The decrease in macrophage-induced killing may result from a reduction in phagocytosis because Tat and Nef have the ability to down-regulate mannose receptors [214, 215]. HIV-infected individuals have a reduced ability to activate alveolar macrophages upon exposure to *Pneumocycstis jirovecii* [216]. The decrease in macrophage activation may result from the ability of HIV to switch the activation status of macrophages from M1 to M2 (reviewed in [30]), which could impair the ability of macrophages to kill microbes. HIV may induce the M2 activation status of macrophages by inducing IL-4 and IL-13 production within infected humans.

HIV decreases the ability of macrophages to signal lymphocytes [217-220]. HIV reduces T cell proliferation by down-regulating B7 and CD40L co-stimulatory molecules [220, 221] along with CD3 and MHC [222, 223] on infected macrophages. The reduction in these molecules may lead too less responsive lymphocytes.

3.7.3 T Lymphocytes

T cells are important for both HIV proliferation and killing [88, 118]. CD4 T cells can be infected with HIV. At the same time CD4 T cells can release cytokines that activate infected cells and they can kill infected cells through death receptors. CD8 T cells can release cytokines and become cytolytic.

3.7.3.1 CD4 T cells

As mentioned above, HIV can infect CD4 T cells. CD4 T cells can release either Th1 cytokines (IFN-γ, TNF and IL-2) or Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) [88, 118]. Th1 responses predominantly activate other cells and are proinflammatory while Th2 responses are generally anti-inflammatory and induce a humoral response. Th1 responses help activate fellow T cells and

macrophages. This is a double-edged sword because it increases HIV killing but it can also increase viral replication since HIV preferentially infects and replicates in activated CD4 T cells [143].

The loss of CD4 T cells in HIV-infected individuals may be a result of direct infection that leads to cellular death [167, 224], cell death induced by viral proteins [225] and a decrease in the proliferation of naïve T cells [226]. Not only does HIV kill CD4 T cells it can also decrease their responsiveness to stimulation [183, 227]. The decrease in responses to recall antigens is correlated to an increase in disease progression [228]. The increase in CD4 T cell death and decrease in their ability to respond to HIV lead to increased viral replication, and increased pathology.

3.7.3.2 CD8 T cells

Cytotoxic CD8 T cells are a major component to the immune response to HIV (reviewed in [229]). Cytolytic T cells can both release cytolytic granules that lyse infected cells and also release activating cytokines [77]. Cytolytic T cell production precedes production of neutralizing antibodies, indicating that this activity is responsible for the initial decrease in viral load [230]. Since these cells cannot become infected with HIV and all cells express MHC-I molecules, CD8 T cells can potentially kill any cell that HIV infects. HIV may reduce the ability of cytolytic T cells to respond to infected cells by causing a defect in T cell receptor signaling [231], altering cellular maturation [232], impairing effector functions [233], and reducing their survival [234].

3.7.4 Humoral Response to HIV

Antibody responses to HIV generally appear within the first 2 weeks [235]. Neutralizing antibodies are responsible for the removal of the majority of viral particles in the blood [236]. The neutralization of viral particles by antibodies leads to viral escape [236], which means the virus rapidly escapes neutralization by generating new emerging variants. Although, the virus continually escapes neutralization, the antibody response to HIV is essential at maintaining viral titers [237]. Anti-HIV antibodies are usually detected within the first 3 weeks of infection [118].

4.0 INTRODUCTION TO THE CO-INFECTION

This chapter is adapted from the original publication with permission:

Diedrich, CR & Flynn, J. HIV/*Mycobacterium tuberculosis* co-infection immunology: How does HIV exacerbate TB? Infection and Immunity. April 2011, p1407-1417, Vol. 79, No. 4. Citation: [238]

The emergence of human immunodeficiency virus-1 (HIV) has exacerbated an already enormous number of cases of tuberculosis (TB) worldwide (Figure 6). TB affects HIV+ individuals throughout all phases of HIV infection and is the leading killer of HIV+ people [239]. Of the 9.4 million new cases of active TB each year, 1.4 million of those individuals are HIV+ [240]. It is widely accepted that HIV causes a depletion of CD4 T cells, which is likely to contribute to the susceptibility of co-infected persons to TB, as this T cell subset is important in control of TB. However, HIV has effects on other cells, including macrophages, and influences cytokine production, which may also prevent a host from containing an initial or latent *Mycobacterium tuberculosis* infection. In this review, we highlight gaps in the human co-infection literature that must be addressed to gain a more complete understanding of the interaction between the pathogens *M. tuberculosis* and HIV.

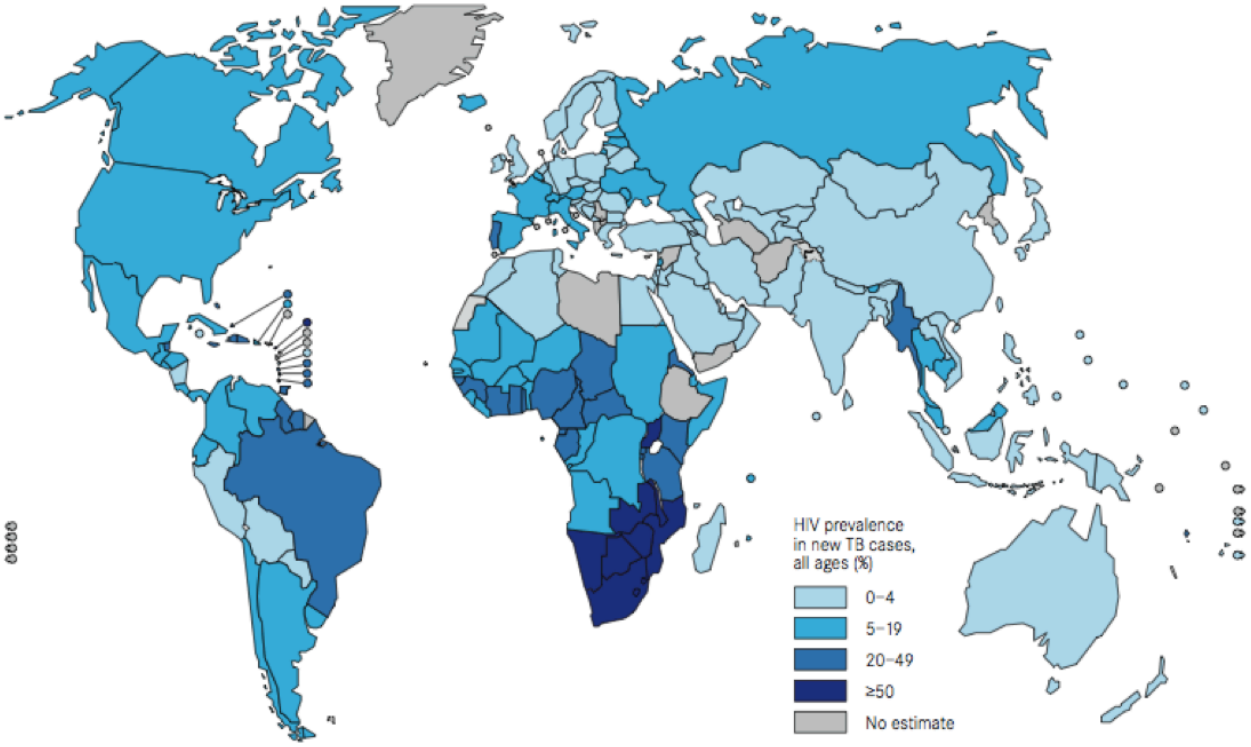

Figure 6. *HIV prevalence in new TB cases globally in 2010.* Map displays percent of HIV-infected individuals with new cases of TB. Figure was reprinted with permission from WHO 2011 Global Tuberculosis Control Report (11).

The current human HIV/TB literature provides a solid foundation for our current understanding of how these pathogens interact *in vitro* and *in vivo*. Several hypotheses have been generated to identify how HIV increases the risk of TB, and how *M. tuberculosis* infection may exacerbate HIV infection. Here, we summarize the data that underlies these hypotheses. However, it must be noted that these hypotheses are based on indirect evidence, extrapolating from the experimentally tractable peripheral sampling to events in *M. tuberculosis*-infected tissues. Although many of these hypotheses are likely valid, confirming the events occurring in the granulomas is a necessary next step. Focusing on confirming these hypotheses at the tissue level in future HIV/TB co-infection studies may identify new mechanisms that drugs and vaccines can target to prevent or cure TB within co-infected people.

4.1 HIV/TB PATHOLOGY

It is well established that HIV impairs the ability to control *M. tuberculosis* infection [16, 241-244]. Clinical studies provide compelling evidence that HIV leads to an increased risk of developing TB shortly after HIV infection. Among miners in South Africa, HIV+ individuals were two to three times more likely to develop TB than HIV- miners within two years of HIV-seroconversion [241, 242] and after 11 years, half of the HIV+ miners developed TB [241]. Although HIV+ individuals in these studies are more prone to developing TB, half of the cases of TB were attributed to time and not HIV due to the high incidence rate of TB among South African miners. It was not determined whether TB was the result of reactivation of latent infection or newly acquired *M. tuberculosis* infection. It is important to differentiate between reactivation and newly acquired TB because the mechanisms by which the human host controls primary and latent infection, and the effects of HIV on these mechanisms, may differ. Evidence from DNA fingerprinting (typing for IS6110 RFLP) studies indicate that HIV+ people in endemic regions, such as South Africa and Malawi, are primarily developing TB from new infection, rather than by reactivation of latent infection [243, 245]. In this type of study, the pattern of IS6110 sequences among *M. tuberculosis* isolates from patients within the cohort indicate whether the TB case is newly acquired as opposed to relapse of latent TB. HIV+ individuals are between 2.2 [245] and 5.5 [243] times more likely to develop TB from a new source compared to HIV-negative individuals. HIV+ individuals are also more likely to become infected with *M. tuberculosis* and die from TB than their HIV- counterparts (Figure 7, [14]).

Not only are HIV+ individuals at greater risk of acquiring *M. tuberculosis* and developing active TB, they have an increased risk of mortality from TB [244, 246]. Although it has been well known over the past 25 years that HIV/*M. tuberculosis* co-infection is remarkably

detrimental [16, 247, 248], the mechanisms by which HIV disrupts the function in both established and newly forming granulomas, leading to the increased morbidity and mortality of co-infected people compared to people with TB alone remains to be determined [249].

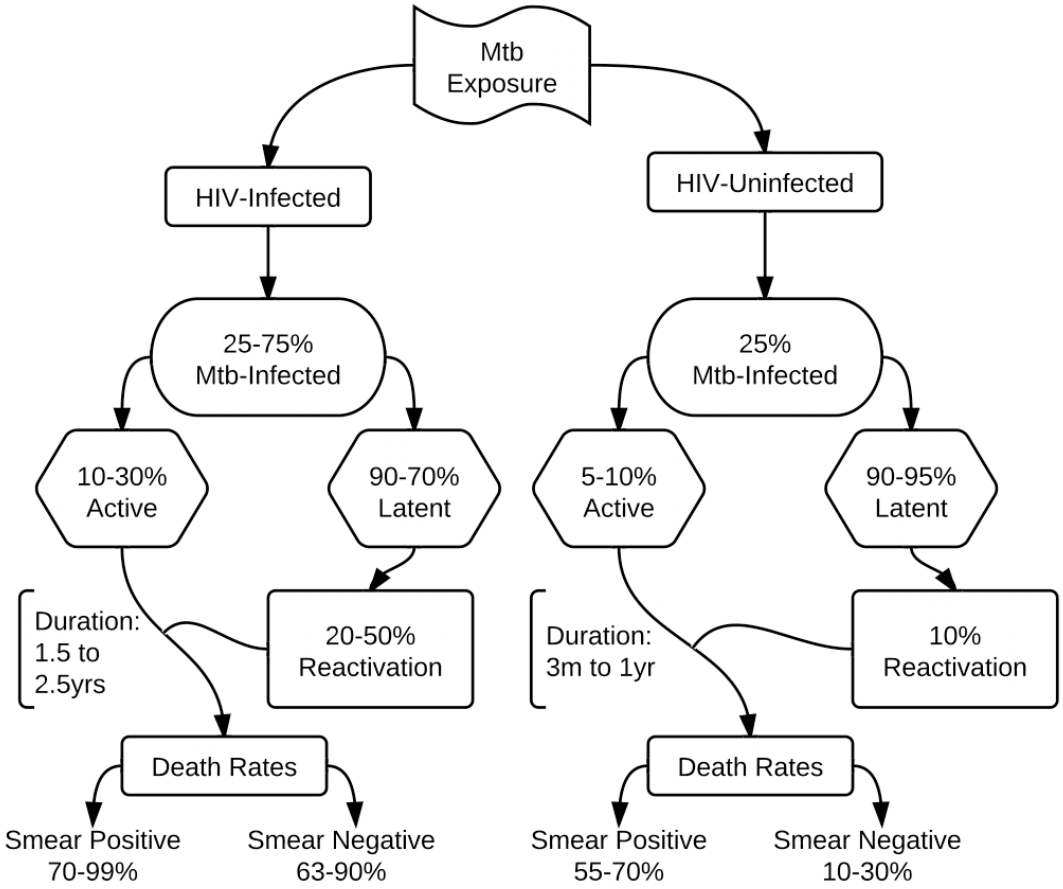

Figure 7. *Flow chart of TB susceptibility and TB death rates in HIV-infected and HIV-uninfected individuals.* HIV-infected individuals are more susceptible to becoming infected with *M. tuberculosis*, developing active and/or reactivated TB, having a longer duration of disease along with an increased TB death rate compared to HIV-uninfected individuals. Smear positive/negative-*M. tuberculosis* culture positive/negative in sputum or BAL. Mtb- *M. tuberculosis.* Percentages are estimates based on multiple sources (14, 16, 243, 245, 285).

4.2 THE EFFECTS OF HIV ON THE *M. TUBERCULOSIS* GRANULOMA

It has been proposed that the increase in pathology associated with HIV/*M. tuberculosis* co-infection is caused by a functional disruption of the local immune response within the granuloma [249-253]. These disruptions presumably decrease the ability of the granuloma to contain *M. tuberculosis*, leading to increased bacterial growth with more mycobacterial dissemination and severe pathology. The cause for the disruption can be divided into general and overlapping processes: 1) an increase in viral load within involved tissue leading to 2) a decrease in total number of CD4 T cells along with 3) a disruption of macrophage function and 4) a perturbation of *M. tuberculosis*-specific T cell function and that lead to functional and detrimental changes within granulomas. Here we review the available data for these HIV-induced changes**.**

4.3 HIV REPLICATION AT SITES OF *M. TUBERCULOSIS* INFECTION

Hypothesis:

HIV replication increases at sites of M. tuberculosis infection, which leads to a reduction in containment of M. tuberculosis.

HIV preferentially replicates within activated CD4 T cells and macrophages. Because CD4 T cells and macrophages are major components of the granuloma, and some proportion of these T cells are likely to be activated (an ideal situation for HIV uptake and replication), sites of *M. tuberculosis* infection are considered ideal for HIV replication. Increased viral load within

involved tissue would likely cause a disruption in equilibrium between granuloma function and mycobacterial growth. The available literature supports that *M. tuberculosis* leads to increased viral replication *in vitro, ex vivo* and *in vivo.*

4.3.1 *M. tuberculosis* increases HIV replication in stimulated co-infected macrophages *in vitro*

Multiple studies have been performed to determine whether *M. tuberculosis* influences HIV replication. The data on replication of HIV within *M. tuberculosis* co-infected macrophages is controversial supporting both increases [254-258] and decreases [254] in viral replication. *In vitro* studies have demonstrated the importance of macrophage activation state and the presence of pro-inflammatory cytokines in inducing HIV replication. THP-1 macrophage cell lines in contact with lymphocytes or neutrophils induce viral replication [256, 257]. These co-infected macrophages increase HIV replication when CCAAT enhancer binding protein beta (C/EBPb) transcription factor is inhibited and an increase in NF-kB production is induced, which binds to HIV long terminal repeat and initiates viral transcription. HIV replication decreased in the presence of neutralizing antibodies against TNF and IL-6, and increased in the presence of antibodies against IL-10 and TGF-β [255], which further supports that co-infected activated macrophages increase HIV replication. However, the increase in viral replication may be *M. tuberculosis* strain-specific, since clinical strains of *M. tuberculosis* can manipulate replication of HIV to different degrees. For example, the strain CDC1551, which is a clinical strain, but considered less virulent [259], induces more HIV replication than the more virulent clinical HN878 strain in co-infected peripheral blood mononuclear cells (PBMC) [258]. *M. tuberculosis* has also been shown to decrease HIV replication in co-infected macrophages. Monocyte-derived

macrophages incubated with heat-inactivated *M. tuberculosis* prior to HIV infection prevented viral replication despite an increase in CCR5, a coreceptor used by HIV to infect cells [254]. However the majority of data supports the increase in HIV replication and this may be deleterious to the co-infected individual because the increase in HIV replication enhances transmission of HIV to autologous T cells *in vitro* [260] and *M. tuberculosis* specific CD4 T cells *in vivo* [261]. The increase in HIV transmission may be caused by an increase in T cell proliferation and viral release occurring when T cells are incubated with HIV/M tuberculosis co-infected macrophages compared to macrophages infected with HIV alone [260].

4.3.2 *M. tuberculosis* microenvironments increase HIV replication *ex vivo* and *in vivo*

The immune environment created by *M. tuberculosis* also promotes viral replication *ex vivo*. *M. tuberculosis* causes an increase in inflammatory cytokines *in vivo*, which can lead to activation of T cells and macrophages, which induces replication of HIV. Garrait et al, determined that pleural fluid from individuals with TB incubated with HIV-infected PBMC induces more replication then when incubated with pleural fluid from individuals without TB [262]. This increase in replication was dependent on IL-6 and TNF, which supports the notion that activated cells induced by proinflammatory environments may increase HIV replication. This supports work that confirmed that HIV replication increases in activated CD4 T cells [263] and CD14+ macrophages [264], which are prevalent at sites of *M. tuberculosis* infection.

High viral titers are inversely proportional to peripheral CD4 T cell counts and correlated with susceptibility to various opportunistic infections [265-267] and advancement to AIDS. One study concluded that there was a 5 to 160-fold increase in plasma viral titers during acute infection with *M. tuberculosis* [268] while another determined that viral titer was 2.5 times

higher in HIV+ individuals upon TB diagnosis [269]. A transient increase in viral titer may occur in co-infected people during acute TB due to an increase in activated CD4 T cells. However, the majority of clinical research has demonstrated that plasma viral titer does not correlate with susceptibility to active TB in co-infected people [270] or monkeys infected with SIV and *M. tuberculosis* [253, 271]. Likewise, treatment of TB does not necessarily lead to a reduction in plasma viral load [272]; further supporting that peripheral viral load does not by itself represent susceptibility to TB.

Although little correlation between plasma viral titer and reactivation of TB has been observed in co-infected individuals, high viral loads within involved tissues have been suggested as a cause of the functional disruption within the granuloma [249, 268, 273]. Nakata et al, determined that HIV replicates within the lungs by measuring viral load and p24 in bronchoalveolar lavage (BAL) cells from co-infected individuals [274]. BAL represents the airway environment, which may be an initial site of *M. tuberculosis* replication, and is also a site of *M. tuberculosis* replication during active TB, but may not accurately represent events within the granulomas in the lung parenchyma. BAL cells from individuals co-infected with HIV and *M. tuberculosis* sampled from the airways of involved lungs (radiographic evidence of TB infiltrate) had higher viral titers and p24 than cells from the airways of uninvolved lungs in the same persons [274]. In this same study, viral load within BAL cells was higher than in plasma. This study was one of the first to demonstrate that HIV may replicate more at sites of disease. Other studies have confirmed that sites of *M. tuberculosis* infection have increased viral replication in co-infected patients [273, 275, 276]. Pleural fluid from co-infected subjects have higher viral titers [276] and increased HIV heterogeneity [273] compared to plasma from the same patients. Increases in viral titer and heterogeneity within *M. tuberculosis*-involved tissue

may increase viral fitness [275] and decrease the ability to contain both infections. On the contrary, granulomas from cynomolgus macaques co-infected with SIVmac251 and *M. tuberculosis* displayed similar SIV viral loads compared to uninvolved tissues, albeit with substantial variability [271]. Since these monkeys had very low plasma and PBMC viral loads, they may not represent exactly what is occurring within co-infected humans.

These studies provide a basic framework for our understanding of how *M. tuberculosis* manipulates HIV replication. However, no clinical studies have demonstrated that granulomas provide this ideal environment for HIV replication, which emphasizes the need for clinical researchers to determine whether the granuloma environment is influencing virus replication directly, and the need for animal models of co-infection.

4.4 CHANGES IN T CELL NUMBER WITHIN GRANULOMA

Hypothesis:

HIV induces active or reactivated TB by reducing CD4 T cells within granulomas.

CD4 T cells are essential for containment of *M. tuberculosis* and long-term survival of infected mice, which was demonstrated by a significant decrease in survival time and increase in bacterial burden in MHC-II-/- and CD4-/- mice [56]. HIV and SIV cause substantial reductions in peripheral, mucosal and gut CD4 T cells shortly after infection by preferentially infecting activated CD4 T cells [158, 167, 277, 278] and resting memory CD4 T cells [166, 279]. Studies have determined that SIV, and presumably HIV, kill up to 60% gut CD4 T cells within the first 10 days of infection, with an 80% reduction of these cells by 2 weeks post infection [158, 277].

The affected cells are mostly effector memory T cells [167, 279], which are abundant in these sites. Because HIV depletes these cells within the periphery, gut and mucosal tissue [278, 280], it has been hypothesized that HIV-induced depletion of CD4 T cells within granulomas leads to a direct disruption of the containment of *M. tuberculosis* infection [249, 281].

4.4.1 HIV-induced decreases in peripheral CD4 T cells correlates with susceptibility to TB

Peripheral CD4 T cell count is a standard measure of disease progression in HIV infected individuals, and this has been reported for many HIV/*M. tuberculosis* co-infection studies [270, 271, 282-284]. HIV+ individuals are more susceptible than HIV- people to TB regardless of peripheral CD4 T cell counts [16, 285], although susceptibility increases with decreasing peripheral CD4 T cells. HIV+ individuals with <200 CD4 T cells/μl blood are more susceptible to TB than HIV+ individuals with >500 CD4 T cells/μl blood regardless of anti-retroviral therapy [270]. Similarly, an acute and transient decrease in peripheral CD4 T cells post-SIVmac251 inoculation in monkeys with latent TB significantly correlated with time to development of reactivation TB [271]. The reduction in CD4 T cells was not confined to the periphery, as co-infected monkeys that reactivated latent TB within 17 weeks of SIVmac251 inoculation had a trend of fewer BAL CD4 T cells compared to monkeys that reactivated after 26 weeks. A decreased frequency of BAL CD4 T cells has also been noted in HIV+ individuals with TB compared to HIV- individuals with TB [281, 284, 286] and also in HIV+ individuals without TB that live in areas with high TB incidence rates compared to HIV- individuals in the same community [286].

4.4.2 Depletion of T cells in TB granulomas of AIDS patients and SIV co-infected monkeys

Histological analysis was used to provide the first data on CD4 counts in granulomas in AIDS patients with TB [252]. Lymph node biopsies from patients with AIDS and tuberculous adenitis had fewer CD4 T cells than individuals without AIDS and tuberculous adenitis. In the absence of CD4 T cells within the granulomas, CD8 T cells were distributed throughout the granuloma without being confined to the periphery, as is normally observed. This suggests that CD4 T cells help maintain the architecture and integrity of the granuloma during co-infection. The reduction in CD4 T cells within granulomas of AIDS patients is not surprising because one characteristic of AIDS patients is <200 CD4 T cells/μl blood and likely reflects a long duration of CD4 T cell depletion. Another study demonstrated that similar numbers of granulomas were observed in HIV+ and HIV- patients with pleural TB [287]. No difference in the number of bacilli assessed by staining or culture positive tissues were observed between the two groups. However, individuals with <100 CD4 T cells/μl blood were more likely to have acid-fast bacilli within biopsied granulomas than individuals with >100 CD4 T cells/μl. CD4 T cell count threshold may be needed in preventing bacterial growth in co-infected individuals. Taken together, these studies demonstrate that the peripheral decrease in T cells correlates to a decrease within the granuloma during AIDS. If this correlation also occurs throughout the entire HIV infection course, this may also explain why co-infected individuals with fewer CD4 T cells are more prone to developing active TB [270]. Many co-infected people present with TB well before the development of AIDS so these granulomas may not represent what is occurring in the majority of co-infected people. We observed significantly fewer CD4 and CD8 T cells within lung granulomas of co-

infected monkeys compared to granulomas from those with active TB alone [271]. The decrease in lung T cell numbers was independent of peripheral CD4 T cell counts, which means that HIV may selectively kill T cells directly involved in maintaining granulomas (i.e. activated T cells at the site) prior to loss of peripheral T cells and signs of AIDS.

Taken together these studies demonstrate that CD4 (and possibly CD8) T cells play a very important role in preventing development of TB in co-infected individuals. However, more studies are needed to confirm that T cell depletion is occurring within granulomas. Future studies may also provide a correlation between peripheral, airway and granuloma T cell counts, which may be used as a biomarker for disease progression.

4.5 CHANGES IN MACROPHAGE FUNCTION

Hypothesis:

The ability of HIV to manipulate macrophage function inhibits killing of intracellular M. tuberculosis.

Alveolar macrophages are presumably the first group of cells infected with *M. tuberculosis* and are the primary immune cells within the airways. They can act as a reservoir for both HIV and *M. tuberculosis*. Following entry of *M. tuberculosis* into the parenchyma, monocytes migrate to the lungs and differentiate into different macrophage types within the granuloma. All of these macrophage types may be susceptible to HIV infection, as well as *M. tuberculosis* infection. HIV envelope phenotyping has suggested that HIV infects activated (HLA-DR+) alveolar macrophages (CD14+, CD36+) as well as lymphocytes (CD26+) in pleural fluid [264] or

airways [288] in co-infected individuals. Since HIV has been shown to infect macrophages *in vivo*, HIV is likely to disrupt the function of *M. tuberculosis*-infected macrophages [212, 257, 288-290], leading to granuloma dysfunction and increased bacterial growth and dissemination.

4.5.1 HIV decreases responsiveness to *M. tuberculosis ex vivo*

Macrophage apoptosis appears to be a critical immune response to *M. tuberculosis* during co-infection [212, 289, 291, 292]. Although it is not fully understood, HIV infection of alveolar macrophages from healthy adults [289] or HIV+ adults [212] is associated with reduced *M. tuberculosis*-induced apoptosis compared to macrophages infected with *M. tuberculosis* alone. Exogenous HIV Nef protein added to *M. tuberculosis*-infected macrophages inhibits ASK1/p38 MAPK signaling, which leads to a decrease in TNF release and TNF-dependent apoptosis [293], suggesting that infectious virus is not necessary for inducing this functional change within a macrophage. This is important because HIV is a retrovirus, with an error prone reverse transcriptase that causes numerous site mutations that render most viral buds noninfectious [294]. Since phagolysosome fusion is inhibited in *M. tuberculosis* infected alveolar macrophages from HIV+ individuals [290], apoptosis may be used as a last resort of infected macrophages. This allows other activated macrophages to engulf the nearby apoptotic bodies, which may lead to the killing of the mycobacteria, and enhanced induction of T cell responses [295]. *M. tuberculosis* induced apoptosis in macrophages is complex and may not always be beneficial to the host. Some evidence suggests that an increase in apoptosis occurs in alveolar macrophages from AIDS patients with pulmonary TB compared to individuals with only pulmonary TB [296]. An increase in apoptosis may be beneficial to the pathogens because it would allow them to exit

macrophages capable of killing. This may also lead to increased dissemination of *M. tuberculosis* and HIV.

HIV appears to manipulate both apoptosis [212, 289] and the ability of macrophages to acidify M tuberculosis-infected phagosomes [290, 297]. These changes in macrophage function may increase the risk of developing active or reactivated TB in co-infected patients. One limitation to many of the studies addressing how HIV and *M. tuberculosis* change macrophage function is the use of cell lines or monocyte-derived macrophages [256, 268, 298] rather than alveolar macrophages [212, 257, 289, 290, 299] or macrophages from granulomatous tissue. Having said that, it is possible that alveolar macrophages may respond differently than macrophages recruited to granulomas, as these environments are very different and represent different stages of the infectious process.

4.6 CHANGES IN *M. TUBERCULOSIS*-SPECIFIC T CELL RESPONSES

Hypothesis:

HIV impairs the function of M. tuberculosis-specific T cells within involved tissue.

T cell mediated responses are essential to protection against disease due to both *M. tuberculosis* and HIV. T cells release cytokines, including IFNγ, TNF, IL-2, as well as a variety of cytolytic molecules that are important in controlling both *M. tuberculosis* and HIV. HIV can exhaust HIV-specific and nonspecific T cells [300, 301], which has led to the hypothesis that HIV reduces the number and functionality of *M. tuberculosis* (*M. tuberculosis*)-specific T cells in co-infected individuals [282, 283, 286, 302, 303].

4.6.1 HIV decreases peripheral *M. tuberculosis*-specific T cell responses

Numerous studies have examined *M. tuberculosis*-specific T cell responses in individuals infected with *M. tuberculosis* by stimulating PBMC, BAL or pleural fluid cells with purified protein derivative (PPD) or culture filtrate protein (CFP) (both are mixtures of mycobacterial proteins and lipids), killed *M. tuberculosis*, or peptide pools from immunogenic *M. tuberculosis*-specific proteins, ESAT-6, CFP10 and Ag85 [261, 282, 283, 302-305]. Zhang and colleagues demonstrated that PBMC stimulated with heat killed *M. tuberculosis* from co-infected individuals proliferated significantly less, released less IFNγ and expressed less IL-2 and IL-12 mRNA than TB-only patients [302]. PBMC from HIV/PPD+ (latently infected) individuals that were stimulated with whole *M. tuberculosis* lysate, ESAT6 or Ag85B proliferated less and released less IFNγ than PBMC from HIV- PPD+ individuals [303]. Likewise, PBMC stimulated with killed *M. tuberculosis* released less TNF without a decrease in IFNγ release in co-infected individuals with active TB compared to people with TB alone [282]. These decreases were not observed in mitogen- or *Candida albicans* antigen-stimulated cells, which supports that HIV is specifically manipulating *M. tuberculosis*-specific T cells. It should be noted that a few studies have demonstrated that co-infected individuals can have a high number of peripheral IFNγ-releasing *M. tuberculosis*-specific T cells even with a low number of CD4 T cells [306-308]. However, the majority of these data support, at least peripherally, that HIV impairs the ability of T cells to respond to *M. tuberculosis*. The reduction in the observable number of peripheral *M. tuberculosis*-specific CD4 T cells may result from their direct infection by HIV in co-infected individuals [261].

One inherent limitation of HIV/TB co-infection clinical research is that it is difficult to assess changes in an immunologic response before and after HIV infection. One excellent study addressed this limitation by examining changes in the number of *M. tuberculosis*-specific peripheral CD4 T cells in individuals with latent *M. tuberculosis* infection before and after HIV seroconversion [283]. They determined that within 3 months post HIV seroconversion a dramatic decrease of peripheral *M. tuberculosis*-specific memory (CD27+CD45RO+) CD4 T cells releasing IFNγ occurred in 4 out of 5 individuals. Although only 5 individuals with latent TB became HIV seropositive during this study, it is the first to demonstrate that HIV specifically reduces *M. tuberculosis*-specific T cells over time. Changes in the peripheral responses have provided evidence to support the hypothesis that HIV depletes and/or functionally disrupts *M. tuberculosis*-specific T cells. However, since TB is rarely a systemic disease it remains to be seen whether these peripheral changes are replicated within involved tissue. An alternative hypothesis is that HIV causes increased *M. tuberculosis* replication in the tissues, and the peripheral cells migrate to the lungs in response to increased antigen, which would present as a reduction in peripheral responses but may not indicate a true loss of specific responses.

4.6.2 HIV reduces *M. tuberculosis*-specific T cell responses in the airways

BAL cells from HIV+ individuals (previously vaccinated with BCG, an avirulent vaccine strain of *M. bovis*) incubated with BCG resulted in significantly fewer IFNγ and TNF releasing BCG-specific CD4 T cells than HIV- individuals [286]. This depletion also occurred in IFNγ+TNF+IL-2+ polyfunctional CD4 T cells within the HIV group. Although these individuals did not have any signs of TB at the time of collection or in the past, this study demonstrates that

HIV specifically impairs the function of both single and polyfunctional mycobacteria (BCG)-specific T cells even without active TB.

The reduction in BAL T cell responses to mycobacteria also occurs in individuals co-infected with HIV and *M. tuberculosis*. AIDS patients with pulmonary TB have a reduced ability to produce IFNγ mRNA in isolated BAL cells compared to individuals with pulmonary TB alone [305]. The functional changes in the context of HIV are not limited to cytokine release. A reduction in proliferation of pulmonary lymphocytes from BAL stimulated with either PPD or an avirulent *M. tuberculosis* strain was observed in individuals with AIDS and TB compared to individuals with TB alone [304]. T cell responses in AIDS patients may not recapitulate what is occurring within HIV+ individuals prior to the significant depletion of CD4 T cells associated with AIDS. However, the BAL studies suggest that HIV disrupts multiple pulmonary T cell functions that may be required to prevent reactivation of latent *M. tuberculosis* infection.

4.6.3 HIV changes cytokine profile within granulomas

In situ hybridization and immunohistochemistry have been used to identify changes in cytokine expression within co-infected granulomas [250, 251]. Although these techniques cannot determine changes in function of *M. tuberculosis*-specific T cells, they provide an overall summary of how cells are responding within the context of granulomas. Bezuidenhout et al., determined that the same number of granulomas within HIV+ and HIV- individuals with pleural TB express Th1 (IFNγ, IL-12, TNF) or Th2 (IL-4, IL-10) mRNA [251]. However, it was determined that granulomas within HIV+ patients expressed more IFNγ, TNF, IL-12 and IL-4 mRNA than granulomas from HIV- individuals. The increase in TNF mRNA expression correlated with an increase in necrotic granulomas within the co-infected patients. This does not

necessarily mean that the *M. tuberculosis*-specific T cells are producing more cytokines in co-infected granulomas. It is possible that the increase in HIV antigens within the granulomas causes an increase in HIV-specific T cell activity too, which cannot be determined without antigen specific functional assays. The increase in cytokine mRNA expression may also be the result of more cells within the granulomas of co-infected individuals compared to HIV- individuals. If the increase in cytokine mRNA leads to increased inflammation, excessive pathology or changes in granuloma function and architecture may occur that inhibit the control of *M. tuberculosis* infection. Contrary to the previous result, another immunohistochemistry study determined that granulomas from HIV+ individuals with TB expressed less TNF and had more extensive necrosis than granulomas from individuals with TB alone [250]. The decrease in TNF expression may be due to a functional disruption or a decrease in the number of T cells and infected macrophages within the granulomas. Due to the highly invasive nature of granuloma-based studies, and the difficulties in obtaining autopsy tissues (and selection bias to these samples), the one solution is the use of a realistic animal model. These studies may elucidate the mechanistic changes that occur within the granuloma as a result of HIV infection, and identify targets for preventive or intervention therapies.

A significant amount of evidence supports the hypothesis that HIV reduces *M. tuberculosis*-specific T cell functions. However, most of these studies have confirmed these changes within the periphery or BAL cells, which may interact differently within the structured environment of a granuloma. No clinical studies have examined functional T cell changes within granulomatous tissue, which could be addressed with an animal model.

4.6.4 Anti-retroviral treatment increases *M. tuberculosis*-specific T cell responses

Anti-retroviral treatment has been used to treat individuals that are co-infected with both HIV and *M. tuberculosis*. Wilkinson et al determined that anti-retroviral treatment in co-infected individuals led to an increase in the percentage of naive (CD27+CD45RA+) CD4 T cells after 36 weeks post anti-retroviral therapy and a sustained increase in central memory (CD27+CD45RA-) by 12 weeks post treatment [309]. The increase in central and naïve CD4 T cells correlate with an increase in ESAT-6/CFP10-specific T cells 48 weeks post anti-retroviral treatment. Surprisingly, a decrease in IFNγ release was observed when PPD was used as a stimulator in this study. Another study followed co-infected patients for 12 months and found an increase in polyfunctional effector memory (CD27-CD45RO+) and terminal memory (CD27-CD45RO-) CD4 T cell responses to PPD [310]. Although anti-retroviral therapy increases T cell responses in co-infected patients, these responses are significantly lower than individuals with TB alone [311]. Although anti-retroviral therapy increases *M. tuberculosis*-specific T cell responses in co-infected individuals this increase in *M. tuberculosis*-specific T cell responses may not always ameliorate TB pathology and may actually exacerbate TB (see section on IRIS below) [312, 313].

4.7 IRIS FURTHER COMPLICATES THE CO-INFECTION

Highly active anti-retroviral treatment (HAART) ameliorates the symptoms of HIV-induced disease through a dramatic reduction in plasma viremia and restoration of CD4 T cell levels [314]. Individuals on HAART are still more susceptible to TB than HIV- individuals [270]. This

susceptibility to TB is inversely proportional to peripheral CD4 T cell count [270]. Individuals co-infected with HIV and TB on HAART have a delayed increase in *M. tuberculosis*-specific T cell responses and may not reach levels observed in HIV- adults [311].

Co-infected individuals on HAART may have excessive inflammation during immune reconstitution, and they may suffer from TB-associated immune reconstitution inflammatory syndrome (IRIS), which occurs in two forms. Paradoxical TB-IRIS occurs in patients on TB treatment before HAART. Unmasking TB-IRIS occurs in patients who are not on TB treatment when they start HAART, and may represent either reactivation of latent infection or enhanced symptoms from TB that was not previously diagnosed as active disease or was subclinical [315]. This is believed to be the result of increased inflammation in the tissues, which can enhance the symptoms of TB, or possibly even trigger reactivation. The available data on IRIS in *M. tuberculosis*-infected persons strongly suggest that excessive inflammation in the setting of subclinical or latent *M. tuberculosis* infection is detrimental to control of the infection [312, 313, 316, 317]. The excessive inflammation may be caused by an increase in antigenic burden, perhaps by reconstituting CD4 T cell effector function in the granuloma which can kill *M. tuberculosis* and release antigen [318], dysregulation of cytokine responses [316] and/or an increase in T cell migration and activation at the site of infection [312, 313, 317]. Higher concentrations of TNF, IL-6 and IFNγ were observed in patients with TB-IRIS compared to individuals with TB alone [316]. The increases in cytokine release may be due to the increase in Th1 responses such as IFNγ releasing T cells [313, 317] and T cell activation (HLA-DR+) [312] observed in individuals that develop IRIS shortly after HAART initiation. One possibility for the increase in Th1 function may be caused by a defect in regulatory T cell function [319], however recent studies have reported no difference in the number of regulatory T cells between co-

infected individuals with and without IRIS [320, 321]. It should be noted that it is not known whether these mechanistic changes are the cause or the result of IRIS, so no predictive clinical biomarker has been identified.

Although the mechanisms that lead to IRIS-associated TB are not fully understood, and have been reviewed more fully elsewhere [315, 322, 323] this unfortunate side-effect of HAART demonstrates that preventing TB is not as straightforward as simply replacing CD4 T cells in the periphery or even in the granuloma [315]. Instead, a balance of pro- and anti-inflammatory responses is necessary for optimal control of *M. tuberculosis* at the granuloma level. The resurgence of immune responses following HAART is likely deficient in reconstituting that balance in some individuals. Again, data are lacking on granulomas and tissues in TB-IRIS patients, and currently, we have no animal model where this phenomenon can be studied.

4.8 ANIMAL MODELS: THEIR POTENTIAL TO ADDRESS GAPS IN HUMAN HIV/TB CO-INFECTION LITERATURE

The available human data has shaped our current understanding of how HIV manipulates *M. tuberculosis* infection and disease. Building on the solid base of knowledge these studies have provided will substantially increase our understanding of the co-infection. A priority should be to increase the number of studies that focus on tissue events, especially at the granuloma level in HIV/M tuberculosis co-infected people. The difficulties in obtaining such samples are obvious. Appropriate and relevant animal models may be the next best choice for determining the events that occur in the tissue and granulomas of co-infected individuals. The TB field has several experimental animal models. The advantages of an animal model include the ability to control

the timing, dose, and strain of infection, to sample or necropsy at pre-determined time points, and to obtain tissue at necropsy. Currently there are only a few animal models available for studying interactions between HIV and TB. These models may be able to address some of the gaps in the human HIV/TB literature.

4.8.1 Mouse models

Mouse models have been invaluable for addressing immunological and pathogenesis questions in TB. Genetic similarities and the availability of genetically manipulated strains, reagents, low cost and relatively easy maintenance makes them ideal for most TB research facilities. A disadvantage of the mouse model is that TB in mice is a chronic infection that differs from both active and latent TB in humans. Murine models have demonstrated the importance of IFNγ, TNF, activated macrophages and CD4 T cells, among other factors, in controlling TB [56, 71, 111]. However, mice are not susceptible to HIV and there is not a suitable homologous murine virus so wild type mice are not ideal candidates for co-infection research.

The most basic HIV/*M. tuberculosis* mouse model is one in which mice are rendered CD4 T cell deficient, either through antibody-mediated depletion or genetic manipulation [56, 111]. Because CD4 T cells are depleted during HIV infection it is logical to study CD4 deficient mice as a model for HIV/TB co-infection. CD4 T cell deficient mice are more susceptible to advanced TB than wild type mice, supporting the importance of CD4 T cells in containing primary and chronic TB. However, the disease associated with HIV is not entirely caused by depletion of CD4 T cells. Viral particles can induce non-specific apoptosis [324], disruption of lymph node architecture [325], T cell anergy [300], and affect macrophages, all of which have

been associated with HIV pathology. These aspects of HIV infection cannot be recapitulated in CD4 T cell depleted mice.

To address how viral proteins, specifically HIV Nef, manipulate immunological responses Nef transgenic mice have been developed [326-328]. These mice express Nef in CD4 T cells, macrophage and dendritic cells and subsequently develop AIDS-like disease characterized by CD4 T cell depletion as well as lung, heart and kidney diseases [327, 328]. This transgenic mouse model demonstrates that Nef expression within CD4 T cells is a major determinant of pathogenicity of HIV infection [326]. Nef expression within CD4 T cells causes increased activation and apoptosis, which eventually leads to their depletion. Future TB studies may be able to use this transgenic mouse to determine how Nef expression changes immunologic responses to TB.

Another mouse model that may increase our understanding of how HIV manipulates TB pathology are humanized bone marrow–liver–thymus (BLT) mice reconstituted with human hematopoietic stem cells, which produces human lymphoid tissues [224]. Human CD4 T cells reconstitute the gastrointestinal and female reproductive tract, causing these mice to be susceptible to rectal [224] and vaginal [329] HIV inoculation. HIV infection results in systemic viral loads, a depletion of systemic CD4 T cells and T cell activation similar to what is observed in humans with HIV [224, 329]. Infecting these mice with HIV and *M. tuberculosis* may identify how HIV behaves within granulomas. Prophylaxis has been shown to reduce infection rates within these mice [329], which indicates that anti-retroviral effectiveness in the context of a HIV/*M. tuberculosis* co-infection may be studied in these mice. The use of HAART in co-infected mice may be able to address how HIV induces functional changes in *M. tuberculosis*-specific T cells within granulomas, but also how the virus changes granuloma formation and

architecture. BLT mice may be used to identify immunologic targets that can be examined within more expensive primate models or clinical studies.

4.8.2 Nonhuman primate models

Nonhuman primates (NHP) have helped elucidate our understanding of HIV [330-332] and *M. tuberculosis* [72, 91, 112, 113] infections. Serial blood, BAL and LN biopsy samples can be obtained from NHP during the course of infection and all tissues are available at necropsy, addressing an inherent limitation of clinical studies. This model may be ideal for approaching questions that cannot be answered in clinical studies.

Several NHP models of HIV/TB co-infection have been developed [253, 271, 333-336]. SIV-infected rhesus macaques have been inoculated with BCG [333, 334, 336, 337] or *M. tuberculosis* [253]. These models have recapitulated the decrease in peripheral mycobacterial-specific T cell responses observed HIV/TB co-infected humans. The similarities between these results and human co-infection studies demonstrate the validity of the NHP as an animal model for co-infection. We recently reported a cynomolgus macaque model of SIV-induced reactivation of latent TB, which should be very useful in understanding how HIV manipulates TB immunology and pathology [271]. This model examined aspects of the co-infection that haven't been addressed in human studies. For example, the severity of the initial but transient reduction in peripheral T cell numbers during acute SIV was correlated with time to reactivation and reductions in T cell numbers also occurred within lung granulomas of co-infected monkeys compared to monkeys with active TB without SIV [271]. Data extrapolated from this model and clinical studies helped elucidate a potential mechanism for the reactivation of latent TB granuloma (see chapter 8). These models have the potential to be used for immunomodulation,

vaccine and antiretroviral-based studies to study efficacy of therapies against TB in the context of the co-infection.

Animal models will allow us to assess HIV-induced changes in *M. tuberculosis*-specific immune responses that may lead to reactivation of TB and increased susceptibility to TB in HIV+ individuals. Although every animal model has its limitations we hope that these new mouse and NHP models will provide evidence supporting or refuting the various hypotheses about how HIV manipulates TB pathology. Increasing our basic understanding of how these pathogens interact *in vivo* will help us uncover possible treatments for co-infected people.

4.9 HOW CAN TISSUE-BASED STUDIES IMPROVE TREATMENT?

Future studies may demonstrate that a high level of HIV replication within the granuloma correlates to granuloma dysfunction and mycobacterial growth. This is important because it is unknown whether anti-retrovirals reduce viral load within granulomas or even if they penetrate granulomas in the appropriate concentrations in co-infected patients. Drug concentration in granulomas can be quantified by direct measurement in animal models, and this may be necessary to determine the best treatment for co-infected persons, taking into account penetration of granuloma, viral load, and type of granuloma. A further important factor is that understanding the correlation between viral titer within BAL and plasma with viral titers in granulomatous tissue will provide a better opportunity to assess the efficacy of HAART in co-infected individuals, and possibly identify biomarkers for drug efficacy. These studies will be essential for development of tractable biomarkers in the blood that translate to granuloma dysfunction and predict outcome in co-infected individuals.

However, if clinical and animal-based studies demonstrate that HIV does not replicate specifically within granulomas, the functional change to the immune response may occur in the lymph nodes, where priming of the initial and perhaps ongoing T cell responses occurs. The thoracic lymph nodes are a common site of *M. tuberculosis* infection, and may actually be a site for reactivation of latent infection [72]. This may be validated by correlating changes in *M. tuberculosis*-specific T cell function with viral load within these lymph nodes. If a positive correlation is present, drug effectiveness may be determined by examining changes in viral load within these tissues during drug treatment.

4.10 CONCLUSION

The mechanisms by which HIV disrupts TB granuloma function and lead to increased morbidity and mortality have been extrapolated from clinical and animal studies, but remain poorly understood. Changes in T cell and macrophage function within granulomas need to be examined in future clinical and animal studies to elucidate possible mechanisms by which HIV disrupts TB immune pathology. These studies may provide insight into potential drug and/or vaccine therapies. In addition, it is important to understand the parallels between easily obtained samples (like blood) and the tissue responses, so that one can correctly interpret the blood-based data. Only through studies that correlate blood and tissue responses can we begin to search for biomarkers for disease status that can be used in vaccine and drug studies.

5.0 STATEMENT OF THE PROBLEM

TB is the leading killer of individuals infected with human immunodeficiency virus-1 (HIV). Immunocompromised individuals have a significantly greater risk of developing active TB than individuals that are HIV-uninfected. Although clinical reports have elucidated a significant amount of information about how the co-infection manipulates our immune system, there are some drawbacks to only studying humans. These reports have been limited because of the paucity of available time points, limited availability of leukocyte samples, the undefined status of the immune system prior to infection, the dose of the inoculum, the ethical obligation to commence treatment, and the extent to which post mortem tissue samples are available.

Because our current understanding about HIV/*M. tuberculosis* co-infection has predominantly been derived from clinical and *in vitro* reports, there exists a need for an animal model recapitulating the co-infection to fill in gaps in knowledge. Cynomolgus macaques (*Macaca fasicularis*) have been used as animal models of human infection with *M. tuberculosis* and simian immunodeficiency virus (SIV) infection. In this study, we developed a non-human primate model of co-infection by infecting cynomolgus macaques with latent TB with SIVmac251. We also developed an *in vitro* and *ex vivo* SIV/*M. tuberculosis* co-infection method to determine how SIV manipulates both macrophage and *M. tuberculosis*-specific T cell function.

The co-infection animal model allowed us to examine multiple immunologic and microbiologic aspects over the entire course of disease that would not have been possible in humans. We focused on examining changes in peripheral T cells over time, changes in cytokine concentrations within plasma, how SIV manipulates granuloma composition and architecture, viral concentrations in the periphery over time and within tissue at necropsy and how SIV changes T cell effector functions within granulomas and thoracic lymph nodes. For the first time we demonstrated that SIV can induce the reactivation of latent TB. In this model we provide evidence that initial T cell depletion after SIV inoculation in monkeys with latent TB perturbs the control of the infection, and directly corresponds to the reactivation of TB, even though peripheral T cell numbers quickly return to pre-SIV levels. This model addressed my first specific aim and part of my second aim.

We also developed *in vitro* and *ex vivo* experiments to examine how HIV manipulates both macrophage and *M. tuberculosis*-specific T cell function. We used peripheral T cells and monocytes, monocyte-derived macrophages and thoracic lymph node homogenates from *M. tuberculosis*-infected macaques (without SIV) along with exogenous SIV to determine how the virus changes immunologic responses to *M. tuberculosis*. Here we determined that SIV reduces *M. tuberculosis*-specific T cell responses through the manipulation of antigen presenting cells. This manipulation of *M. tuberculosis*-specific T cell function results from SIV inducing *M. tuberculosis*-infected macrophages to produce IL-5, which specifically caused a reduction in *M. tuberculosis*-specific CD4 T cells releasing TNF. These studies suggest a novel role for IL-5 and address specific aims 2 and 3.

5.1 SPECIFIC AIM 1: ESTABLISH A CYNOMOLGUS MACAQUE MODEL OF *M. TUBERCULOSIS*/SIV CO-INFECTION

Hypothesis:

SIVmac251 will reactivate latent TB in cynomolgus macaques.

A clinically relevant *M. tuberculosis*/SIV co-infection model will enable a detailed investigation throughout the course of disease and provide information about how HIV reactivates latent TB.

5.2 SPECIFIC AIM 2: EXAMINE CHANGES IN *M. TUBERCULOSIS*-SPECIFIC T CELL EFFECTOR FUNCTIONS THAT RESULT FROM *M. TUBERCULOSIS*/SIV CO-INFECTION

Hypothesis:

SIV will reduce the responsiveness of M. tuberculosis-specific T cells.

T cell effector functions are crucial in controlling *M. tuberculosis*. HIV has the ability to kill and reduce the responsiveness of both CD4 and CD8 T cells. HIV-induced manipulation of *M. tuberculosis*-specific T cell responses may decrease containment of *M. tuberculosis*.

5.3 SPECIFIC AIM 3: DETERMINE HOW SIV MANIPULATES MACROPHAGE FUNCTION THAT RESULTS FROM *M. TUBERCULOSIS*/SIV CO-INFECTION

Hypothesis:

Co-infected macrophages will have a reduced ability to stimulate M. tuberculosis-specific T cells.

Macrophages are a major component of granulomas and their function is essential in containing *M. tuberculosis*. Macrophages both kill intracellular pathogens and can activate T cells. HIV and *M. tuberculosis* can both reduce normal macrophage function. This dysfunction is exacerbated during the co-infection.

6.0 REACTIVATION OF LATENT TUBERCULOSIS IN CYNOMOLGUS MACAQUES INFECTED WITH SIV IS ASSOCIATED WITH EARLY PERIPHERAL T CELL DEPLETION AND NOT VIRUS LOAD

This chapter is adapted from the original publication:

Diedrich CR, Mattila JT, Klein E, Janssen C, Phuah J, et al. (2010) Reactivation of Latent Tuberculosis in Cynomolgus Macaques Infected with SIV Is Associated with Early Peripheral T Cell Depletion and Not Virus Load. PLoS ONE 5(3): e9611. doi:10.1371/journal.pone.0009611. Citation: [271].

6.1 ABSTRACT

HIV-infected individuals with latent *Mycobacterium tuberculosis* (*M. tuberculosis*) infection are at significantly greater risk of reactivation tuberculosis (TB) than HIV-negative individuals with latent TB, even while CD4 T cell numbers are well preserved. Factors underlying high rates of reactivation are poorly understood and investigative tools are limited. We used cynomolgus macaques with latent TB co-infected with SIVmac251 to develop the first animal model of

reactivated TB in HIV-infected humans to better explore these factors. All latent animals developed reactivated TB following SIV infection, with a variable time to reactivation (up to 11 months post-SIV). Reactivation was independent of virus load but correlated with depletion of peripheral T cells during acute SIV infection. Animals experiencing reactivation early after SIV infection (<17 weeks) had fewer CD4 T cells in the periphery and airways than animals reactivating in later phases of SIV infection. Co-infected animals had fewer T cells in involved lungs than SIV-negative animals with active TB despite similar T cell numbers in thoracic lymph nodes. Co-infected animals had fewer IFN-γ producing T cells within the thoracic lymph nodes than monkeys with active TB. Granulomas from these animals demonstrated histopathologic characteristics consistent with a chronically active disease process. These results suggest initial T cell depletion may strongly influence outcomes of HIV-*M. tuberculosis* co-infection.

6.2 INTRODUCTION

Approximately 90% of human *Mycobacterium tuberculosis* (*M. tuberculosis*) infections are clinically latent and likely represent an immune response that successfully limits bacterial growth, resulting in persistence within multi-cellular structures called granulomas [338]. While granulomas are composed of many different cell types, macrophages and T cells are important components that collaborate to limit bacterial replication and prevent dissemination. The immune response of immunocompetent individuals can prevent active tuberculosis for years or decades, and latently infected individuals have only a 5-10% lifetime risk of developing reactivated tuberculosis (TB) [339]. Immunosuppressed individuals have a significantly greater chance of

developing active disease, and TB is the leading killer of individuals infected with human immunodeficiency virus (HIV) [14]. In contrast to most opportunistic infections, which present in the later stages of HIV infection, TB afflicts HIV-positive individuals throughout the course of infection, even while CD4 numbers are well preserved [340-342]. While factors explaining the high rates of reactivated TB in co-infected humans remain unclear, depletion of CD4 T cells [281] and increased virus loads [343] within granulomatous tissue may be contributors.

Co-infections in humans, and the accompanying immune responses, are inherently difficult to investigate and studies are frequently confounded by uncontrolled variables. Our current understanding of immune responses to HIV-*M. tuberculosis* co-infection comes predominantly from human clinical studies [249, 343]. As with all clinical studies involving human subjects, there are limitations to studies that can be performed with HIV-*M. tuberculosis* co-infected individuals. Some challenges include difficulty determining which infection occurred first and when, limited availability of pre- and post-infection samples, restrictions on unnecessary invasive procedures to obtain tissue samples, and limited availability of post mortem tissue samples for immunologic analysis. Additionally, most HIV-TB clinical studies are in individuals with active tuberculosis, and cannot fully explore the events that precede or occur during reactivation. Human studies also have numerous uncontrolled variables including the undefined status of the immune system prior to infection and the presence of other undiagnosed co-infecting pathogens that may have an effect on the host immune response. Consequently, a biologically relevant animal model of HIV-*M. tuberculosis* co-infection where the amount and sites of sampling could be increased and the confounding variables minimized would be an extremely valuable asset.

Good animal models for HIV and TB exist, but there is not a model which recapitulates HIV-infection in an individual with latent TB. Macaques are frequently used to model HIV by infection with simian immunodeficiency virus (SIV) or SHIV, a HIV-SIV chimera. Depending on the macaque species and the virus type used, these animals can be excellent models for human infection and disease [174, 344-350]. Macaques are also valuable in studying tuberculosis [113, 116, 336, 351-353]. Cynomolgus macaques infected with a low number of *M. tuberculosis* bacilli develop clinical signs and pathology similar to humans with active TB or develop subclinical latent infections, with equal proportions of each infection outcome observed [113, 116]. Moreover, latency can be maintained for significant time periods. In our experience working with cynomolgus macaques over the past decade, only two of approximately 85 latently infected monkeys spontaneously reactivated [13,19, Flynn and Lin unpublished data]. Thus, cynomolgus macaques with latent TB have a <5% chance of spontaneously reactivating TB within a few years of infection and can maintain this latent state for years [113, 354].

Nonhuman primates have been used to examine interactions between SIV and mycobacteria. Macaques co-infected with SIV and *M. tuberculosis* (strain H37Rv) [253] or SIV and *M. bovis* BCG [334, 336] have been used to examine how mycobacteria induce AIDS-like symptoms. Rhesus (*M. mulatta*) or pigtail (*M. nemestrina*) macaques were inoculated with mycobacteria after SIV infection or simultaneously co-infected with BCG and SIV [334, 336]. These studies indicated that SIV could be immunosuppressive and sometimes exacerbate mycobacterial disease. Despite the range of model systems available, similar studies into disease processes underlying reactivated TB in HIV-infected humans with latent TB have yet to be done.

In the current study we use cynomolgus macaques infected with SIVmac251, a virulent HIV-like virus, to develop a novel model of HIV-induced reactivated TB. We used this model to

examine immunologic, microbiologic and virologic changes in the peripheral blood and tissues that have not been extensively investigated in human or nonhuman primate HIV/SIV-*M. tuberculosis* co-infection studies. Co-infected animals showed a spectrum of disease severity, with the animals that reactivated <17 weeks post-SIV infection experiencing more severe pathology than animals that reactivated >26 weeks post-SIV infection. We found that time to reactivation correlated with significant peripheral CD4 T cell depletion but not virus load during the acute phase of SIV infection. The co-infected animals had fewer T cells in lung tissue than SIV-negative macaques with active TB, and a trend towards fewer T cells in the pulmonary lymph nodes than SIV-only macaques. Co-infected animals also had fewer IFN-γ releasing T cells within thoracic lymph nodes compared to monkeys with active TB. We also present evidence of rapid impaired local control of latent *M. tuberculosis* infection, following SIV infection. These studies pave the way for detailed investigations of factors underlying reactivated TB in HIV-*M. tuberculosis* co-infected humans that were not previously possible.

6.3 MATERIALS AND METHODS

6.3.1 Ethics Statement

All experimental manipulations and protocols were approved by the University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee. The animals were housed and maintained in accordance with standards established in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

6.3.2 Experimental Animals

Fifteen adult (>4 years of age) cynomolgus macaques (*Macaca fascicularis*) of Chinese origin were used for these studies (Covance, Alice, TX; USA Valley Biosystems, West Sacramento, CA). Prior to the study, animals were tested to ensure they were free of *M. tuberculosis*, SIV, SHIV or simian retrovirus D infection. Animals were also given physical and clinical examinations including differential blood cell counts, erythrocyte sedimentation rate (ESR), serum chemistry profile and thoracic radiography to ensure they were free of underlying disease processes. All animals were given 14-day prophylactic Bactrim (sulfamethoxazole and trimethoprim) treatments to eliminate potential *Pneumocystis* colonization infection. SIV-infected animals were housed under BSL-2 conditions while *M. tuberculosis*-infected animals were housed under BSL-3 conditions. Bronchoalveolar lavage (BAL), gastric aspirate and other procedures were performed as previously described [113, 116]. We used historical controls from previous studies [18,21, Flynn and Lynn unpublished data] on animals with active and latent TB to minimize the number of animals used in this study.

6.3.3 *M. tuberculosis* and SIV infection

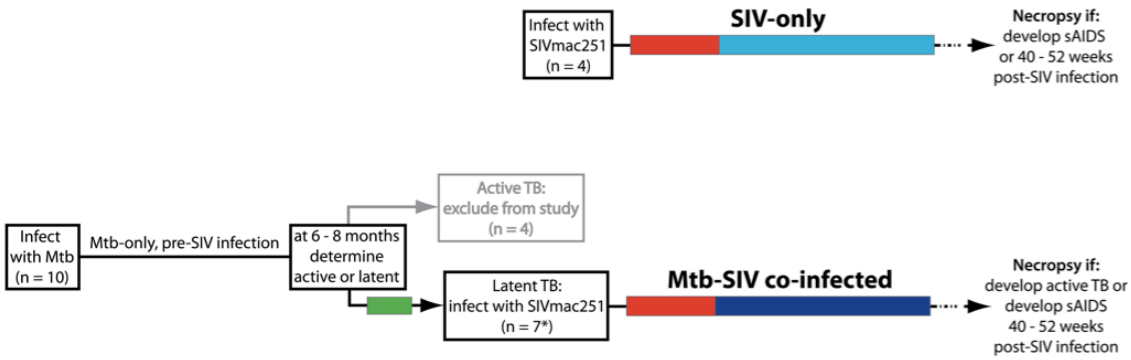

Figure 8. *Schematic representation of experimental design*. Two groups of animals were established, a SIV-only control group and a Mtb-SIV co-infection group. Historical controls based on other studies were used as Mtb-only controls for active or latent TB. Green indicates the time where Mtb-only, pre-SIV infection baseline data were acquired. Red indicates 0–8 weeks post-SIV infection where blood was drawn weekly, BAL cells and gastric aspirates were acquired every four weeks and lymph node biopsies were done at four and eight weeks post-SIV. Light blue (SIV-only) indicates where monthly blood draws and BAL procedures were performed. Dark blue (Mtb-SIV co-infected) indicates where blood draws for ELISPOT assays and virus load determination were performed every other week, BAL cells and gastric aspirates were acquired every four weeks and lymph node biopsies were performed at weeks 12 and 24 post-SIV infection. No SIV-only animals experienced any sAIDS-like symptoms.

The experimental design of these studies is described in (Figure 8). Ten animals were infected with ~25 CFU Erdman strain *M. tuberculosis* via intra-bronchial instillation as previously described [116]. *M. tuberculosis* infection was confirmed in all ten animals by conversion of negative to positive tuberculin skin test and peripheral blood mononuclear cells (PBMC) responses elevated from baseline in lymphocyte proliferation (LPA) and PBMC enzyme-linked immunosorbent spot (ELISPOT) assays. Animals were classified as latent or active 8-10 months post infection with the criteria for latency defined as TST positive but with no signs of clinical disease as previously described [113, 116]. Of the original ten animals infected with *M. tuberculosis*, six were classified as latent and selected for SIV infection. A seventh latent monkey (10405) and infected with the same dose and strain of *M. tuberculosis* from another study, was added to this group to increase the number of animals.

For SIV infection, concentrated SIVmac251 stock (kindly provided by Dr. Keith Reimann, Beth Israel Deaconess Medical Center, Harvard University) was diluted in RPMI medium, and 10^6-10^7 TCID50 units of virus injected intravenously. Seven animals with latent TB and four *M. tuberculosis*-negative animals were infected with SIV, with the four *M. tuberculosis*-negative animals designated as the SIV-only control group. Historical data from latent and active control monkeys were used for comparison to minimize the number of animals needed for this study. Some data from these monkeys, as well as full characterization of pathology and disease outcome, has been published previously [113]. Criteria for assessing reactivation included prolonged weight or appetite loss, elevated erythrocyte sedimentation rate (ESR), *M. tuberculosis*-positive gastric aspirate or BAL cultures, or radiographic evidence of lung involvement, as shown in (Table 1).

6.3.4 Necropsy procedures

Animals were humanely euthanized and necropsied as previously described [116] when indicators of active TB were present (in co-infected monkeys) or at the end of the study (11 months post-SIV). Necropsies were conducted by veterinarians with substantial experience examining *M. tuberculosis*-infected cynomolgus macaques (E.K., C.J.). Lung lobes, liver, spleen, and kidney were examined for evidence of tuberculous disease. Similarly, thoracic, axillary and inguinal lymph nodes were examined for the presence of granulomas. All gross pathology associated with tuberculous disease was recorded and quantified using a previously established scoring system validated to differentiate latent from active tuberculosis in this model [113]. Tissue (granulomas, lymph nodes, uninvolved lung from each lobe, extrapulmonary organs) was divided into pieces for histology and RNA isolation, and the remainder was mechanically

homogenized into single-cell suspension for immunologic and microbiologic analysis and virus load determination. 30-40 samples per monkey were examined histologically and used for bacterial, virologic and immunologic assays. In several monkeys, gut tissue was obtained for analysis of T cell frequencies. Bacterial numbers in tissues were quantified by plating dilutions of tissue homogenate onto 7H10 agar plates and the number of colony forming units (CFU) in the original sample calculated after 4-6 weeks of incubation at 37°C/5% CO_2.

6.3.5 Immunologic analysis

Blood was drawn from SIV-only and co-infected animals every week prior to and for the first eight weeks post infection. Thereafter, blood was drawn from *M. tuberculosis*-SIV co-infected animals every other week and SIV-only animals monthly. PBMC were isolated via percoll gradient centrifugation as previously described [331]. Axillary or inguinal lymph nodes were biopsied at pre-infection, 4, 8 and 16 weeks post SIV infection. PBMC and lymph node cells were subjected to flow cytometry and IFN Axillary or inguinal lymph nodes were biopsied at pre-infencluded autologous monocyte-derived dendritic cells, as antigen-presenting cells, as previously described [116, 331].

6.3.6 ELISPOT assays for *M. tuberculosis*- and SIV-induced IFNγ responses

ELISPOT assays were performed as previously described [116, 331] with 150,000 PBMC or lymph node cells per well using ELISPOT reagents with known cross reactivity against macaque

IFNγ (MabTech, Mariemont, OH) [18]. *M. tuberculosis* antigens used in ELISPOT were peptide pools (overlapping 20-mers; 10 ug/mL) from CFP-10 (Rv3872) and ESAT-6 (Rv3875) synthesized by Sigma-Genosys (Woodlands, TX). Peptide pools for viral antigens gag, pol, env, tat, nef and rev (overlapping 20-mers) all used at 10 ug/mL, based on SIVmac239) were obtained from the AIDS Research and Reference Reagent Program (National Institutes of Health, Germantown, MD). Phorbol 12,13-dibutyrate (PDBu) and ionomycin (50 nM and 10 uM final concentration respectively; Sigma) were used as positive controls. Media-only wells were used as negative controls. Cells in ELISPOT assays were incubated with antigens for two days at 37°C/5% CO_2 prior to being developed as previously described [331] and read using an ELISPOT plate reader (Cellular Technology LTD, Cleveland, OH). All conditions were performed in duplicate wells. ELISPOT data were normalized and expressed as SFU per 10^6 cells.

6.3.7 Flow cytometry

All antibodies used for flow cytometry were direct conjugates against human proteins and obtained from BD Biosciences (San Jose, CA) unless otherwise noted. Approximately $1x10^6$ PBMC or tissue cells were stained using combinations of the following antibodies: CD3 (clone SP34-2), CD4 (clone L200), CD8 (clone DK25 [Dako; Carpinteria, CA], clone SK-1, clone OKT8 [eBioscience; San Diego, CA]) CD29 (clone HUTS-29) and CD69 (clone FN50) (clone 2H7 [eBioscience]). Cell phenotypes were read with a LSR II flow cytometer (BD Biosciences) and positively-stained populations gated using fluorochrome-matched isotype antibodies as negative controls using the FlowJo software package (Tree Star Inc., Ashland, OR).

6.3.8 Plasma cytokine determination during acute SIV

Frozen plasma was saved every week for the first 8 weeks post SIV inoculation in the co-infected and SIV-only infected animals. Plasma was thawed and sterile filtered filtered (0.22μm). Bio-plex nonhuman primate beads were used according to manufacturer's instructions. Cytokines analyzed were: IFN-γ, TNF, IL-10, IL-15 and IL-18. Samples were read on a Luminex 100 IS Bio-Plex System machine (Luminex Corporation, Austin, TX).

6.3.9 Virus load determination

Quantitative real time reverse transcriptase (qRT-PCR) for the SIV *gag* gene was used to measure virus loads as previously described [331, 355]. RNA was isolated from $1x10^6$ percoll gradient-isolated PBMC or lymph node cells, and from $2x10^5$ BAL or tissue cells using the Qiagen RNeasy kit (Valencia, CA). Plasma virus load was determined by isolating RNA from 200 uL of plasma using the Purelink Viral RNA/DNA mini kit (Invitrogen, Carlsbad, CA) as per manufacturer's instructions.

6.3.10 Statistical analysis

Data were analyzed using Prism (Graphpad Software, San Diego, CA). Pair-wise comparisons between groups (e.g. latent and co-infected animals) was performed using the Mann-Whitney test with $p<0.05$ considered statistically significant.

6.4 RESULTS

6.4.1 CD4 T cells were transiently depleted in the periphery of SIV-infected animals coincident with peak virus load in PBMC.

We measured viral titers in SIV-infected animals to determine how SIV infection correlated with reactivation or whether *M. tuberculosis* infection influenced SIV loads. SIV titers in plasma, PBMC, and lymph node cells were similar between co-infected monkeys and SIV-only monkeys (**Figure** 9). Peak virus loads in PBMC and plasma occurred at 2-3 weeks post SIV infection and then declined to low levels for the duration of the study. The greatest virus burden in BAL cells from co-infected monkeys occurred two weeks after peak virus load in the peripheral blood. Virus loads in cells from peripheral lymph nodes were not significantly different between time points, but these sites maintained moderate virus loads (10^4-10^6 copies/10^6 cells).

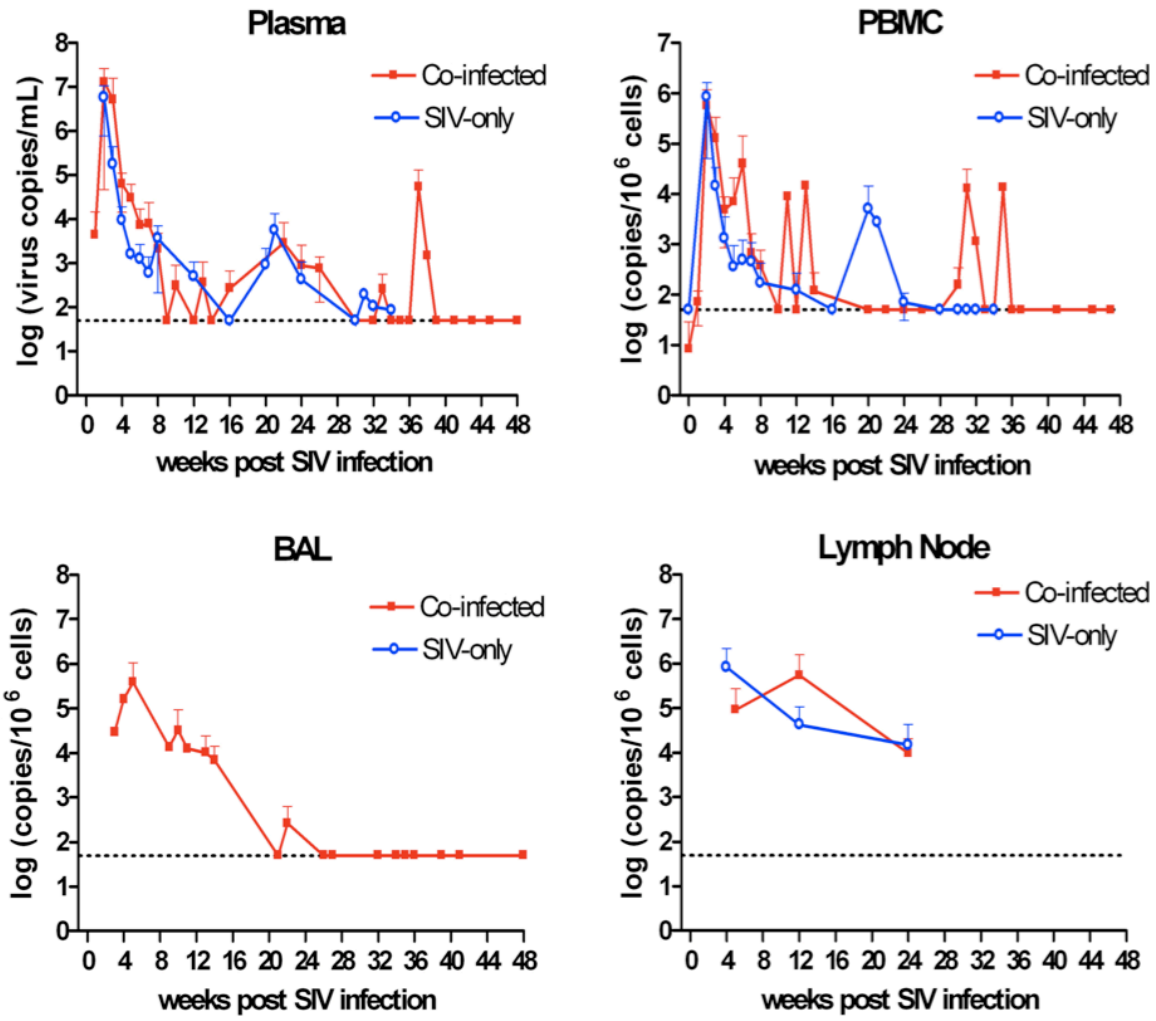

Figure 9. *SIV loads in plasma, PBMC and lymph nodes of Mtb-SIV co-infected and SIV-only monkeys.* No significant differences in plasma, peripheral blood mononuclear cells (PBMC), bronchial alveolar lavage cells (BAL) or peripheral lymph node viral titer were observed between co-infected cynomolgus macaques (red line, n = 7) or the SIV-only group (blue line, n = 4). We were unable to obtain enough BAL cells from SIV-only monkeys to reliably measure virus load. The horizontal dashed line represents the detection limit for viral copy numbers. Lower error bars have been removed for clarity.

6.4.2 SIV causes reactivation of latent tuberculosis

We previously described the clinical, microbiologic, radiologic and immunologic parameters used to define latent infection and active disease in macaques and demonstrated that these are validated by quantitative measurements of bacterial burden and gross pathology at necropsy [113, 116]. Briefly, latent monkeys are asymptomatic and lack radiographic or microbiologic evidence of *M. tuberculosis* infection beyond three months post infection [113, 116]. Reactivation is defined as evidence of active TB either by microbiologic, radiographic or clinical parameters after a period of stable latent infection [113]. Based on these criteria, three of the seven co-infected monkeys with latent TB had findings consistent with reactivated TB 12-17 weeks post-SIV infection while the remaining four monkeys reactivated between 26-48 weeks post-SIV (Table 1). Monkey 1807 was necropsied after positive BALs and had relatively low necropsy and bacterial number scores, suggesting that positive cultures from BALs do not necessarily correlate with significant disease burden, but may signify the first sign of reactivation [113]. Considering this, Monkey 1907 was necropsied approximately 28 weeks after the first positive BAL and with additional positive clinical results to confirm reactivation was underway. While a spectrum of disease was observed, we loosely categorized the monkeys according to time of reactivation: early reactivation (reactivated TB in <17 weeks post SIV infection) and later reactivation (reactivated >26 weeks post SIV).

Table 1. Clinical evidence for reactivated TB in co-infected monkeys

Monkey	Positive clinical indicator of TB (week observed)[a]	Necropsy[a]
2407	GA[b] (4,9), increasing ESR (4,9,12), chest x-ray (9, 12)	12
1407	increasing ESR[b] (4,9,13), chest x-ray (14)	15
1207	GA (10), chest x-ray (6)	17
1807	BAL[b] (16,17)	26
1907	BAL (17,34), GA (37,44), chest x-ray (37)	45
3007	increasing ESR (41,45), progressive weight loss (37,41,45)	47
10405	chest x-ray (47)	48

[a]indicates weeks post-SIV infection.
[b]GA–gastric aspirate, ESR–erythrocyte sedimentation rate, BAL–bronchoalveolar lavage.

6.4.3 Relationship between peripheral T cell depletion and virus loads and time to reactivation

SIV infection affects lymphocyte numbers, and changes in CD4 and CD8 T cell numbers were followed in the PBMC and axillary or inguinal lymph nodes (referred to here as peripheral lymph nodes) of co-infected monkeys over the course of SIV infection to better understand the dynamics between lymphocyte numbers and reactivation. All co-infected monkeys experienced declines in numbers of CD4 and CD8 T cells in the PBMC (Figure 10) and peripheral lymph nodes (Figure 11) between 2-4 weeks post SIV infection, a time corresponding with peak viremia (Figure 10A). After 8 weeks, T cells recovered to pre-SIV levels in all monkeys. No

differences in CD4 or CD8 frequencies were found among the SIV-only, early and late reactivators (Figure 11). Peripheral T cell numbers in co-infected animals were averaged during acute SIV infection (weeks 1-8 post-SIV) and plotted against the week that either indicators of reactivated TB were noted or the week that necropsy occurred to address the relationship between T cells and reactivation.

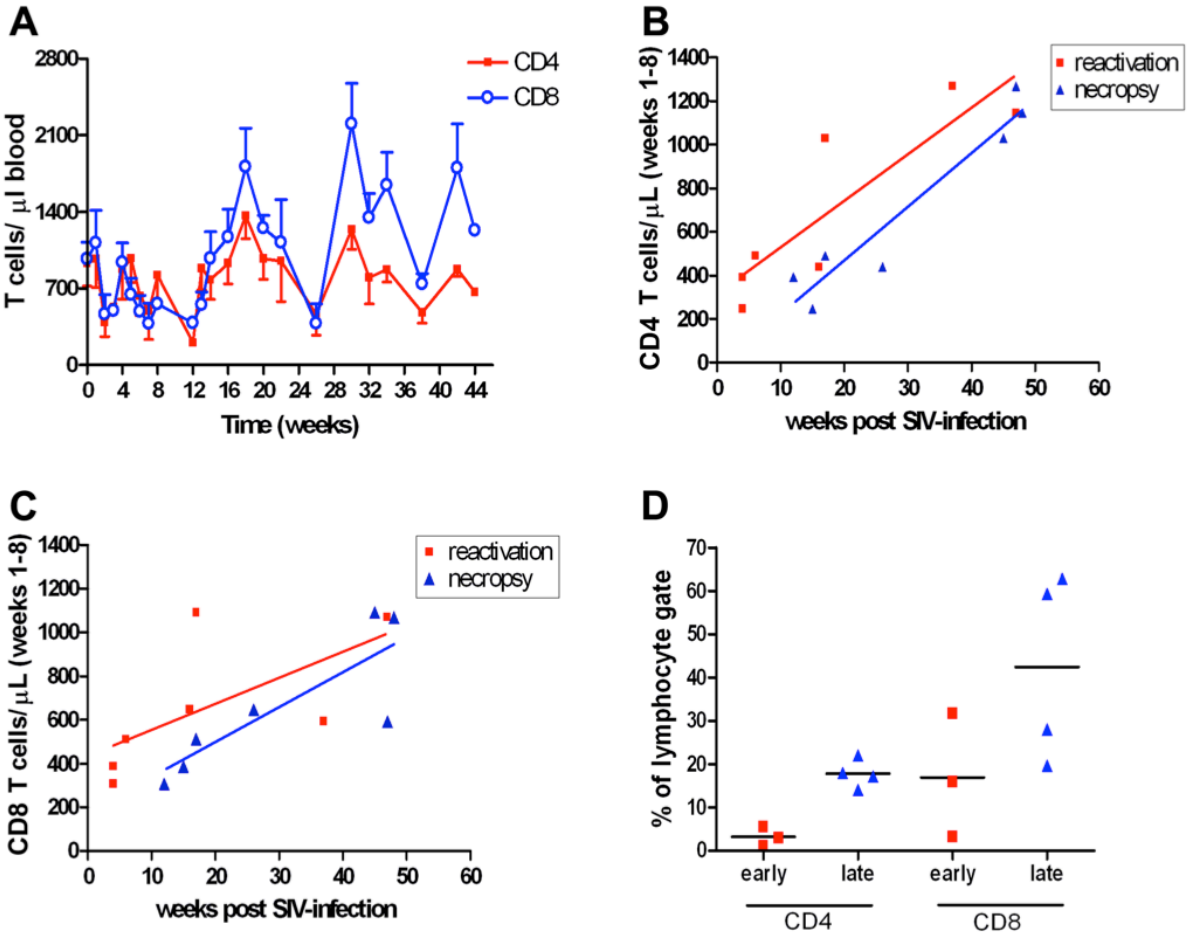

Figure 10. *Acute CD4 T cell depletion correlates with reactivated TB*. A. Mean CD4 and CD8 T cell numbers from whole blood of co-infected macaques. Upper (CD8) and lower (CD4) error bars have been omitted for clarity. B. Mean CD4 T cell numbers over weeks 1–8 correlate with reactivation (red line: P = 0.011, R2 = 0.756) and necropsy time (blue line: P = 0.0007, R2 = 0.9172). C. Mean CD8 T cell numbers do not correlate with reactivation (red line: P = 0.112, R2 = 0.426) but do correlate to necropsy time (blue line: P = 0.020, R2 = 0.692). D. Animals that reactivate <17 weeks post-SIV infection have a trend toward lower frequencies of CD4 T cell in the BAL at 10 weeks post SIV infection than monkeys >26 weeks post SIV infection Man-Whitney (P = 0.057). 1207 (early) BAL cells were sampled 8 weeks post SIV infection.

Necropsy time was based on the aggregate clinical signs in a monkey. There was a significant correlation between extent of depletion of CD4 T cell numbers 2-8 weeks post-SIV and time to reactivation ($P = 0.011$, $R^2 = 0.755$) and necropsy time ($P = 0.0007$, $R^2 = 0.9172$) (Figure 10B). The correlation between reactivation signs and early depletion of CD8 T cell was not significant ($P = 0.112$, $R^2 = 0.425$) but extent of early depletion of CD8 T cells did correlate with time of necropsy ($P = 0.0202$, $R^2 = 0.6923$; Figure 10C). Although depletion in the periphery is an indicator of the overall effect of SIV infection, T cell depletion in the local granuloma environment is more likely to have an influence on reactivation. It was not possible to directly sample granulomas to measure T cell frequencies over the course of SIV infection (except at necropsy, see below), so we sampled the airways and analyzed BAL cells instead. The early-reactivating monkeys had lower frequencies of CD4 T cells ($p=0.0571$) in the airway at 10 weeks post-SIV than monkeys that reactivated later (Figure 10D).

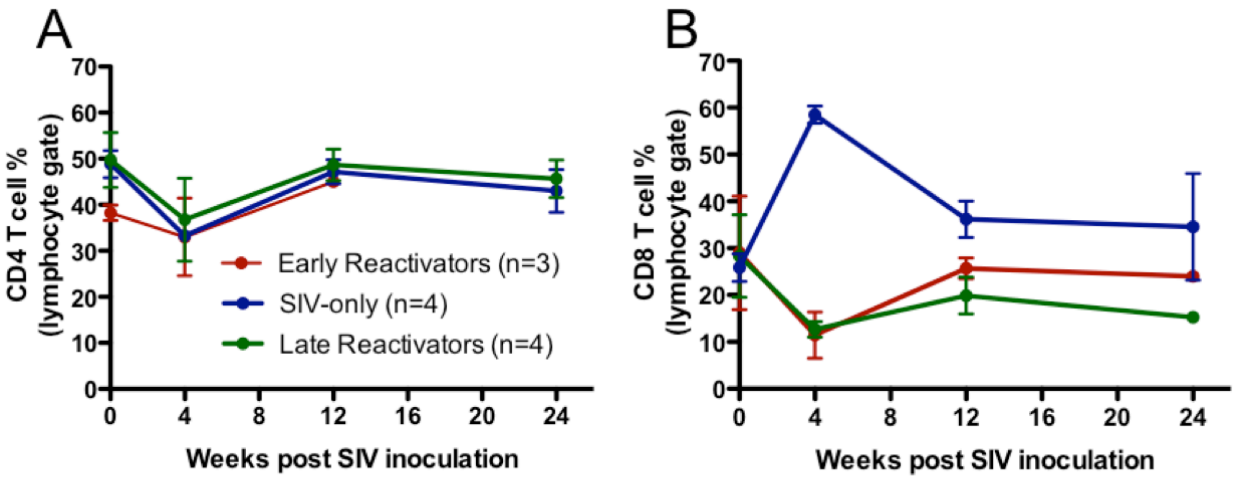

Figure 11. *Changes in T cell frequencies in the peripheral lymph nodes of co-infected and SIV-only infected macaques.* Peripheral (inguinal or axillary) lymph node biopsies were performed on co-infected monkeys at 0, 4, 12, and 24 weeks post SIV inoculation. Average CD4 (A) and CD8 (B) T cell frequencies are displayed for co-infected and SIV-only infected macaques. No significant difference was observed over time or among the three groups.

6.4.4 Peripheral IFNγ responses increase in response to SIV infection

Previous studies using IFNγ ELISPOT indicate macaques with active or reactivating TB have more IFNγ-producing PBMC than animals with latent TB [116, 354]. Given this, we performed IFNγ ELISPOT to determine whether changes in the frequency of *M. tuberculosis*- or SIV-specific T cells within PBMC could predict reactivation in co-infected animals (Figure 12). Unexpectedly, numbers of *M. tuberculosis*- and SIV-specific T cells increased sharply in all monkeys between 7-8 weeks post SIV infection. This increase in SIV- and *M. tuberculosis*-specific T cells quickly declined in all late reactivators and one early reactivator. The number of SIV-specific T cells within the SIV-only group did not change considerably between 8 and 32 weeks post SIV infection.

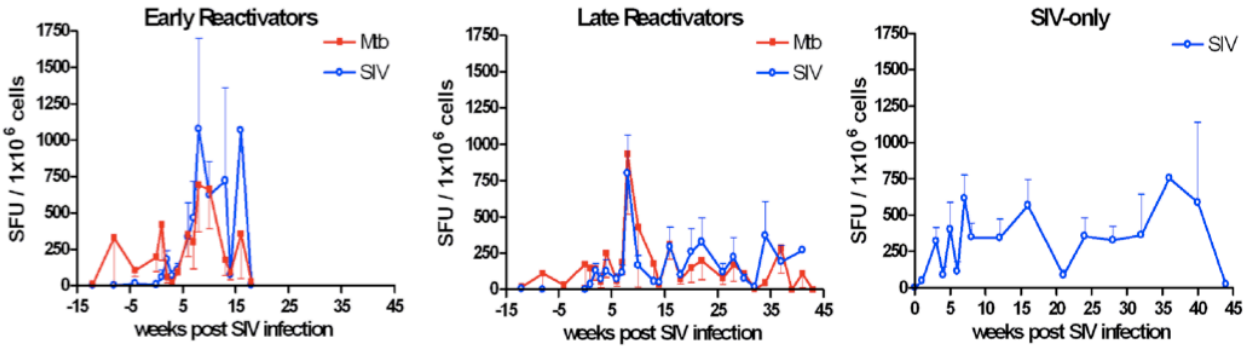

Figure 12. *Co-infected monkeys experience an increase in numbers of M. tuberculosis-specific T cells following early after SIV infection.* IFN-γ ELISPOTs were performed on PBMCs stimulated with *M. tuberculosis* or SIV antigens. IFN-γ was measured in spot forming units (SFU) per 1×10^6 cells.

The increase in *M. tuberculosis*-specific T cells coincided with an increase in the expression of late (CD29) but not early (CD69) activation markers on both CD4 and CD8 T cells (Figure 13). We hypothesized that the initial burst of *M. tuberculosis*-specific IFNγ responses

shortly after SIV infection may reflect a transient perturbation of the local control of infection at the level of the granuloma, resulting in an increase in antigen load due to bacterial replication.

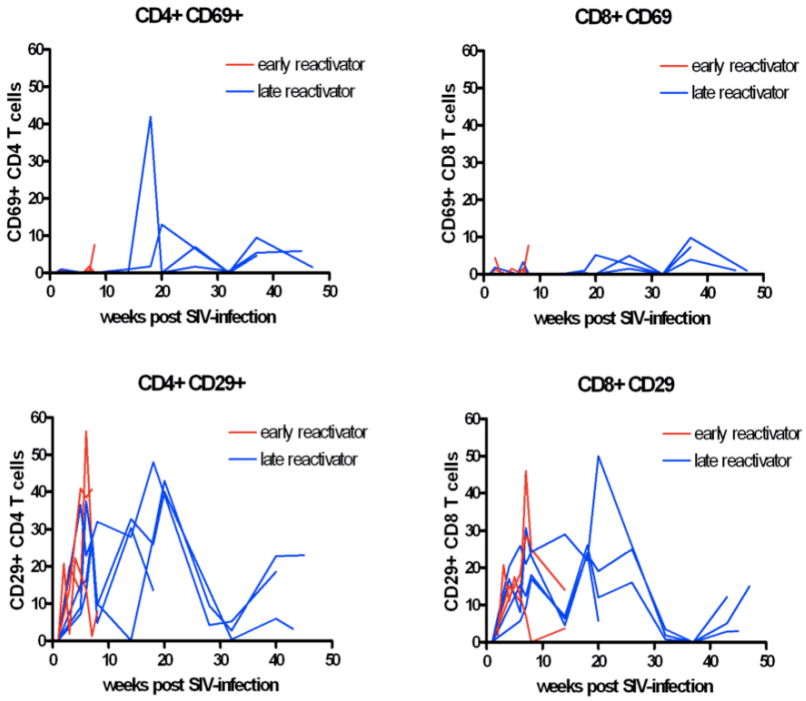

Figure 13. *T cell activation markers increase following SIV infection.* Changes in the expression of early (CD69) and late (CD29) activation markers are represented in CD4 or CD8 peripheral T cells over time.

6.4.5 SIV causes an acute spike in plasma concentrations of IFN-γ, TNF and IL-10 in the co-infected monkeys.

After SIV inoculation we observed a trend in the increase of IFN-γ, TNF and IL-10 in the co-infected monkeys (Figure 14). This increase was not significant but occurs 3 weeks post SIV inoculation when compared to the day of inoculation. We did not observe any difference in cytokine concentrations between the early reactivators (red) and the late reactivators (blue)

within the co-infected group. Cytokine concentrations in SIV-only monkeys appear to be more sporadic than the co-infected monkeys (Figure 14).

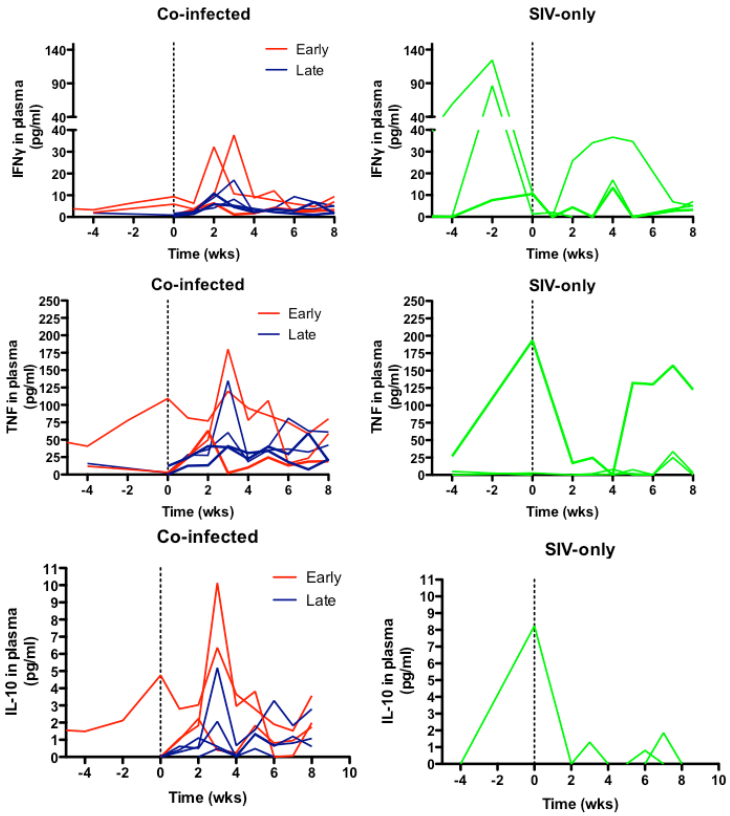

Figure 14. *Changes in cytokine concentrations in plasma during acute SIV in co-infected and SIV-only infected animals*. Protein concentrations of IFN-γ, TNF and IL-10 were determined in SIV-only infected monkeys (N = 4, green) along with co-infected monkeys that reactivated early (N = 3, red) and late (N = 4, blue) during the first 8 weeks of SIV infection.

6.4.6 Co-infected monkeys had more pathology, higher bacterial numbers, and more dissemination than latent monkeys

Each co-infected monkey displayed clinical indicators of TB following SIV infection that are not seen in monkeys with latent TB. Reactivated disease was confirmed at necropsy by pathology-

and microbiology-based measurements that characterize the extent of visible and microscopic disease. Gross pathology at necropsy was quantified by a metric referred to as the necropsy score [113] that reflects the grossly visible tuberculous disease including the number and size of visible granulomas in each lung lobe and thoracic lymph node and extent of dissemination beyond the thoracic cavity. The monkeys that reactivated early post-SIV infection (<17 weeks) had more TB-related pathology than monkeys that reactivated later post-SIV infection, and thus had a trend towards higher necropsy scores (P= 0.0571, Figure 15A). We also scored the total bacterial burden for each monkey, and the percent of tissues sampled at necropsy that were *M. tuberculosis* culture-positive. A bacterial number score [113] was used to reflect the total bacterial load and was obtained by summing the log-transformed number of colony forming units (CFU) from all sampled tissues. The percent positive score measured the proportion of sampled tissues that are *M. tuberculosis* culture positive, thereby indicating the extent of bacterial dissemination in visibly involved and uninvolved tissues. The co-infected monkeys had gross pathology, percent positive and bacterial number scores that were similar to SIV-negative monkeys with active TB and significantly higher than what is seen in latent monkeys (Figure 15B). These metrics, therefore, support the pre-necropsy clinical data indicating that the co-infected monkeys had disease consistent with reactivated TB.

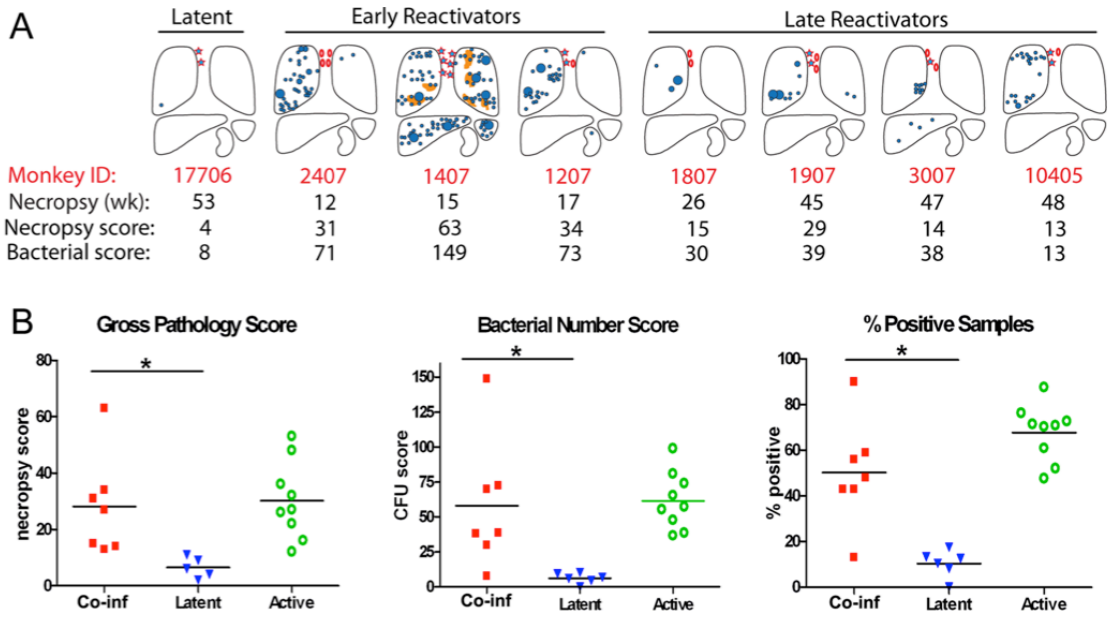

Figure 15. *Pathology associated with reactivation in co-infected monkeys.* A. Gross pathology at time of necropsy in co-infected monkeys. Weeks indicates time post-SIV infection for co-infected monkeys or post-Mtb infection for monkey 17706. Necropsy score represents the gross pathology score, CFU score indicates the bacterial number score. Lungs, thoracic lymph nodes, spleen, liver and kidney are represented. Granulomas are represented by blue circles, TB pneumonia by orange patches, enlarged thoracic lymph nodes by open ovals, and granulomatous thoracic lymph nodes by stars. Monkey 17706 is representative of pathology found in latent monkeys. B. Reactivation was quantified by gross pathology score, bacterial number score and percentage of tissue samples positive for Mtb in homogenized tissue. Co-infected monkeys are statistically different than latent monkeys (Mann-Whitney, gross pathology P = 0.0025, bacterial number score P = 0.004, % positive samples P = 0.005). Latent monkeys are also statistically different than active monkeys in gross pathology (Mann-Whitney, P = 0.001, bacterial number score P = 0.0004, and % positive P = 0.0004).

6.4.7 Histology reveals a mixture of chronic and active granulomas in co-infected monkeys

The granulomas seen in active and latent monkeys have been described previously and are extremely similar to those seen in human TB [113, 116, 356]. To summarize, monkeys classified as having latent infection typically have at least one granuloma in one lung lobe and associated draining thoracic lymph node(s). These granulomas are generally caseous with partial or total

mineralization, often combined with extensive peripheral fibrous connective tissue deposition [113]. Completely fibrotic (sclerotic) lesions are occasionally observed. In contrast, monkeys with active TB frequently have a range of lesion types, including caseous with or without peripheral fibrosis, non-necrotizing (primarily epithelioid macrophages with a lymphocytic component), and suppurative (with significant neutrophilic infiltrate) [113]. Mineralized or completely fibrotic lesions indicative of more chronic immunologic responses to subclinical (latent) infection are rarely observed in monkeys with active TB.

Table 2. Granulomas observed in *M. tuberculosis*-only and co-infected monkeys

	Total number of monkeys	Granulomas examined	Caseous	Non-necrotic	Mineralized	Completely fibrotic
Active	4	224	62%	36%	1%	1%
Mtb-SIV: *early*	3	169	39%	57%	3%	1%
Mtb-SIV: *Late*	4	82	15%	52%	7%	26%
Latent	5	23	4%	0%	82%	13%

In general, the co-infected monkeys had a mix of active and chronic lesions (Table 2). Monkey 1407, which reactivated early post-SIV infection, had extensive widespread active lesions with little evidence of chronic disease. Monkeys 2407 and 1207, which also reactivated early post SIV infection, had pathology consistent with disseminated active TB (Figure 16A), but additionally showed some chronic appearing lesions, including a few solid fibrotic lesions. Certain chronic caseous granulomas had signs of reactivation foci around the perimeter (Figure 16B) as indicated by the presence of macrophage and lymphocyte-rich "satellite" granulomas associated with reactivation [113, 354]. Monkeys that reactivated later post-SIV infection experienced a range of involvement, with 3007 and 1907 having more active-type lesions than 10405 and 1807, although these monkeys as a group had fewer active-appearing lesions than the animals that reactivated earlier following SIV-infection. Additionally, there were more mineralized granulomas reflecting the original latent infection. However, a striking finding was

the presence of substantially more completely fibrotic granulomas, especially in 3007, (Figure 16C,D and Table 2) than is normally noted in active or latent monkeys. In our experience to date, these completely fibrotic granulomas are usually seen in monkeys that had active disease but were then treated with anti-tuberculous drugs for 1-2 months (Flynn and Lin unpublished data), strongly suggesting that this phenotype represents healing of active granulomas. These granulomas are occasionally seen in latent monkeys, but are present in a higher proportion of granulomas examined histologically from the co-infected monkeys (Table 2). Table 2 also demonstrates that many more granulomas were found in the co-infected monkeys than in *M. tuberculosis*-only latent monkeys, which reflects reactivation and likely the spread of infection. Taken in the context of the co-infection, a possible interpretation of the increased presence of completely fibrotic granulomas is that SIV infection perturbed the local control of the latent infection, leading to subclinical reactivation in some monkeys and spread of infection in the lungs, with subsequent healing of some granulomas as the host controlled virus load and regained control of the *M. tuberculosis* infection.

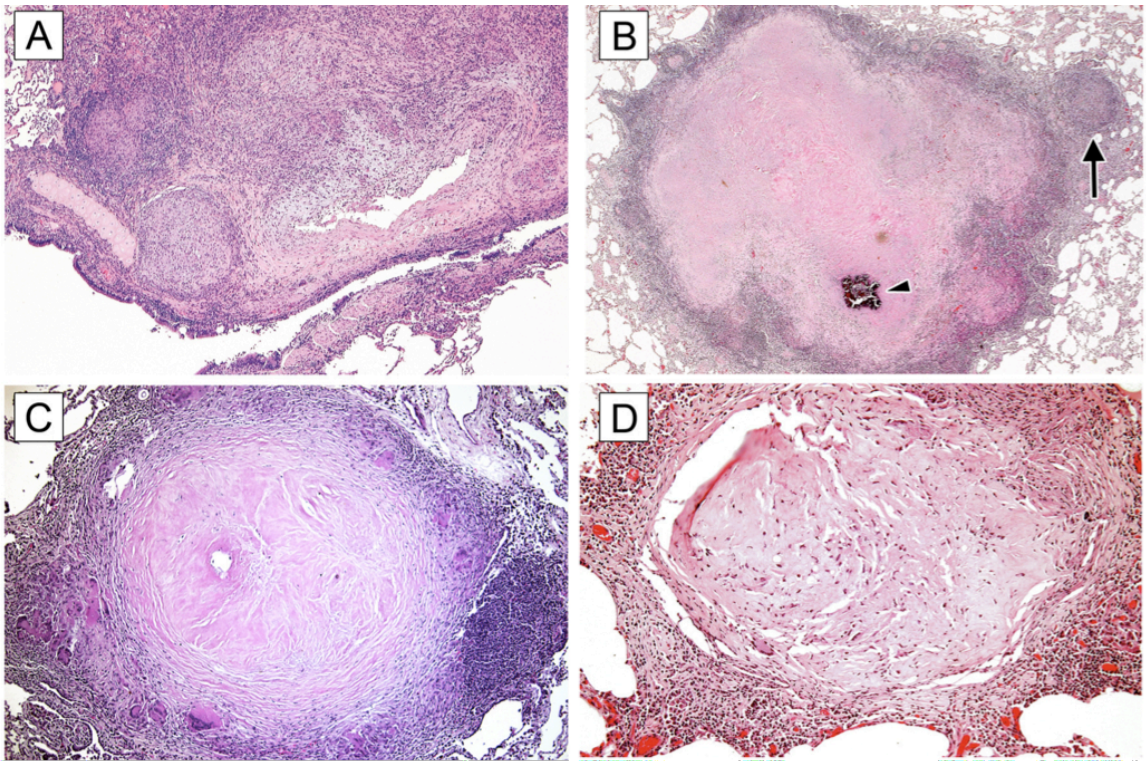

Figure 16. *Histopathology associated with co-infected monkeys*. A. Actively disseminating tuberculous disease characterized by granulomatous inflammation infiltrating into the wall of a large bronchus and adjacent vessel. Hematoxylin and eosin (H&E) stain, 5× magnification. B. A "satellite" nodule (arrow) comprised of non-necrotizing granulomatous inflammation extends from the outer margin of a more chronic caseous granuloma, suggesting loss of immunological containment and reactivation. Arrowhead indicates mineralized area. H&E stain, 2× magnification C. A chronic granuloma with extensive central fibrous organization in conjunction with a dense circumscribing margin of peripheral fibrosis. H&E stain, 10× magnification. D. A solid fibrotic granuloma comprised of maturing fibroblasts depositing a collagenous matrix. H&E stain, 10× magnification.

6.4.8 *M. tuberculosis*-specific IFNγ production in thoracic lymph nodes was reduced by SIV infection

IFNγ ELISPOTs were performed on tissue homogenates of lung lobes and thoracic lymph nodes at necropsy to determine whether SIV infection impaired anti-mycobacterial IFNγ responses (Figure 17 and 18). Lung tissues were separated into tissue containing grossly visible

granulomas (involved) and those without (uninvolved) (Figure 17). Uninvolved lung tissue did not demonstrate a strong response to either *M. tuberculosis* or SIV peptides in any monkeys, except 1907 which had *M. tuberculosis*-culture positive tissues that did not contain visible granulomas, but likely had microscopic disease. Two monkeys had very high frequencies of *M. tuberculosis* and SIV-specific IFNγ-producing cells in involved lung tissue; these monkeys also had the most disease and highest bacterial burdens. Previously, we demonstrated that IFNγ producing T cells in the tissues of monkeys are higher in monkeys with more disease, likely reflecting the amount of antigen present in the tissues, due to higher numbers of bacilli [113]. IFNγ responses from the thoracic lymph nodes in co-infected monkeys to *M. tuberculosis* antigens (ESAT-6 and CFP-10) was lower than monkeys with active TB without SIV (Figure 18A). Co-infected monkeys had similar frequencies of SIV-specific IFNγ-releasing cells compared to SIV-only monkeys in thoracic draining lymph nodes (Figure 18B).

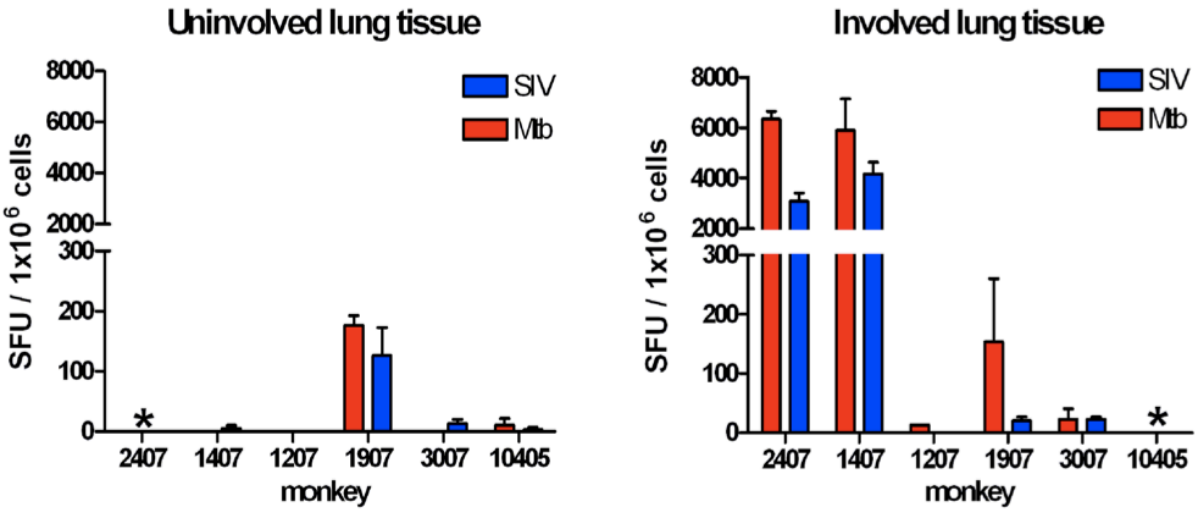

Figure 17. *Antigen specific T cells in involved and uninvolved tissue of co-infected monkeys.* Tissue homogenates were isolated from the granulomatous (involved) or tissues without grossly present granulomas (uninvolved) in lung. Monkeys not represented because too few cells were recovered from tissues at necropsy are indicated by asterisks.

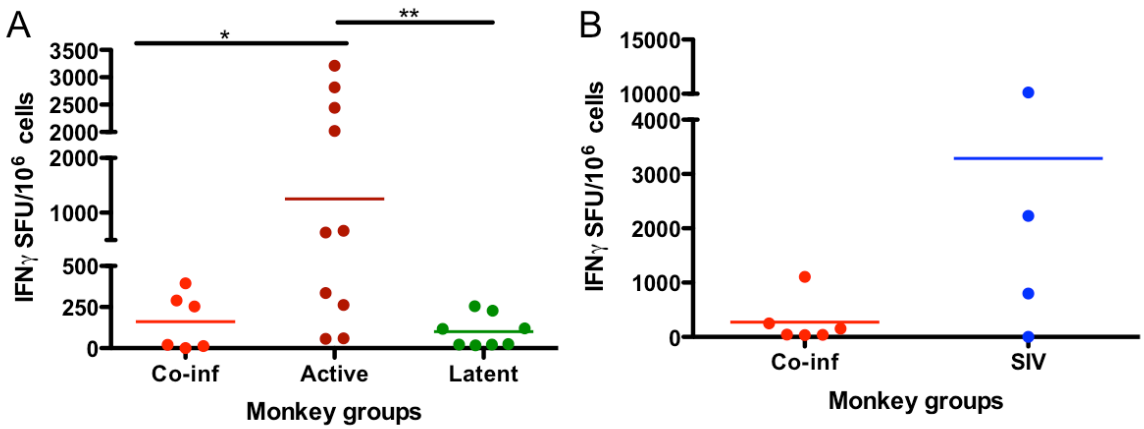

Figure 18. *SIV causes a significant decrease in IFN-γ releasing M. tuberculosis-specific T cells in thoracic lymph nodes of co-infected monkeys compared to monkeys with active TB without SIV.* Tissue homogenates were isolated from thoracic lymph nodes of co-infected monkeys (N = 6), monkeys with active (N = 10) or latent (N = 8) TB without SIV or monkeys infected with SIV only (N = 4). IFN-γ release was measured for *M. tuberculosis*-specific (CFP10/ESAT6) responses (A) or SIVpool (B) overlapping peptide pools. Kruskal-Wallis with Dunn's multiple comparison test with significance set at $P<0.05$ [* = $P < 0.05$ and **$P = < 0.01$] was used to determine significance among Co-infected (N =6), Active (N = 10), and latent (N = 8) (A) and Mann-Whitney test (B) was used to determine significance between Co-infected (N = 6) and SIV (N = 4).

6.4.9 Correlation between mycobacterial CFU and viral titer

It has been reported that TB-involved lung lobes have higher virus loads than uninvolved lobes in humans [274], suggesting that *M. tuberculosis*-related inflammation infection can increase viral replication. To address this, we directly sampled granulomas and other tissues at necropsy to measure bacteria and virus loads. While we found a broad range of *M. tuberculosis* and SIV loads, both between monkeys and within the same monkey, there was no correlation of viral titers with bacterial loads in either granulomas or thoracic lymph nodes (Table 3).

Table 3. Virus and bacterial burden in uninvolved and involved tissues

Table S1. *Virus and bacterial burden in uninvolved and involved tissues.*

Tissue	Early Reactivators			Late Reactivators			
Uninvolved lung	**1207**	**1407**	**2407**	**1807**	**1907**	**3007**	**10405**
CFU/gram tissue	4.0×10^{2}	1.3×10^{2}	1.4×10^{5}	1.2×10^{2}	2.5×10^{3}	4.2×10^{2}	7.4×10^{2}
SIV titer (copies/10^{5} cells)	9.1×10^{2}	<50	1.2×10^{4}	1.2×10^{3}	5.0×10^{4}	3.0×10^{3}	<50
Involved lung	**1207**	**1407**	**2407**	**1807**	**1907**	**3007**	**10405**
CFU/gram tissue	2.0×10^{4}	3.7×10^{4}	2.5×10^{3}	6.3×10^{3}	7.4×10^{3}	4.4×10^{4}	6.0×10^{2}
SIV titer (copies/10^{5} cells)	1.3×10^{4}	<50	<50	6.5×10^{3}	3.0×10^{5}	2.7×10^{3}	<50
Uninvolved lymph node[a]	**1207**	**1407**	**2407**	**1807**	**1907**	**3007**	**10405**
CFU/gram tissue	7.3×10^{2}	8.7×10^{4}	1.9×10^{3}	5.8×10^{2}	0	4.4×10^{3}	0
SIV titer (copies/10^{5} cells)	1.7×10^{3}	3.7×10^{2}	2.0×10^{4}	1.4×10^{5}	3.7×10^{5}	5.4×10^{4}	<50
Involved lymph node[a]	**1207**	**1407**	**2407**	**1807**	**1907**	**3007**	**10405**
CFU/gram tissue	7.8×10^{3}	1.4×10^{5}	NA[b]	1.3×10^{4}	1.6×10^{3}	4.0×10^{4}	0
SIV titer (copies/10^{5} cells)	6.4×10^{2}	1.7×10^{2}	NA[b]	4.0×10^{5}	5.8×10^{5}	1.2×10^{4}	6.0×10^{1}

[a] Lymph nodes are lung-draining thoracic lymph nodes

[b] NA indicates tissues not available for analysis

6.4.10 Co-infected monkeys had fewer T cells in involved tissues than SIV- or *M. tuberculosis*-only animals

To determine that SIVmac251 depleted CD4 T cells in mucosal (uninvolved) tissue, we compared CD4 and CD8 T cell frequencies in gut tissue in monkeys with infected with *M. tuberculosis* (no SIV), co-infected monkeys and an SIV-only monkey. There was substantial depletion of CD4 T cells in the colon (25.3% *M. tuberculosis*, 5.7% co-infected) and ileum/jejunum (14.0% *M. tuberculosis*, 1.6% co-infected, 8.6% SIV only). CD8 T cells were only slightly decreased in the co-infected monkey gut tissues compared to controls. These data indicate that SIVmac251 is capable of reducing T cells in the mucosal tissue, as has been previously reported for rhesus macaques [357] and for humans (with HIV) [358].

We measured CD4 and CD8 T cell numbers in draining lymph nodes and TB-involved lung from co-infected monkeys to better understand how SIV infection affects cell numbers in tissues infected with TB. A trend of fewer CD4 T cell numbers in the thoracic lymph nodes of co-infected monkeys compared to SIV-only monkeys was observed (Figure 19). T cell numbers in lung tissue were also examined although we did not obtain sufficient numbers of T cells from lung tissue from SIV-only monkeys for comparison, probably because these monkeys did not have pulmonary infections with other pathogens. As previously reported, T cell numbers are higher in involved lung tissue from active monkeys compared to latent monkeys [113]. Here, the co-infected monkeys had T cell numbers similar to the number observed in latent monkeys (Figure 19) even though they had significant disease and increased bacterial numbers, similar to active TB monkeys. This observation suggests co-infected monkeys may have critical defects in their ability to mount or maintain T cell-mediated immune responses at the site of infection.

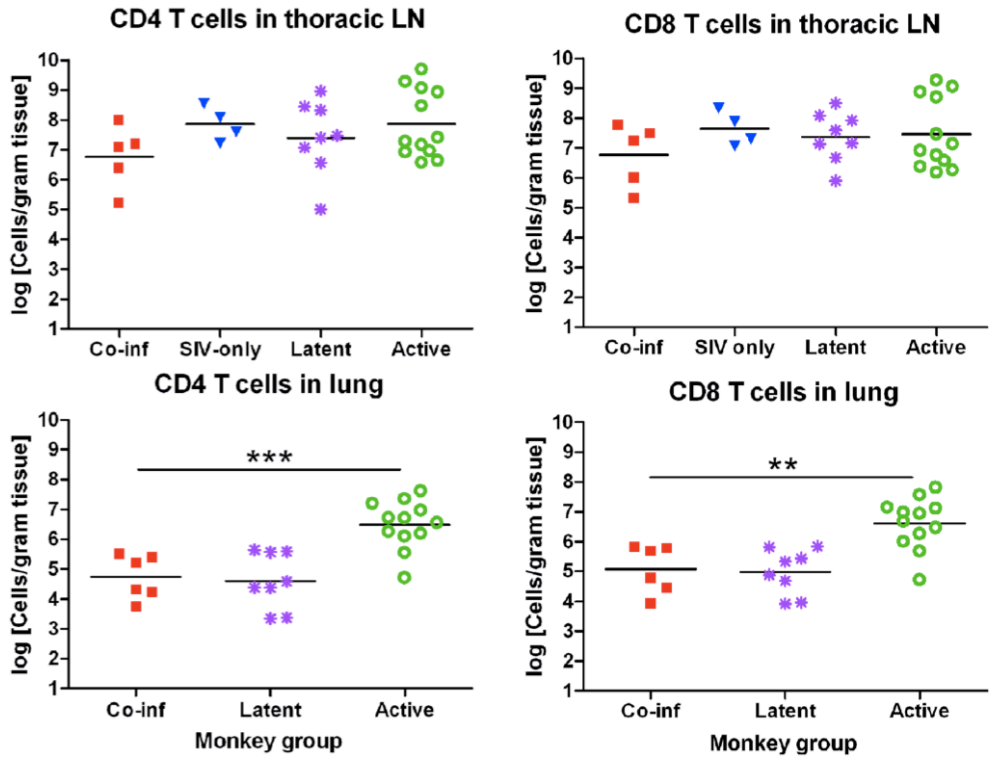

Figure 19. *CD4 and CD8 T cell numbers in lung and thoracic lymph nodes.* CD4 and CD8 T cell numbers in lung-draining thoracic lymph nodes (LN) are not significantly different between co-infected animals and animals with latent or active TB. T cell numbers in co-infected lung tissues were similar to animals with latent TB but significantly lower than animals with active TB (Mann-Whitney, CD4 P = 0.0023; CD8 P = 0.0078). Latent monkeys have fewer lung T cells than active monkeys (Mann-Whitney, CD4 P = 0.0014; CD8 P = 0.0018). Data points represent average T cell numbers from either thoracic LN or lung tissue from each monkey. Not all co-infected monkeys are not present due to limited tissue availability.

6.4.11 Co-infected monkeys did not have higher tissue viral loads than SIV-only macaques

Viral titers in the thoracic lymph nodes, peripheral lymph nodes and spleen co-infected monkeys and SIV-only monkeys were not significantly different at time of necropsy (Figure 20). However, co-infected monkeys did have a trend of higher viral loads on average when compared to monkeys infected with SIV alone (Figure 20).

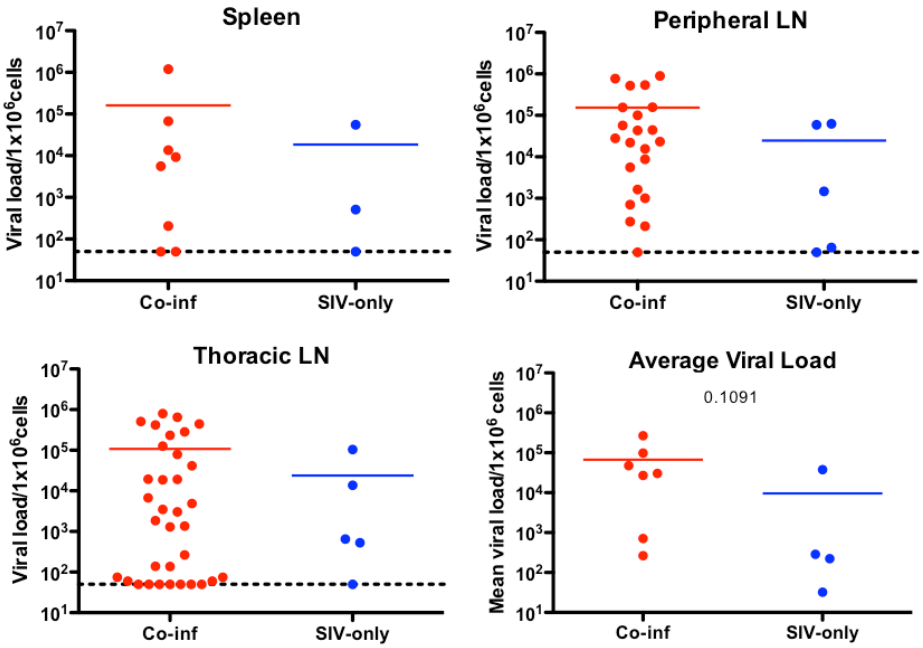

Figure 20. *No significant differences in tissue viral loads between co-infected and SIV-only infected monkeys.* Viral loads were measured in all homogenized tissues gathered from necropsy. Viral loads were measured in spleen, peripheral lymph, and thoracic lymph nodes. The sum viral load divided by the total number of excised tissues is also represented. Each dot represents an individual homogenate from spleen (Co-inf N = 8; SIV N = 3), peripheral (Co-inf N = 22; SIV N = 5) and thoracic lymph node (Co-inf N = 34; SIV N = 5) graphs. Each dot in average viral load graph represent sum viral load from all excised tissue divided by number of excised tissue from a signal monkey (Co-inf N = 7; SIV-only N = 4). Dotted line represent the detection limit (<50 copies/10^6 cells). Mann-Whitney statistical analysis was used with significance set at $P < 0.05$. P value that represents a trend is represented.

6.5 DISCUSSION

Reactivated TB is a major source of morbidity and mortality among HIV-infected individuals. While most opportunistic infections commonly occur when CD4 T cell numbers have significantly declined, TB occurs throughout the entire spectrum of HIV disease, including when CD4 T cells numbers are well preserved and stable [340-342]. Despite the severe risk of TB in HIV-infected persons, research into factors underlying early reactivation has been hampered by

lack of appropriate animal models combining latent TB and HIV infection. In this study, cynomolgus macaques with latent TB were infected with SIVmac251 to investigate the factors that could lead to reactivation of latent TB. All the monkeys with latent TB co-infected with SIV experienced clinical signs, pathology and bacterial load consistent with reactivated disease while the *M. tuberculosis*-negative SIV-infected monkeys did not develop simian AIDS or opportunistic infections within 11 months of infection. SIV-negative cynomolgus macaques with latent TB have a <5% chance of undergoing reactivation and can maintain latent TB without reactivation for more than three years (Flynn and Lin unpublished data). These observations suggest that while these monkeys can control either SIV or latent *M. tuberculosis* infection independently, SIV-induced immune dysfunction disrupted the immunologic pressure constraining mycobacterial growth, inducing reactivation. To our knowledge, this is the first animal model of latent TB where the immunologic and microbiologic responses to SIV co-infection can be directly investigated.

Although individuals are at a high risk of developing TB within the first year of HIV-seroconversion [242], clinical studies of HIV-*M. tuberculosis* co-infection indicate that depletion of peripheral CD4 T cell numbers is not predictive of when individuals are most likely to experience TB [342]. However HIV+ individuals with low peripheral CD4 T cells have been shown to be at greater risk of developing active TB [270]. All the monkeys experienced T cell depletion during acute SIV infection, with reactivated TB occurring during the period when the animals had regained normal and stable peripheral T cell numbers. Our data suggests that rather than the T cell numbers during chronic SIV infection being a major factor in reactivation, the extent of initial depletion and post-acute recovery may affect ultimate control of latent *M. tuberculosis* infection. Monkeys 1407 and 2407, which reactivated early post-SIV, had fewer

peripheral T cells than other monkeys and didn't recover from the initial depletion as well as monkeys that reactivated later. Similarly, 1807 and 1907, which also had fewer peripheral T cells following SIV infection, displayed signs of active disease earlier than the other late reactivators, supporting the hypothesis that early perturbations of the immune response may ultimately affect disease progression. Prior to the development of the SIVmac251/*M. tuberculosis* animal model experiments, we infected two latent cynomolgus macaques with SHIV89.6 (Santosh and Flynn unpublished data). These monkeys had significant sustained CD4 T cell depletion in the periphery as has been reported for cynomolgus macaques infected with SHIV89.6 [331], yet did not experience reactivation by seven months post-SHIV infection (Santosh and Flynn unpublished data). Although SHIV89.6 induces a dramatic loss of peripheral CD4 T cells it did not cause significant decreases of mucosal CD4 T cells (Santosh and Flynn unpublished data). Taken together, these data support the conventional paradigm that peripheral CD4 T cell populations are imperfect indicators of an individual's susceptibility to TB, and instead indicate that early T cell depletion by SIV (or perhaps HIV) that depletes both peripheral and mucosal CD4 T cells may predict risk of reactivation.

Although the link between peripheral cells and maintenance of latency is not clear, depletion in the periphery shortly after SIV infection may reflect significant depletion of T cells in the granuloma. Monkeys 1407 and 2407, which didn't recover well from acute SIV infection and reactivated early, displayed more disease than later reactivators, possibly indicating that significant disruption occurred within granulomas and the subsequent inability to replace T cells lead to reactivated TB. This link between events in the periphery and the local granuloma environment is supported by the decreases observed in the frequency of CD4 T cells in the airways from the early-reactivating macaques. The acute phase of SIV infection was

accompanied by increased numbers of *M. tuberculosis*-specific IFNγ-producing T cells in the periphery. This may be caused by SIV-induced impairment of granuloma function leading to increased mycobacterial growth and dissemination, which results in increased amounts of bacterial antigen released. Taken together, these data suggest that systemic T cell depletion permits resumption of bacterial growth and perhaps reactivation. Indeed, HIV-induced depletion of *M. tuberculosis*-specific CD4 T cells in the granuloma, and subsequent disruption of cellular architecture critical [27] for bacterial containment, have been hypothesized as factors contributing to increased reactivation rates in co-infected humans [249, 359].

The extensive histological examination of granuloma-containing tissue from animals with widely different amounts of disease expanded our basic understanding of how HIV may change the pathology of tuberculosis. Granulomas from the earliest-reactivating animals were most similar to animals with active disease [113] whereas macaques that reactivated later had more mineralized granulomas, which are most often associated with latent disease, with evidence for some localized dissemination and reactivation. The lungs of latent monkeys normally contain few granulomas, and they are usually either completely calcified lesions or caseous ones with significant mineralization, with an occasional completely fibrotic granuloma in some monkeys. The most striking finding in the co-infected monkeys was the presence of numerous completely fibrotic granulomas in the lungs, which are rarely seen in latent infection [113]. We have observed this granuloma type in the setting of short-term drug treatment in the macaque model (Flynn and Lin unpublished data), and thus associate this fibrosis with a healing response. Our interpretation of this unusual pathology is that the initial events surrounding SIV infection caused a depression in the local immune response (the granulomas) in latent animals, allowing increased bacterial growth, spread to other areas of the lung, and formation of additional

granulomas. After this initial event, some animals may regain partial control of the infection, and these new granulomas begin to heal and become fibrotic. Thus, the interaction of SIV with the latent TB granuloma is a dynamic, and can lead to various outcomes. The cytokine milieu in the SIV-infected lungs may also promote more fibrotic-type healing, leading to the abundance of this type of lesion; this hypothesis requires further study.

There was a trend of fewer total CD4 T cells and significantly fewer IFN-γ releasing T cells in the thoracic lymph nodes of co-infected monkeys compared to SIV-only monkeys, indicating that the presence of mycobacteria, and perhaps the increased T cell activity in the lymph nodes, leads to more depletion of these cells in the lymphoid tissue, and may contribute to enhanced disease. Co-infected monkeys had fewer CD4 and CD8 T cells within TB-involved lung tissues when compared to SIV-negative monkeys with active TB. This contradicts what normally happens in animals with active TB, which have more T cells in involved tissues than latent monkeys. Significant reductions in T cell numbers within the lung tissue of the co-infected monkeys suggest that SIV is interfering with migration of T cells to the site of infection as has been seen with HIV [360]. Alternatively, SIV may be inducing T cell death within the granuloma like that observed in HIV-*M. tuberculosis* co-infected humans [251]. Exposure to *M. tuberculosis* antigens is linked to apoptosis in HIV-infected cells [282], which could mechanistically explain why tissues in co-infected animals were depleted when compared with animals only infected with *M. tuberculosis*. To our knowledge, this study provides the first evidence demonstrating quantitative T cell depletion within granulomatous tissue, supporting hypotheses for mechanisms by which HIV disrupts the maintenance of latent *M. tuberculosis* infection [249, 343].

Several groups have investigated the effects of HIV-*M. tuberculosis* co-infection on the replication of both pathogens [254, 256, 257, 288] and the relationship between virus load and disease progression [273, 275, 281]. We found similar peripheral and tissue virus loads in co-infected and SIV-only monkeys, suggesting that virus loads were not influenced by *M. tuberculosis* infection. Granulomatous tissue might be an ideal environment for HIV replication as abundant T cells, increased cell-cell interaction, and high TNF expression promote cell-cell transmission and virus replication [249, 256, 257]. We were unable to confirm this, however, and could not correlate virus loads with *M. tuberculosis* burden in individual granulomas (Table 3). Differences in viral titer did not appear to be the cause of reactivation either, as viral titers (peak and during the chronic phase) were similar between the early and late reactivators. Similar findings have come from the HIV-TB clinical literature [361, 362], highlighting the clinical relevance of the cynomolgus macaque co-infection model. Our viral titers were relatively low following the acute infection, however, and it is possible that higher viral titers in a different macaque species or with a different virus strain could lead to different results. Even with the low virus loads observed in these cynomolgus macaques they all experienced reactivation of latent TB.

We have developed a tractable animal model of reactivated TB in HIV-co-infected humans. Although numerous clinical studies have been undertaken to investigate HIV-*M. tuberculosis* co-infection, most examine patients with active TB and many are either retrospective or limited by the number and types of tissues and time points that could be sampled. This novel model will be a useful tool for investigating interactions between *M. tuberculosis* and HIV, and the immunologic events that lead to reactivation of latent TB or exacerbation of primary infection.

6.6 ACKNOWLEDGEMENTS

We thank Dr. Keith Reimann for supplying the SIVmac251. SIV peptides were obtained from the NIH AIDS Research and Reference Reagent Program (NIAID, NIH). SHIV experiments were performed with help from Dr. Santosh N. Pawar. We gratefully acknowledge the technical assistance of Mark Rodgers, Catherine Cochran, Kelly Wyatt, Jennifer Kerr, Melanie O'Malley, Jamie Tomko, Dan Fillmore and Paul Johnston.

7.0 SIV REDUCES TNF RELEASE BY *M. TUBERCULOSIS*-SPECIFIC T CELLS THROUGH A MONOCYTE-DERIVED IL-5 DEPENDENT MECHANISM

An adapted version of this chapter is in preparation:

Collin R Diedrich, Joshua T Mattila, Kyle H Rhode, Dawn M O'Dee, David G Russell, JoAnne L Flynn. SIV reduces TNF release by *M. tuberculosis*-specific T cells through a monocyte-derived IL-5 dependent mechanism. In preparation.

7.1 INTRODUCTION

The mechanisms responsible for the increased susceptibility of HIV-infected persons to *M. tuberculosis* are not currently known, but multiple hypotheses have been proposed [238, 249, 271, 363], including loss of CD4 T cells, as described in Chapter 4. HIV-induced manipulation of *M. tuberculosis*-specific effector T cell function and inhibition of the ability of macrophages to kill *M. tuberculosis* are two possible contributors to this increased susceptibility to TB [238, 363].

Co-infected individuals have significantly fewer peripheral *M. tuberculosis*-specific T cells that can release IFN-γ, TNF, and IL-2 or proliferate compared to individuals with active TB alone [261, 282, 283, 302, 303]. HIV decreases IFN-γ mRNA production and proliferation of *M. tuberculosis*-specific T cells within the airways of AIDS patients with TB compared to individuals with active TB without AIDS [304, 305]. HIV also reduces the function of other

mycobacterial-specific T cell responses by reducing IFN-γ–TNF–IL-2 polyfunctional BCG-specific CD4 T cells within the airways of BCG-vaccinated individuals without signs of TB compared to HIV-uninfected controls [286]. The cause of *M. tuberculosis*-specific T cell dysfunction in HIV-co-infected individuals is not known, but may result from the ability of HIV to exhaust T cells [177]. HIV may be able to directly manipulate *M. tuberculosis*-specific T cells due to its close proximity to these cells within granulomas since SIV replication occurs within granulomas of macaques [271] and HIV is present at sites of active TB in humans [274, 276, 364]. Taken together, the close proximity of HIV and *M. tuberculosis* within the granuloma provides an ideal environment for HIV to directly manipulate the function of *M. tuberculosis*-specific T cells and *M. tuberculosis*-infected macrophages.

In Chapter 6, I described the development of an animal model of HIV/*M. tuberculosis* co-infection, where we demonstrated that SIVmac251 induces reactivation of latent TB in cynomolgus macaques [77, 271]. We correlated changes in peripheral T cell counts during the acute phase of SIV to the reactivation of latent TB [271] and hypothesized that the outcome of latent infection (i.e. reactivation or control of the latent infection) in a co-infected individual is strongly influenced by the host response to the acute phase of HIV infection. Our hypothesis is that during the acute phase of infection HIV almost immediately disrupts *M. tuberculosis*-specific T cell function within the granulomas. Multiple studies demonstrate that HIV manipulates T cell effector function [261, 282, 283, 302, 303] but they do not address how long it takes HIV to inhibit *M. tuberculosis*-specific T cell responses or how and where the inhibition takes place. The cause of the reduction in T cell function is not known, but it may be a result of direct T cell manipulation or changes in antigen presenting cell function.

We developed *in vitro* and *ex vivo* models to investigate the mechanisms HIV-induced *M. tuberculosis*-specific T cell dysfunction to overcome the difficulties of studying this phenomenon *in vivo*. Using T cells and monocytes from SIV-uninfected monkeys with active or latent TB, we demonstrate here that exogenously added SIV causes a significant decrease in the frequency of TNF and IFN-γ releasing *M. tuberculosis*-specific T cells. This was due to SIV manipulating monocytes and not the T cells directly. *M. tuberculosis*/SIV-co-infected monocytes produced significantly more IL-5 than monocytes infected with *M. tuberculosis*-alone. By manipulating levels of IL-5 in our *in vitro* system, we determined that the reduction in *M. tuberculosis*-specific TNF-releasing CD4 T cells was due, at least in part to the SIV-induced production of IL-5 by monocytes. This study provides evidence for a novel role for IL-5 and its ability to reduce *M. tuberculosis*-specific T cell functions in co-infected individuals.

7.2 METHODS

7.2.1 *M. tuberculosis* infection

Cynomolgus macaques were infected with ~25 or ~200 CFU Erdman strain *M. tuberculosis* via intrabronchial instillation as previously described [113] for other ongoing studies. Ninety percent of monkeys infected with 200 CFU developed active TB, while 50% of the monkeys infected with 25 CFU developed active TB. Blood samples from monkeys with active or latent infection were used for the *in vitro* studies described here.

7.2.2 PBMC and tissue isolations

Blood was drawn from *M. tuberculosis*-infected monkeys (without SIV) enrolled in other studies. PBMCs were isolated via Percoll gradient centrifugation as previously described [72, 112, 113]. Animals were humanely euthanized and necropsies performed as previously described [72, 112, 113]. Thoracic lymph nodes from *M. tuberculosis*-infected monkeys were homogenized into single-cell suspensions with Medimachines (BD Bio- sciences, San Jose, CA) as described previously [72, 112, 113].

7.2.3 Magnetic separation

Magnetic beads were used to separate CD3+ T cells (Miltenyi Biotec, 130-092-012) and CD14+ monocytes (Miltenyi Biotech, 130-091-097) from PBMC of SIV-uninfected and *M. tuberculosis*-infected cynomolgus macaques. Manufacturers instructions were followed for magnetic separations. Isolated monocytes were plated ($5x10^5$ to $1.2x10^6$ cells/ml) in RPMI media supplemented with 10% human AB serum (Gemini Bio-Products, West Sacramento, CA), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO), and 1% HEPES (Sigma) in a 5ml polypropylene round-bottom tube (Becton Dickinson), 24 well flat bottom plate or 96 well round bottom-plate (Becton Dickson), depending on the assay run. Isolated CD3 T cells were resuspended in supplemented RPMI +10% hAB serum at a density of $5x10^6$ cells/ml in 24-well flat bottom plate. Cells were incubated overnight at 37C with 5% CO_2 before adding back to the CD3-depleted PBMC or monocytes (see below).

7.2.4 Infections and Stimulations

7.2.4.1 PBMC Stimulations

Freshly isolated PBMCs were resuspended in supplemented RPMI media at a density of $5x10^5$ to $1x10^6$ cells/ml and transferred to FACS tubes for stimulation with *M. tuberculosis* culture filtrate protein-10 (CFP10; overlapping peptide pools; 10μg/ml) or phytohemagglutinin (PHA; 10μg/ml) for 5-6 hours at 37C at 5% CO_2. After the first 1 hour of stimulation, brefeldin A was added to the cells and incubated for an additional 4-5 hours. After stimulation cells were washed and stained.

7.2.4.2 *M. tuberculosis* -infection of CD3-depleted PBMC

For *M. tuberculosis*-infected cells, CD3 T cell-depleted PBMC were incubated for 24 hrs with *M. tuberculosis* (MOI 0.5) in 500μl of supplemented RPMI+10% hAB serum, then washed and resuspended in 500μl of fresh media. One aliquot of *M. tuberculosis*-infected cells was incubated with SIVmac251 (MOI 0.1) for 12 to 16 hrs. Analysis by trypan blue exclusion showed that SIV did not increase cell death during this incubation, compared to SIV-uninfected cells. One aliquot of cells was not infected with SIV or *M. tuberculosis*. The CD3 T cells were added back to the CD3-depleted PBMC and resuspended in a total of 250μl of supplemented RPMI +10% hAB with brefeldin A (GolgiPlug; BD Bioscience) for 5 hours. After stimulation cells were washed and stained (see flow cytometry methods, 7.2.5, below).

7.2.4.3 *M. tuberculosis*-infection of monocytes

Isolated CD14+ monocytes isolated from PBMC and incubated overnight in RPMI + 10% hAB in 5ml round bottom tubes overnight. Monocytes were aliquoted into 4 groups: Media, SIV, *M.*

tuberculosis and *M. tuberculosis*/SIV. T cells were also isolated from PBMC and incubated in RPMI +10% hAB for later use. Two groups of monocytes were infected with *M. tuberculosis* (MOI 0.5) in 500µl for 4 hours, then 1 ml of media was added to each tube and the cells were incubated overnight. After the overnight incubation cells were washed with warm RPMI and incubated in 500µl RPMI. Appropriate cells were incubated with SIVmac251 (MOI 1.0) overnight (12 to 16hrs). The next morning the cells were washed with RPMI and autologus T cells were added to the monocytes at a ~3:1 T cell to monocyte ratio in 250µl of media with brefeldin A. T cells and monocytes were incubated for 6 hrs. After stimulation cells were washed and stained.

7.2.4.4 Using inactivated SIVmac251 in *M. tuberculosis*-infected monocytes

Experiments were performed the same as described above except that an inactivated virus was added to determine whether live SIVmac251 was needed to disrupted *M. tuberculosis*-specific T cell responses. Instead of adding SIVmac251, approximately 0.1pg p28 equivalents of inactivated SIVmac251 Aldrithiol-2 per $1x10^6$ monocytes (Courtesy Dr. Jeff Lifson, AIDS & Cancer Virus Program, Frederick MD) were added to the *M. tuberculosis*-infected monocytes. T cells were then added and experiments resumed as previously described.

7.2.4.5 Lymphocyte proliferation assay

PBMC from *M. tuberculosis*-infected monkeys were isolated and suspended in AIM V media (Invitrogen, Grand Island, NY) at 200,000 cells/well in 200µl. Cells were stimulated with PHA (5µg/ml), CFP10 peptides, or media in triplicate wells for 60 hours at 37°C, 5% CO_2 with or without SIV; for the final 18 hours, [3H]-thymidine (1µCi/well, Amersham) was added. Cells

were harvested onto filters and radioactive incorporation measured. Data were reported as a stimulation index (SI): fold increase in cpm over unstimulated control.

7.2.4.6 Neutralization of IL-5 and IL-13

Isolated monocytes were placed in a 96 well plate (~$2x10^5$ monocytes per well). Monocytes were infected with *M. tuberculosis* (MOI 0.5) for 4 hours then washed with warm RPMI. Monocytes were washed and the media was replaced with 200µl of RPMI + 10% hAB and incubated overnight. Monocytes were incubated with SIVmac251 (MOI 1.0) the following day in 500µl of RPMI + 10% hAB. Neutralization antibodies to IL-5 (40pg/ml ebioscience 16-7059-81) or IL-13 (64pg/ml Miltenyi biotec 130-093-053) were added to half of the SIV-exposed monocytes at the same time as the addition of SIV. Isotype antibodies were added to a group of co-infected monocytes as the baseline control. Monocytes were incubated overnight (12 to 16hrs) at 37C in 5% CO_2. The following morning cells were washed with warm RPMI. Autologous T cells were added to the monocytes in ~ 3:1 T cell to monocyte ratio in 100µl RPMI + 10% hAB with brefeldin A. T cells and monocytes were incubated for 6hrs.

7.2.4.7 Addition of recombinant IL-5 and IL-13

Isolated monocytes were infected with *M. tuberculosis* (MOI 0.5) for 4 hours then washed with warm RPMI in a 96 well plate (~$2x10^5$ monocytes per well). Media was replaced with 200µl of RPMI + 10% hAB and incubated overnight at 37C in 5% CO_2. The following day monocytes were washed with warm RPMI and resuspended in 150µl of RPMI + 10% hAB. Either recombinant human IL-5 (PeproTech, Rocky Hill, NJ; 40pg/ml/$1x10^6$ cells) or human IL-13

(Miltenyi Biotec; 60pg/ml/1x10^6 cells) were added to the appropriate wells in 50µl of RPMI + 10% hAB. The amount of recombinant protein added to the wells was equivalent to the highest amount of IL-5 or IL-13 produced by co-infected monocytes. Monocytes were incubated overnight at 37C in 5% CO_2. Autologous T cells were added to the monocytes at a ~3:1 T cell to monocyte ratio in 100µl RPMI + 10% hAB with brefeldan A. T cells and monocytes were incubated for 6hrs.

7.2.5 Flow Cytometry

All antibodies used for flow cytometry were direct conjugates against human proteins and obtained from BD Biosciences (San Jose, CA) unless otherwise noted. Approximately 1x10^6 PBMC or tissue cells were stained using combinations of the following antibodies: CD3 (clone SP34-2), CD4 (clone L200), CD8 (clone DK25 [Dako; Carpinteria, CA], clone SK-1, clone OKT8 [eBioscience; San Diego, CA]), IFN-γ (clone B27; BD Bioscience), IL-2 (clone MQ1-17H12; eBioscience), and TNF (clone mab11; eBioscience). Cell phenotypes were read with a LSR II flow cytometer (BD Biosciences) and positively stained populations gated using fluorochrome-matched isotype antibodies as negative controls using the FlowJo software package (Tree Star Inc., Ashland, OR).

7.2.6 Luminex

Isolated monocytes (3x10^6 to 1x10^6 cells) were divided into 4 groups (Media, SIV, *M. tuberculosis*, *M. tuberculosis* + SIV) incubated in 24 well plates in 1 ml of RPMI +10% hAB overnight. The following day the *M. tuberculosis*-infected monocytes were infected with *M.*

tuberculosis (MOI 0.5) for 4 hours incubation, then media was replaced with 1 ml of RPMI + 10% hAB. The appropriate monocytes were then incubated with SIVmac251 (MOI 1.0) overnight. The media was removed, filtered (Millipore, 0.45um) and frozen for Luminex analysis. A 23-plex non-human primate Luminex assay (Millipore; Billerica, MA) was performed on the filtered supernatants following manufacturer's instructions. Proteins analyzed were CD40 Ligand, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12/23 (p40), IL-13, IL-15, IL-17, IL-18, IL-1ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, TGF-α, TNF-α, VEGF. Samples were read on a Luminex 100 IS Bio-Plex System machine (Luminex Corporation, Austin, TX).

7.2.7 RNA isolation and IL-5 detection in monocytes

Isolated monocytes (~$4x10^6$ cells) were divided into 4 groups (Media, SIV, *M. tuberculosis*, *M. tuberculosis* + SIV) incubated in 24 well plates in 1 ml of RPMI +10% hAB overnight. The following day the *M. tuberculosis*-infected monocytes were infected with *M. tuberculosis* (MOI 0.5), for 4 hours, then media was replaced with 1 ml of RPMI + 10% hAB. The appropriate monocytes were then incubated with SIVmac251 (MOI 1.0) overnight, then the supernatant was removed and the RNA was isolated following Trizol Reagents (15596-026, Invitrogen, NY) manufacturing protocols. Isolated RNA was amplified using custom made IL-5 primers (Forward: GAGACCTTGGCACTGCTTTC and Reverse: ACTCTCCGCCTTTCTTCTCC) with AMV RT (M510F, Promega, WI) and PCR master mix (M750B, Promega) in the following program: 42C [30min], 94C [5min] (94C [30s], 55C [30s], 72C [30s])x30, 72C [5min]. Amplified products were run on a 2% agarose gel with 5x loading buffer (BioRad, CA) along with a 100bp molecular ruler (BioRad). Gel was then stained with ethidium bromide and imaged.

Primers were generated from the rhesus macaque mRNA sequence and the product identity was sequenced for confirmation (98% sequence homology).

7.2.8 Statistics

Wilcoxon ranked paired test was used to compare cells from each animal with different in vitro treatments, with $P < 0.05$ considered to be significant. Mann-Whitney analysis was used to compare two different groups with significance set at $P < 0.05$. Kruskal Wallis multiple comparison test with Dunn's post-test were used to compare differences among three unpaired groups. Freidman test was used with Dunn's multiple comparison test to determine significance among 3 or more paired groups with significance set at $P < 0.05$.

7.3 RESULTS

7.3.1 SIV specifically decreases TNF release in CFP10 stimulated PBMC from monkeys infected with *M. tuberculosis*.

To determine whether SIV can cause an immediate disruption in *M. tuberculosis*-specific T cells, we incubated freshly isolated PBMC (from animals infected with *M. tuberculosis*, but not SIV) with SIVmac251 (MOI 0.1) or without the virus overnight. We split the cells into four groups and stimulated the PBMC with PHA, the *M. tuberculosis* antigen CFP10 (peptide pools), *Pneumocystis jirovecii* kexin (peptide pools) or media alone. After the 5-6 hour stimulation we measured TNF and IFN-γ production by intracellular cytokine staining and flow cytometry.

SIVmac251 caused a significant decrease in the release of TNF by CFP10-stimulated CD4 T cells (Figure 21A) while no significant decrease occurred in CD8 T cells (Figure 21D). The decrease in TNF production by CD4 T cells did not occur in 2 out of the 16 monkeys (Figure 20A), which demonstrates the wide range of responses. No change in T cell IFN-γ release was observed (Figure 22A, D). SIV did not cause a significant change in cytokine release by PBMC stimulated with PHA (Figure 21B,E and Figure 22B,E). Interestingly, SIV caused a significant increase in CD4 T cell TNF release (Figure 21C) and no significant change in IFN-γ (Figure 22 C,F) in PBMC that were stimulated with Pneumocystis kexin peptide pools in kexin-antibody responsive (i.e. Pneumocystis-exposed) monkeys. This supports that SIV explicitly inhibits the ability of *M. tuberculosis*-specific T cells to produce cytokines without decreasing the ability of all T cells to respond to a nonspecific T cell activator (PHA) or another pathogen (pneumocystis). To determine if the change in TNF production by CD4 T cells was the result of T cell death induced by SIV we determined changes apoptosis in these assays (Figure 23A,B). PBMC incubated with SIV did not result in an increase in activated caspase 3 in CD4 and CD8 T cells (Figure 23A,B).

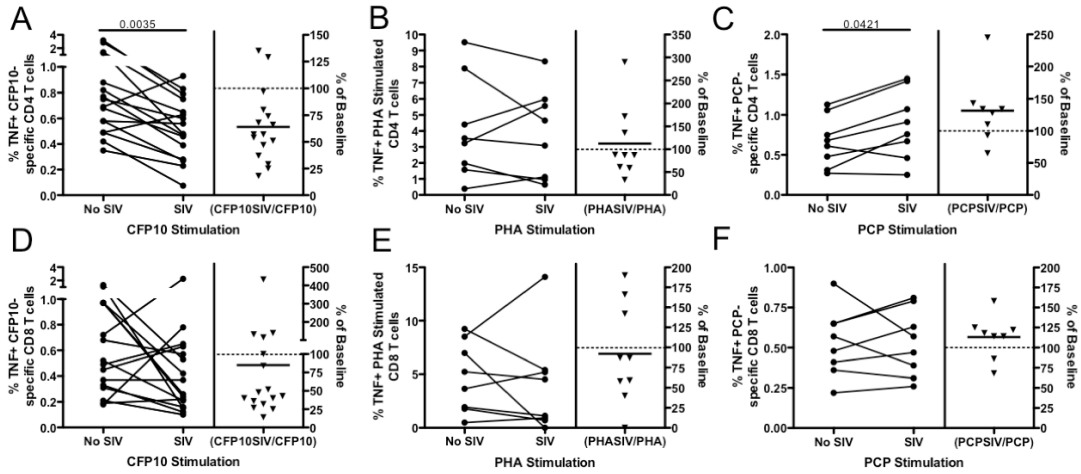

Figure 21. *SIV reduces TNF-production in CFP10-specific CD4 T cells.* PBMC were stimulated with *M. tuberculosis*-specific antigens (CFP10, A and D), phytohaemagglutinin (PHA, B and E) or pneumocystis kexin peptides (PCP, C and F). TNF was measured in CD4 (A-C) and CD8 (D-F) T cells. SIV caused a significant decrease in TNF release in CFP10-specific CD4 T cells (N = 16, A) and an increase in PCP-specific CD4 T cells (N = 8, C). No change was observed in PHA stimulated CD4 T cells (N = 9, B) or CFP10-specific CD8 T cells (N = 16, D), PHA stimulated CD8 T cells (N = 9, E) or PCP-specific CD8 T cells (N = 8, F). Lines represent changes in CD4 or CD8 T cell cytokine responses within an individual monkey. The column on the right side of each graph indicate the percent difference in T cell responses in PBMC incubated with SIV compared to PBMC without SIV (triangles; line represents mean percentage). The dotted line represents the baseline cytokine response in T cells from PBMC without SIV. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. P values that indicate significance are represented.

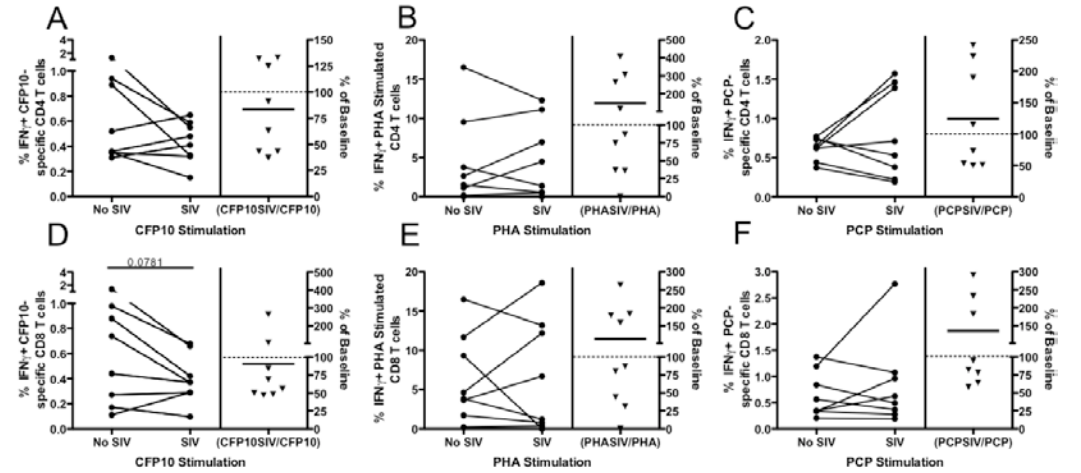

Figure 22. *SIV does not cause a reduction in IFN-γ producing T cells.* PBMC stimulated with *M. tuberculosis*-specific antigens (CFP10, A and D), phytohaemagglutinin (PHA, B and E) or pneumocystis kexin peptides (PCP, C and F). IFN-γ was measured in CD4 (A-C) and CD8 (D-F) T cells. SIV caused a trend in the reduction in IFN-γ release in CFP10-specific CD8 T cells (N = 8, D) without affecting CFP10-specific CD4 T cells (N = 8, A). SIV caused no change in IFN-γ release in PHA stimulated CD4 (N = 9, B) or CD8 (N = 9, E) T cells or PCP-stimulated CD4 (N = 8, C) or CD8 (N = 8, F) T cells. Data are presented similar to figure 21. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. P value that indicates a trend is represented.

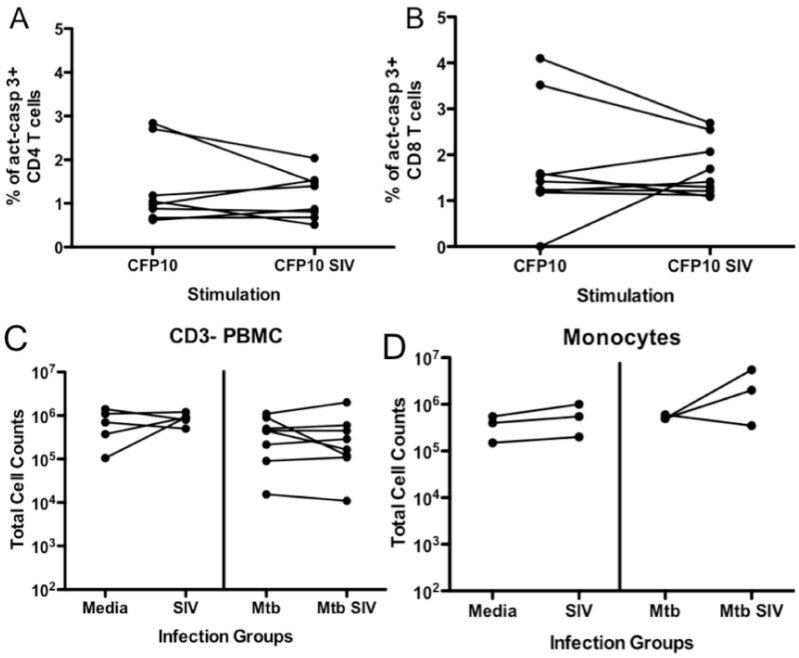

Figure 23. *SIV does not cause an increase in cell death.* Activated caspase-3 in PBMC (A-B) and cell death in CD3- PBMC (C) and monocytes (D) were determined after an overnight incubation with SIV. No difference in activated caspase 3 was observed in CD4 (N = 7; A) or CD8 (N = 8; B) T cells in PBMC incubated with and without SIV overnight. SIV did not reduce number of cells in CD3-PBMC (N = 5-8; C) and monocytes (N = 3; D).

7.3.2 SIV reduces CFP10-specific TNF release in CD4 and CD8 T cells from thoracic lymph nodes of *M. tuberculosis* infected monkeys

To determine whether the results from peripheral T cells are recapitulated in tissue from *M. tuberculosis*-infected animals, SIV (MOI 0.1) was added to lung draining lymph node cells (thoracic lymph nodes) from monkeys with active TB (Figure 24). SIV was incubated with the lymph node cells overnight prior to the addition of CFP10. SIV caused a significant decrease in TNF release from both CFP10-specific CD4 and CD8 T cells (Figure 24A,B) without affecting IFN-γ release (Figure 24C-D) from lung draining lymph node homogenates.

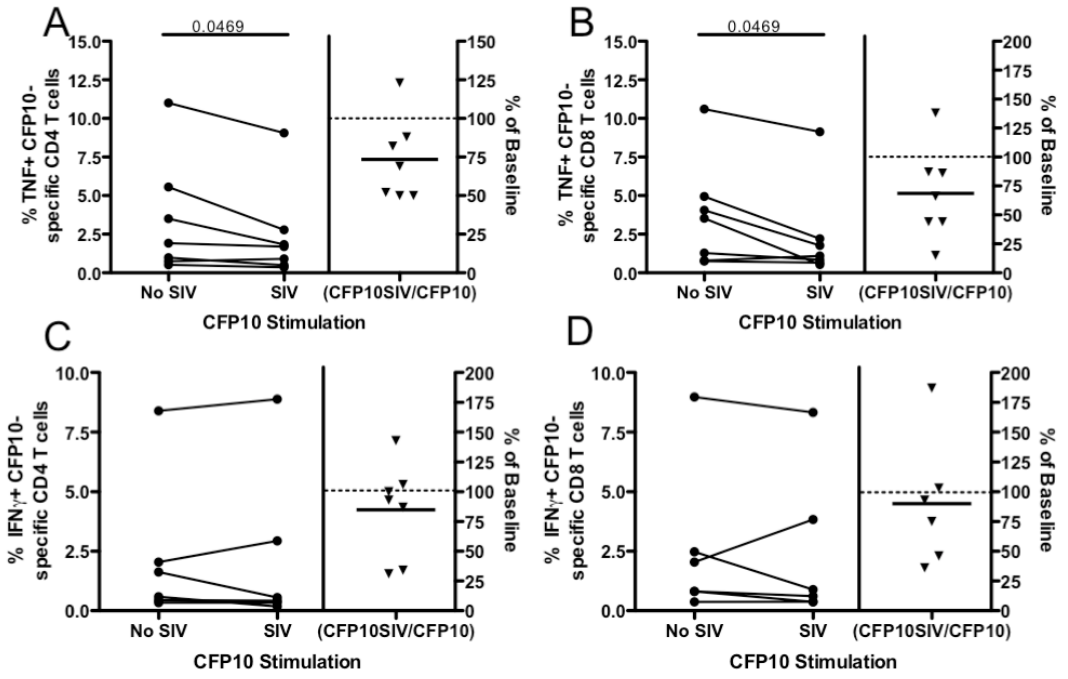

Figure 24. *SIV reduces TNF production in CFP10-specific T cells within thoracic lymph nodes.* CD4 (N = 7, A) and CD8 (N = 7, B) T cells from thoracic lymph nodes stimulated with CFP10 and incubated with SIV had significantly decreased TNF production compared to cells not incubated with SIV. SIV did not manipulate IFN-γ producing CD4 (N = 7, C) or CD8 (N = 7, D) T cells. Data are presented the same as figure 21. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. P values that indicate significance are represented.

7.3.3 SIV induces changes in antigen presenting cells that decrease the ability of T cells to respond to CFP10

We next wanted to determine whether the decrease in TNF release from CD4 T cells was a result of SIV directly manipulating T cells or indirectly manipulating T cells through effects on antigen-presenting cells. We separated peripheral CD3+ T cells from PBMC of *M. tuberculosis* infected monkeys. We exposed either the CD3+ T cells or CD3-depleted PBMC (containing monocytes among other cells) to SIVmac251 (MOI 0.1) or media alone. After an overnight incubation, we combined the cells together as follows: SIV-exposed CD3 T cells with CD3-

PBMC; CD3 T cells with SIV-exposed CD3- PBMC; or CD3 T cells with CD3- PBMC (with no exposure to SIV). Each group of cells was stimulated with CFP10 peptides for 5-6 hrs. We compared the SIV-exposed cell responses to the responses of SIV unexposed cells to quantify the percent decrease in TNF production. A significant decrease in TNF production was observed in both CD4 and CD8 T cells when T cells were added to SIV-exposed CD3- PBMC compared to cells stimulated with CFP10 without SIV (Figure 25). SIV-exposed CD3 T cells did not demonstrate a reduction of TNF production (Figure 25). These data support that SIV is not directly manipulating the CD3+ T cell population, but is affecting another cell type in PBMC, that then results in a reduction in cytokine production from the *M. tuberculosis*-specific T cells.

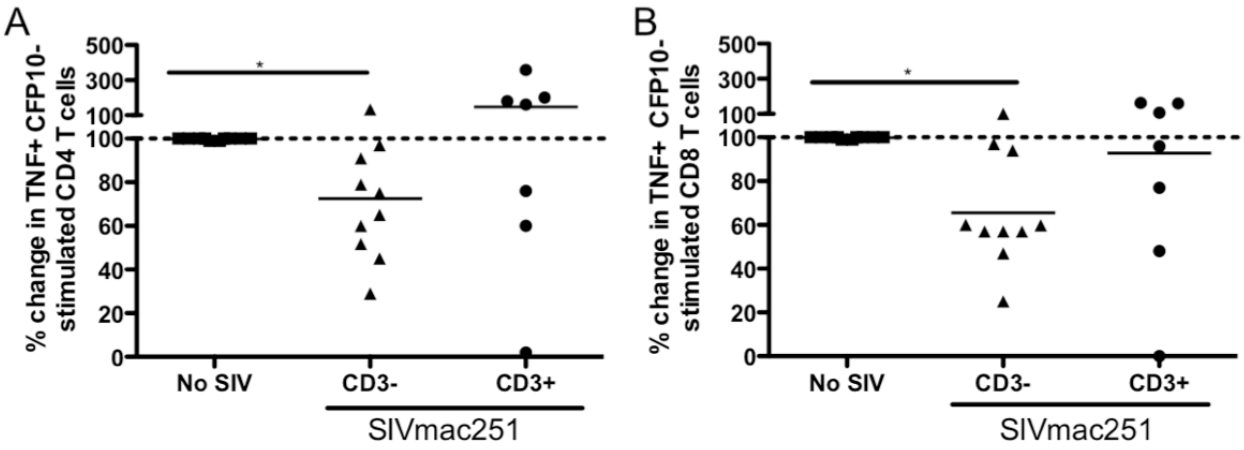

Figure 25. *The reduction in TNF-producing peripheral T cells is caused by SIV manipulating CD3- PBMC.* SIV-incubated CD3- PBMC had reduced TNF production in CD4 (CD3-, N = 10, A) and CD8 (CD3-, N = 10, B) T cells. The incubation of SIV in T cells did not reduce TNF production in CD4 (CD3+, N = 7, A) or CD8 (CD3=, N = 7, B) T cells. The dotted line represents 100% baseline of TNF production by T cells when SIV is not added during the incubation (No SIV, N = 10). Kruskal-Wallis with Dunn's multiple comparison test with significance set at $P < 0.05$ (*).

7.3.4 SIV causes a reduction in responsiveness of CD4 T cells to *M. tuberculosis*-infected cells

The previous experiments used CFP10 peptide pools to demonstrate the SIV-induced reduction of TNF production by CD4 T cells. The addition of these peptides allows the antigen presenting cells to signal T cells without processing *M. tuberculosis*. We next asked whether SIV could decrease the ability of T cells to respond to *M. tuberculosis*-infected cells, rather than just cells presenting CFP10 peptides. CD3+ T cells and the CD3-PBMC were incubated overnight followed by infection of the CD3- PBMC with *M. tuberculosis* (MOI 0.5) for 24hrs. Half of the *M. tuberculosis*-infected cells were then exposed to SIVmac251 (MOI 0.1) overnight. Autologous T cells were then added to each group of cells. SIV incubation with *M. tuberculosis*-infected CD3-depleted PBMC reduced the ability of CD4 T cells to produce TNF in response to *M. tuberculosis* (Figure 26), similar to our results with CFP10 peptides. We determined that the change in CD4 T cell TNF production did not result from SIV-induced cell death (Figure 23C), which could potentially manipulate cytokine production.

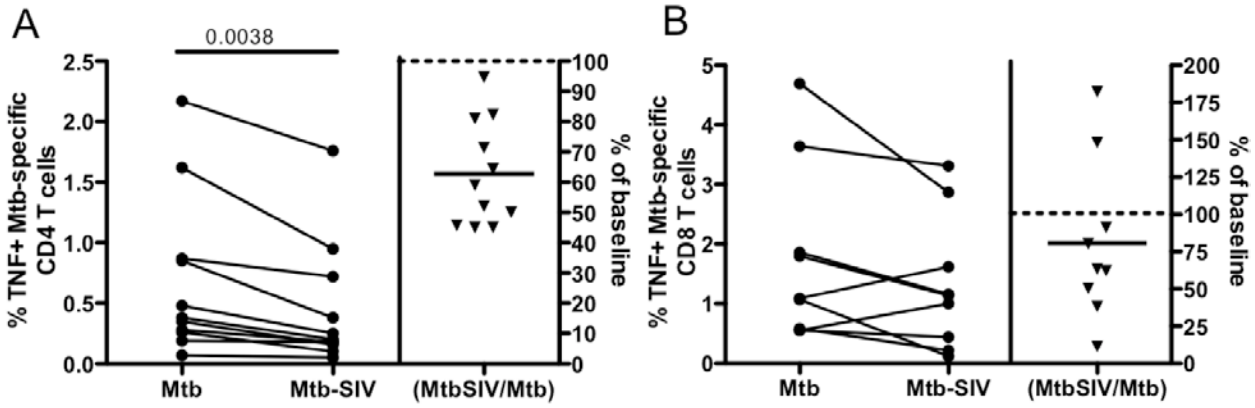

Figure 26. *SIV reduces the ability of CD4 T cells to respond to M. tuberculosis infected CD3- PBMC.* SIV/*M. tuberculosis*-co-treated CD3- PBMC (Mtb-SIV) reduced TNF production in CD4 (A, N = 11) and not CD8 (B, N = 9) T cells compared to T cells incubated with *M. tuberculosis*-treated CD3- PBMC (Mtb). Data are presented the same as Figure 21. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. P value that indicates significance is represented.

7.3.5 T cell dysfunction results from SIV disrupting *M. tuberculosis* infected monocytes

We previously demonstrated that SIV was manipulating CD3- PBMC, which contain multiple cell types, including antigen-presenting cells (APC) that could potentially reduce T cell cytokine production. To determine whether the APC affected by SIV were monocytes, we magnetically separated CD14+ cells and CD3+ T cells from PBMC of monkeys infected with *M. tuberculosis*. After allowing the cells to rest overnight, we infected the adherent CD14+ monocytes with *M. tuberculosis* (MOI 0.5) for 24 hrs. Cells were then washed with RPMI and half of the *M. tuberculosis*-infected cells were inoculated with SIVmac251 (MOI 1.0) overnight. After the overnight incubation CD3+ T cells were added to the monocytes (~3:1 ratio) and incubated together for 5-6 hrs with brefeldin A. The MOI used in the monocyte experiments is 10 fold higher than the MOI used in the CD3- PBMC experiment. We determined that an MOI of 1 in the monocyte-T cell experiment approximately recapitulates an MOI of 0.1 in CD3- PBMC because monocytes make up approximately 10% of CD3- PBMC.

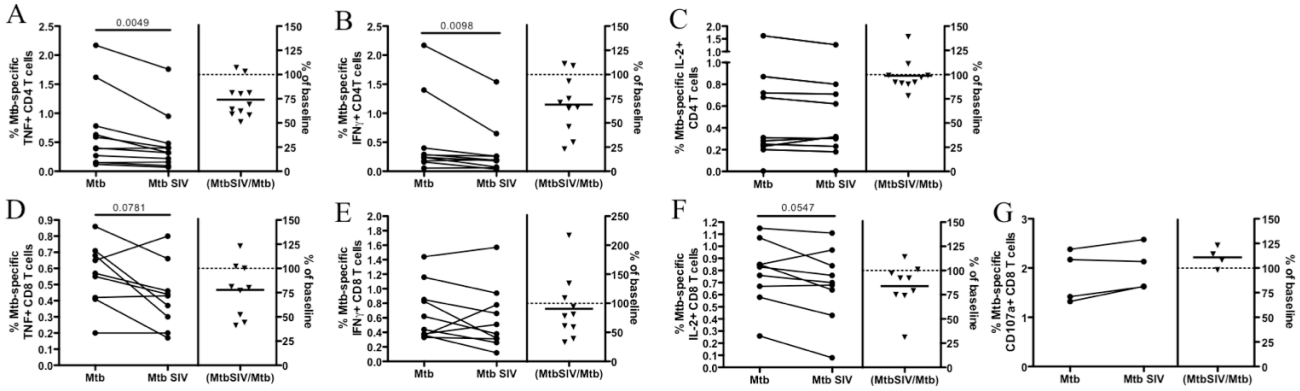

Figure 27. *SIV reduces TNF and IFN-γ production in CD4 T cells though the manipulation of M. tuberculosis-infected monocytes.* SIV/*M. tuberculosis*-treated monocytes (Mtb SIV) led to a decrease in TNF (N = 11, A) and IFN-γ (N = 10, B) production in CD4 T cells without inducing a significant change in CD4 T cell produced IL-2 (N = 10, C) when compared to T cells incubated with *M. tuberculosis*-infected monocytes (Mtb). SIV led to a trend of a decrease in TNF (N = 9, D) and IL-2 (N = 9, F) with no change in IFN-γ (N = 10, E) production in CD8 T cells. Data are presented the same as figure 21. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. P values that indicate significance and trends are represented.

CD4 T cells incubated with monocytes that had been infected with *M. tuberculosis* and cultured with SIVmac251 were inhibited for TNF and IFN-γ production compared to CD4 T cells incubated with monocytes infected with *M. tuberculosis* alone (Figure 27A, B). No change in IL-2 release occurred in CD4 T cells (Figure 27C). SIV did not lead to a significant change in CD8 T cell function (Figure 27D-F), however a trend in the decrease of TNF (Figure 27D; P = 0.0781) and IL-2 (Figure 27F; P = 0.0547) was observed. No significant decrease in CD107a cytolytic marker was observed on CD8 T cells (Figure 27G). Co-infected monocytes had no change in cell count compared to *M. tuberculosis*-only infected monocytes (Figure 23D). We also observed similar SIV-induced changes in cytokine responses within the same monkey in serial repeat experiments, which indicates that in this assay SIV-induced dysfunction *in vitro* is fairly consistent over time (Figure 28). The SIV-induced decrease in TNF-production by CD4 T cells was relatively consistent despite variability in TNF production over time (Figure 28).

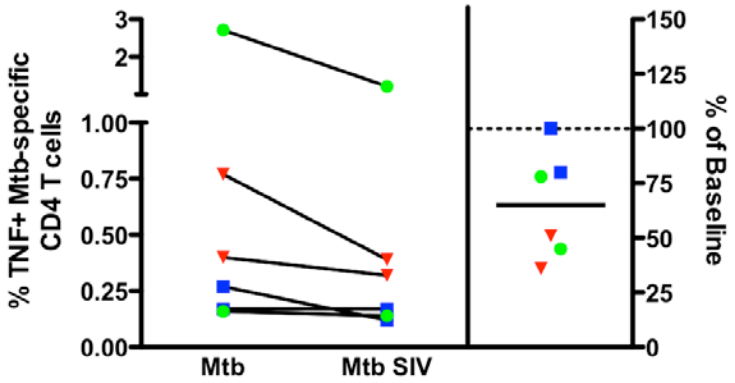

Figure 28. *SIV causes similar reductions in TNF production by M. tuberculosis-specific CD4 T cells over time in the same monkeys.* Changes in TNF production by CD4 T cells are presented at 2 different time points within the same monkey (N = 3). Individual monkeys are represented as green circles, blue squares and red upside-down triangles. Data are presented the same as figure 21.

Monocytes differentiate into macrophages in tissues, including granulomas. A pilot experiment was performed with monocyte-derived macrophages (MDM) instead of monocytes to

confirm the effects of SIV on macrophages as antigen presenting cells. Only 4 animals were used for this experiment. SIV caused a modest reduction in TNF (Figure 29A, N = 4, 84%±24.5) and IFN-γ (Figure 29B, N = 4, 87%±9.3) production by *M. tuberculosis*-specific CD4 T cells. However, SIV did cause a moderate increase in CD8 T cells producing TNF (Figure 29C, N = 4, 109%±12.9) and IFN-γ (Figure 29D, N = 4, 150%±39).

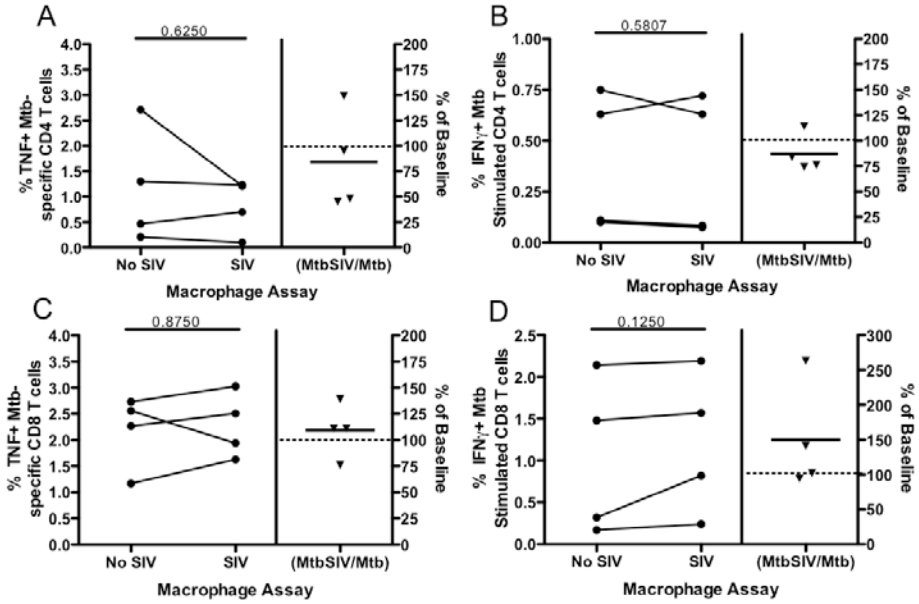

Figure 29. *TNF and IFN-γ production by CD4 T cells is reduced when treating M. tuberculosis-infected monocyte derived macrophages with SIV.* SIV/*M. tuberculosis*-treated macrophages resulted in an 84% ± 24.5 decrease in TNF (N = 4; A) and an 84% ± 9.3 decrease in IFN-γ (N = 4; B) production by CD4 T cells. SIV/*M. tuberculosis*-treated macrophages resulted in a 109% ± 12.9 increase in TNF (N = 4; C) and a 150% ± 39 increase in IFN-γ (N = 4; D) production by CD8 T cells. Data are presented the same as Figure 21. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$.

7.3.6 Non-infectious virus does not cause a significant reduction in CD4 TNF and IFNγ release.

We repeated the same monocyte-T cell experiment from above with inactivated SIVmac251 to determine if SIV requires the infection of monocytes to cause a significant decrease in cytokine

responses. Incubation with inactivated SIV did not result in significant changes in TNF or IFN-γ production by *M. tuberculosis*-specific T cells (Figure 30). The results with the inactivated virus were quite variable with some moneys demonstrating a dramatic decrease in TNF production, while the majority showed no change or an increase in cytokine production. We determined that infectious virus was needed to cause a consistent and significant reduction in CD4 TNF and IFN-γ release (Figure 30).

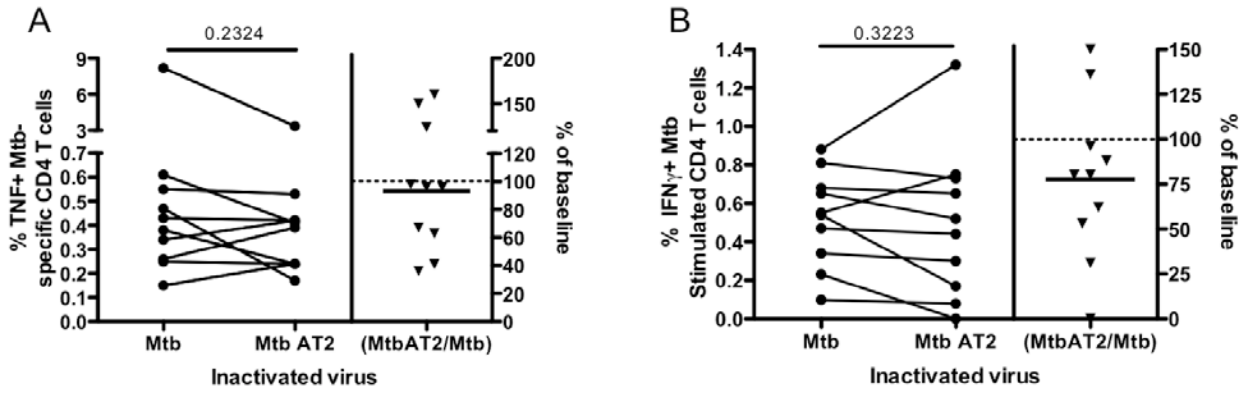

Figure 30. *Inactivated virus does not reduce cytokine production in M. tuberculosis-specific CD4 T cells.* AT2 inactivated SIVmac251 added to *M. tuberculosis*-infected monocytes did not cause a reduction in TNF (N = 10; A) or IFN-γ (N = 10; B) producing CD4 T cells. Data are presented the same as Figure 21. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$.

7.3.7 SIV does not change lymphocyte proliferation or monocyte expression of co-stimulatory molecules or MHCs.

Changes in cytokine production by *M. tuberculosis*-specific T cells may result from a change in the presence of co-stimulatory and MHC molecules on monocytes. In order to determine how SIV causes a decrease in TNF and IFN-γ production in *M. tuberculosis*-specific CD4 T cells we examined monocyte expression of co-stimulatory molecules CD40, CD40L and CD80, and the antigen presentation molecules MHC-I (HLA-A,B,C) or MHC-II (HLA-DR,DQ,DP) (Figure 31).

SIV did not cause a change in co-stimulatory or MHC expression on *M. tuberculosis*-infected monocytes. There was also no difference in the expression levels of these proteins [as assessed by mean fluorescent intensity (MFI)] between co-infected and *M. tuberculosis*-only infected monocytes.

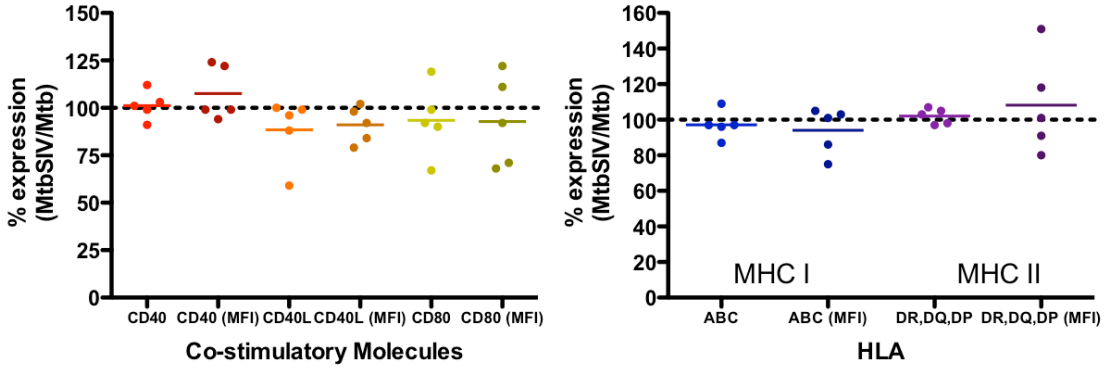

Figure 31. *SIV does not change co-stimulatory molecules or MHC expression on monocytes.* SIV/*M. tuberculosis* co-treated macrophages had the same percentage of monocytes (N = 5) expressing CD40, CD40L, CD80, HLA-ABC and HLA-DR,DQ,DP compared to *M. tuberculosis*-incubated monocytes. No change in mean fluorescent intensity (MFI) of the monocytes was observed. Dotted line represents the baseline level (*M. tuberculosis*-infected monocytes) of expression of each molecule.

Lymphocyte proliferation assay (LPA) was performed to determine whether the decrease in cytokine expression resulted from a decrease in proliferation of CD4 T cells (Figure 32). SIV did not cause a change in proliferation in PBMC incubated with media, PHA or CFP10.

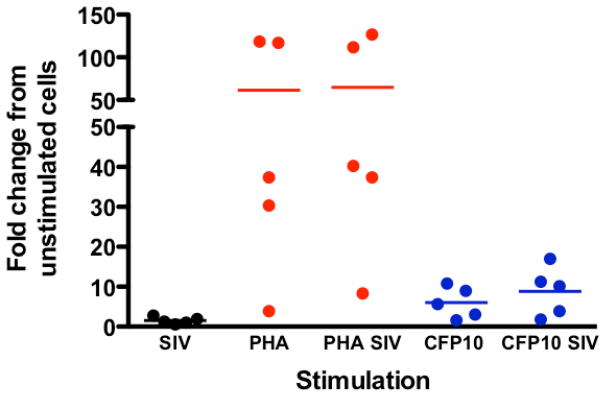

Figure 32. *SIV infection of monocytes does not cause a change in lymphocyte proliferation.* PBMC (N = 5) incubated with media, PHA or CFP10 and SIV did not cause a change in lymphocyte proliferation compared to PBMC without SIV. The fold change is compared to the proliferation of PBMC incubated in media without SIV or stimulators.

7.3.8 SIV-induces a significant increase in monocyte-derived IL-5 production by monocytes incubated with media or *M. tuberculosis.*

The data presented thus far supports that SIV affects the monocyte population, and indirectly inhibits cytokine production from *M. tuberculosis*-specific T cells. One possibility is that SIV modulates cytokine production in the *M. tuberculosis*-infected monocytes, and that the monocyte derived cytokines influence changes in the T cell responses. I took a broad approach to identifying changes cytokine production by monocytes in response to SIV. Monocytes were prepared from 10 monkeys and incubated with media, SIV, *M. tuberculosis*, or SIV+*M. tuberculosis* for 24 hours. A 23-plex Luminex assay was performed on the supernatants of monocytes (Figure 33 and 34). *M. tuberculosis* caused an increase in production of TNF (656% ± 769.1, mean ± S.D; P = 0.0195) and IL-1β (686% ± 573.4; P = 0.002) compared to the media-only groups (Figure 33A,B), which is consistent with published results from human studies [212, 289, 365]. SIV reduced TNF (76% ± 19.2; P= 0.002) and IL-1β (76% ± 14.7; P = 0.0059) release by co-infected monocytes compared to monocytes infected with *M. tuberculosis* alone (Figure 33C-D).

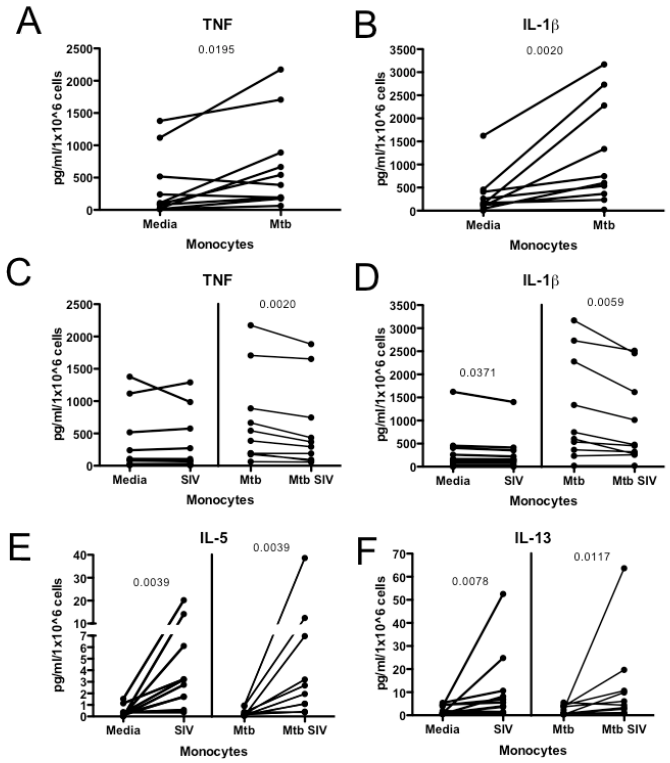

Figure 33. *SIV/M. tuberculosis treated monocytes produce more IL-5 and IL-13 than monocytes incubated with M. tuberculosis. M. tuberculosis* increased TNF (N = 10, A) and IL-1β (N = 10, B) production compared to monocytes incubated with media alone. *M. tuberculosis*/SIV co-treated monocytes reduces TNF (N = 10, C), IL-1β (N = 10, D) and increases IL-5 (E) and IL-13 (F) compared to *M. tuberculosis* incubated monocytes. SIV decreases IL-1β (D) and increases IL-5 (E) and IL-13 (F) production compared to monocytes incubated with media. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. P values that indicate significance are represented.

SIV caused a significant increase in the production of IL-5 by monocytes (SIV-only compared to uninfected monocytes, 3054% ± 4606.7; P = 0.0039 and *M. tuberculosis*/SIV treated cells compared to *M. tuberculosis*-only infected cells, 2496% ± 3777.9; P = 0.0039; Figure 33E). SIV also induced IL-13 production in the absence (1187% ± 1582.8; P = 0.0078 compared to uninfected monocytes) or presence of *M. tuberculosis* coinfection (774% ± 1122, P = 0.0117; SIV/*M. tuberculosis* compared to *M. tuberculosis*-only infected monocytes; Figure 33F). SIV also caused a slight reduction in GMCSF (89% ± 21; P = 0.0371; SIV/*M. tuberculosis* compared

to *M. tuberculosis*-only treated monocytes) and IL-4 (63% ± 36; P = 0.0781; SIV/*M. tuberculosis* compared to *M. tuberculosis*-only treated monocytes) production (Figure 33). SIV did not cause a change in monocyte production of IFN-γ, IL-2, G-CSF, IL-1α, IL-6, IL-8, IL-10, IL-12/23 p40, IL-15, IL-17, MCP-1, MIP-1β, MIP-1α, sCD40L, VEGF or IL-18 (Figure 34).

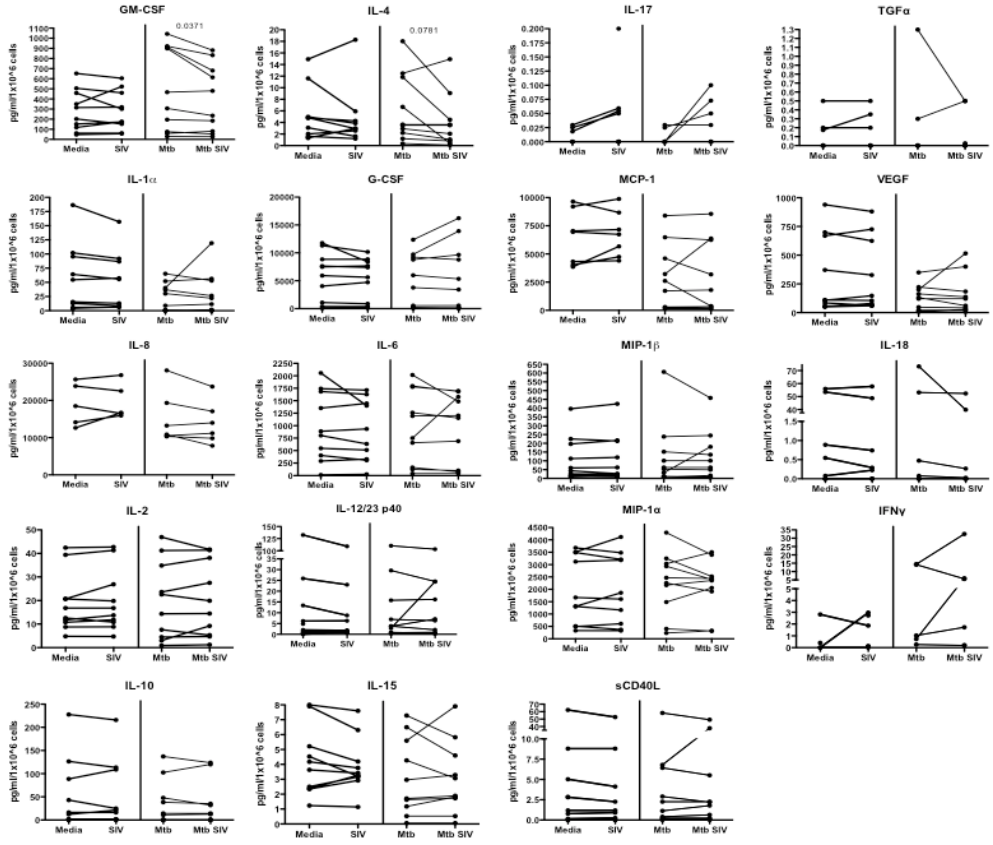

Figure 34. *Changes in SIV-induced cytokine production by monocytes.* GM-CSF, IL-4, IL-17, TGF-α, IL-1α, G-CSF, MCP-1, VEGF, IL-8, IL-6, MIP-1β, IL-18, IL-2, IL-12/23 p40, MIP-1α, IFN-γ, IL-10, IL-15 and sCD40L were measured in supernatants that were extracted from monocytes incubated with media, SIV, *M. tuberculosis*, or *M. tuberculosis*/SIV. *M. tuberculosis*/ SIV co-treated monocytes reduces GM-CSF (N = 10, P = 0.0371), IL-4 (N = 10, P = 0.0781) compared to *M. tuberculosis* incubated monocytes. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. P values that indicate significance and trends are represented.

To confirm monocytes were producing IL-5 we examined IL-5 mRNA production in SIV infected monocytes from two different monkeys after a 12 hour incubation period (Figure 35). All of our SIV infected monocytes contained IL-5 mRNA. To our surprise, we detected IL-5

RNA in the monocytes from monkey 22910, irrespective of SIV infection. From monkey 22410, the media only cells did not express IL-5, but the SIV, *M. tuberculosis*, and co-infected cells did have a detectable IL-5 message. We confirmed the IL-5 bands were 98% homologous to Homo sapien IL-5 mRNA through genomic sequencing followed by a blast search (Figure 36).

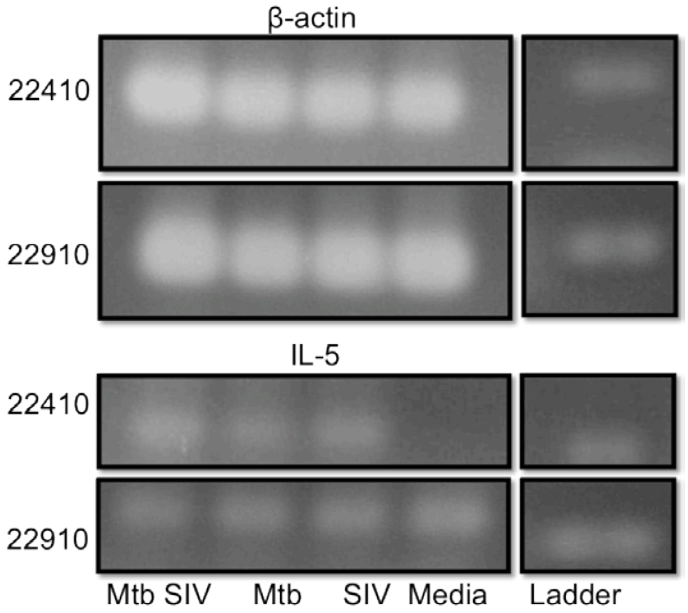

Figure 35. *Monocytes express IL-5 mRNA.* β-actin and IL-5 cDNA from monocytes incubated with media, SIV, *M. tuberculosis* (Mtb) or *M. tuberculosis* and SIV (Mtb SIV) are represented. IL-5 cDNA is present in all monocytes except 22410 monocytes incubated with media alone. Gels are representative of multiple experiments from 2 different monkeys.

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>ref|NM_000879.2| UEGM Homo sapiens interleukin 5 (colony-stimulating factor, eosinophil)
(IL5), mRNA
Length=816

 GENE ID: 3567 IL5 | interleukin 5 (colony-stimulating factor, eosinophil)
[Homo sapiens] (Over 100 PubMed links)


 Score =  335 bits (181),  Expect = 3e-91
 Identities = 188/192 (98%), Gaps = 0/192 (0%)
 Strand=Plus/Minus

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Sbjct  152  CAGTGCCAAGGT  141
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Figure 36. *Sequence homology of IL-5 bands is 98% homologous to Homo Sapien's IL-5.*

7.3.9 Neutralizing IL-5 in monocytes rescues TNF release within CD4 T cells

Since both IL-5 and IL-13 were induced by SIV, neutralizing antibodies to IL-5 or IL-13 were added to monocytes incubated with SIV and *M. tuberculosis* to determine whether either cytokine was responsible for the decrease in T cell cytokine production. Neutralizing antibodies were added to the *M. tuberculosis*-infected monocytes at the same time as the SIV inoculation. After an overnight incubation the cells were washed and autologous T cells were added to the co-infected monocytes. Anti-IL-5 antibody caused an increase in the percentage of CD4 T cells releasing TNF from most monkeys, reversing the SIV-induced reduction in TNF (Figure 37A) and no change in IFN-γ (Figure 37B), ($p < 0.05$, comparing CD4 T cell TNF production incubated with co-infected monocytes treated with anti-IL-5 antibody or an IgG isotype control). Neutralization of IL-13 did not rescue TNF or IFN-γ production by T cells (Figure 37C,D).

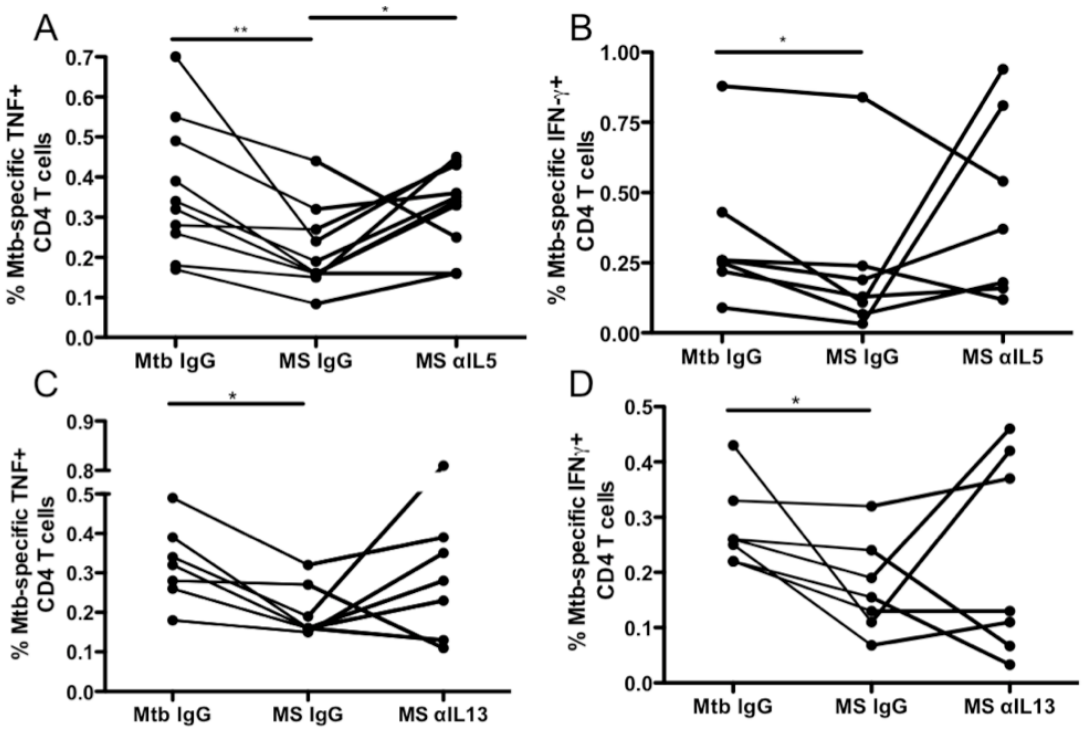

Figure 37. *Neutralization of SIV-induced monocyte production of IL-5 and not IL-13 rescues CD4 T cell TNF production without affecting IFN-γ production.* SIV (MS IgG) causes a significant decrease in CD4 T cell TNF (A) and IFN-γ (B) production compared to *M. tuberculosis* infected monocytes incubated with IgG (Mtb IgG). The addition of IL-5 neutralizing antibody to SIV/*M. tuberculosis* co-treated cultures (MS αIL-5) significantly increased CD4 T cell TNF production (N=10, A) and caused no change in IFN-γ production (N=7, B) compared to co-infected monocytes incubated with an isotype control (MS IgG). The addition of neutralizing IL-13 antibody did not effect TNF (N = 7, C) or IFN-γ (N = 7, D) production of CD4 T cells. Freidman test was used with Dunn's multiple comparison test to determine significance $P < 0.05$ (*) and $P < 0.01$ (**).

7.3.10 IL-5 recapitulates the effect of SIV on *M. tuberculosis*-infected monocytes

To confirm that IL-5 is responsible for the inhibition of TNF release by T cells, recombinant IL-5 was added to monocytes infected with *M. tuberculosis* overnight for 24hrs. Exogenous IL-5 was removed by washing the cells prior to the addition of T cells. A significant decrease in TNF release within CD4 T cells was observed (Figure 38), similar to that seen with SIV-treated

monocytes. There was a similar trend in CD4 TNF release when we added 4pg of rIL-5 to the *M. tuberculosis*-infected monocytes, although it was not significant (Figure 38).

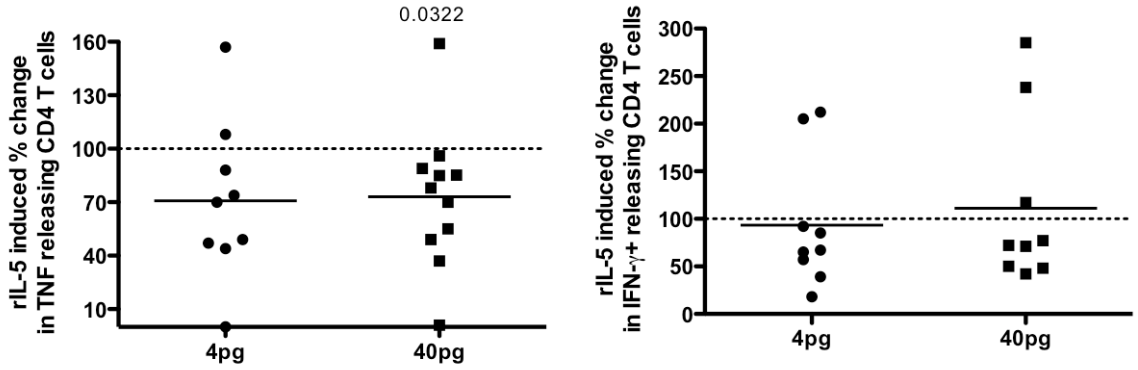

Figure 38. *Adding recombinant IL-5 to M. tuberculosis-infected monocytes recapitulates the affect SIV has on TNF releasing M. tuberculosis-specific CD4 T cells.* The addition of human recombinant IL-5 (rIL-5, 40pg/ml/1x10^6 cells) to *M. tuberculosis* infected monocytes caused a decrease in TNF (N=11, A) and not IFN-γ (N=9, B) production in CD4 T cells when compared to CD4 T cells incubated with *M. tuberculosis* infected monocytes without IL-5. Adding 10 fold less IL-5 (rIL-5, 4pg/ml/1x10^6 cells) to infected monocytes did not significantly decrease TNF (N = 9, P = 0.1289) or IFN-γ (N = 9) production. The dotted line represents cytokine production by CD4 T cells incubated with *M. tuberculosis* infected monocytes. The squares represent the percent of cytokine production in the CD4 T cells incubated with *M. tuberculosis* and IL-5 treated monocytes. Wilcoxon matched-pairs signed rank test was used with significance set at $P < 0.05$. Significant P value is presented for TNF values.

7.4 DISCUSSION

A reduction in *M. tuberculosis*-specific T cell responses in HIV-co-infected individuals has been described in several studies [261, 282, 283, 286, 310], although the mechanisms responsible are not fully understood. In this chapter, we demonstrate that SIV causes a significant decrease in TNF-production by *M. tuberculosis*-specific T cells in PBMC and thoracic lymph nodes. The data support that this effect is due to the interaction of SIV with monocytes. The similar, albeit not identical decrease in T cell responses in both blood and thoracic lymph nodes help validate

the approach of using PBMC to identify mechanisms by which changes might be occurring in *M. tuberculosis*-involved tissue, which is needed to better understand the co-infection [238]. In this study we demonstrated that SIV can manipulate both peripheral and thoracic lymph node T cell responses within the first 24hrs of incubation, which is important because the outcome of disease status in co-infected individuals is likely to be at least partially dictated during the acute phase of HIV infection [77, 271].

The decrease in *M. tuberculosis*-responsive T cells appears to be specific to *M. tuberculosis*. HIV preferentially infects and kills peripheral *M. tuberculosis*-specific CD4 T cells without affecting CMV-specific T cells [261]. Similarly, we demonstrated that SIV did not cause a significant decrease in pneumocystis-specific (PCP, kexin stimulated) or non-specifically activated T cells (PHA stimulated). However, SIV did increase TNF release in PCP-specific CD4 T cells. One reason may be that the Pneumocystis-specific T cells in the monkeys are likely true memory cells, as these monkeys were serologically positive for Pneumocystis but had no signs of active infection, although it remains possible that they were colonized. In contrast, the persistence and replication of *M. tuberculosis* bacilli, even in latently infected animals, may result in a higher percentage of effector T cells, rather than true memory T cells, and these may be differently affected by SIV interactions with the monocytes.

Monkeys co-infected with SIV and *M. tuberculosis* had significantly fewer IFN-γ releasing cells in their thoracic lymph nodes compared to monkeys with active TB without SIV. Thoracic lymph nodes drain the lungs and often contain granulomas, and therefore are useful for investigating how HIV manipulates granulomas. The decrease in T cell responses occurred without a significant loss in total T cell numbers; there was however a trend towards fewer T cells in the lymph nodes of in co-infected monkeys compared to *M. tuberculosis*-infected

monkeys [271]. This study provides the first quantifiable *in vivo* evidence that HIV may inhibit the function of *M. tuberculosis*-specific T cells within granulomatous tissue.

To our surprise the data from our in vitro model system, using PBMC, indicated that SIV was not directly manipulating T cells. SIV causes a significant decrease in T cell functionality through the manipulation of monocytes, the antigen presenting cells. *M. tuberculosis*-specific CD4 T cells released less IFN-γ and TNF when stimulated by SIV/*M. tuberculosis*-infected monocytes, compared to stimulation with monocytes infected with *M. tuberculosis* alone. These data were recapitulated using CFP-10 peptides to stimulate the M. tuberculosis specific T cells, rather than *M. tuberculosis*-infected monocytes. These results suggest that the reduction in *M. tuberculosis*-specific T cell responses that occurs in co-infected individuals may be due to a combination of factors, including manipulation of antigen presenting cells and infection or direct manipulation of CD4 T cells. The manipulation of antigen presenting cells occurs within a 24hr incubation period, which demonstrates that this defect occurs during acute viral infection. This suggests that viral surface proteins, and not viral replication, are manipulating the monocytes in such a way that decreases their ability to signal *M. tuberculosis*-specific CD4 T cells.

HIV [257, 264, 276] and SIV [271] are present in *M. tuberculosis*-infected tissues, which provide an opportunity for HIV to directly manipulate macrophage function within granulomas. HIV has been reported to inhibit the killing of intracellular *M. tuberculosis* by interfering with macrophage production of TNF [289, 293], *M. tuberculosis*-induced macrophage apoptosis [212, 289], and the acidification of vacuoles [290, 297]. As an additional mechanism for immune suppression, we propose that HIV inhibits the ability of macrophages to signal *M. tuberculosis*-specific T cells. HIV can decrease MHC expression [366], co-stimulatory molecule expression [367] and antigen processing [368] within macrophages. Although we did not observe a

significant decrease in MHC or co-stimulatory molecules, the sensitivity of our assay was limiting, since we could not specifically analyze the co-infected cells for expression of these molecules, and instead analyzed the total population of monocytes. Thus, it remains a possibility that SIV is modulating expression of co-stimulatory and antigen presenting molecules on co-infected macrophages.

A limitation of our *in vitro* model was that we were using monocytes for our antigen presenting cells. Monocytes differentiate into macrophages when they migrate to tissues, and therefore are likely present only at low levels in granulomas. We confirmed our monocyte findings using monocyte-derived macrophages from a small subset of monkeys. SIV modulated the ability of macrophages to stimulate cytokine production from *M. tuberculosis*-specific T cells. The *M. tuberculosis*-infected monocytes produce more TNF and IL-1β than uninfected or SIV-only infected monocytes, which is similar to how macrophages respond to *M. tuberculosis* infection. Co-infected monocytes produced less TNF than monocytes infected with *M. tuberculosis*-alone, as was previously reported for HIV [212, 289]. TNF is required for control of M. tuberculosis infection [72] and the combination of reduced monocyte TNF production together with the decrease in TNF production of CD4 T cells could result in a substantial reduction in overall TNF in the granulomas of co-infected persons, which may lead to reactivation. We previously demonstrated that neutralization of TNF in monkeys with latent *M. tuberculosis* infection leads to reactivation within 5-8 weeks [72].

A surprise finding from this work was that the SIV-induced IL-5 production by monocytes was key to the ability of these cells to inhibit TNF production by *M. tuberculosis*-specific CD4 T cells. Increased IL-5 production was observed in SIV-exposed monocytes, regardless of whether the cells were infected with *M. tuberculosis*. Neutralization of IL-5 in co-

infected monocytes reversed the effects of SIV on stimulation of T cells, while addition of recombinant IL-5 to *M. tuberculosis*-infected monocytes replicated the inhibition of TNF production observed with *M. tuberculosis*/SIV-exposed monocytes. This study presents a novel role for IL-5, suggesting that the cytokine may be one of the factors responsible for the reduction of *M. tuberculosis*-specific T cells within co-infected individuals. Interestingly, we observed IL-5 (and SIV) inhibits TNF release but not always IFN-γ. One possible reason for this discrepancy is that T cell kinetics suggests that TNF production occurs before IFN-γ upon mycobacterial stimulation in CD4 and CD8 T cells [369], so IL-5 or SIV may need more time to modulate IFN-γ production. If this is the case, varying stimulation times can address this hypothesis.

IL-5 is considered a Th2 cytokine that is primarily involved in eosinophil maturation and function, along with B cell growth and antibody production [370, 371]. IL-5 has also been shown to be involved in the development of cytolytic T cells in mice [372]. IL-5 is primarily produced by CD4 T cells [373, 374], however macrophages and monocytes have been reported as sources of IL-5 as well [375-378]. Similar to our study, low levels of IL-5 have been detected from macrophage-like cells stimulated with IFN-γ and *Mycobacterium avium* [375], murine macrophages infected with *M. tuberculosis* [377], macrophages/monocytes from HIV-infected individuals [376] and intestinal macrophages from individuals with Crohn's disease [378].

IL-5 is also detected in CFP and PPD stimulated PBMC [379-381] and plasma [382] of HIV/*M. tuberculosis* co-infected individuals. However, the function of IL-5 in the co-infection of HIV and *M. tuberculosis* is not understood. High incidence of TB within co-infected individuals that received BCG vaccine correlated with increased IL-5 production in response to stimulation of PBMC with mycobacterial protein mixtures [PPD and culture filtrate protein (CFP)][379, 381]. A sustained increase in IL-5 production by CFP-stimulated PBMC also occurred in

tuberculin skin test (TST) positive co-infected individuals [381]. Conversely, plasma IL-5 levels significantly increased over 1-3 months of anti-retroviral treatment in HIV/*M. tuberculosis* co-infected individuals, which did not correlate to IFN-γ production [382]. These studies demonstrate that mycobacteria are capable of stimulating PBMCs in co-infected individuals to release IL-5 with no correlation to IFN-γ production.

It is not obvious how SIV-induced IL-5 is modulating the ability of monocytes to stimulate cytokine production from T cells. IL-5 may interact with macrophages directly by decreasing their responsiveness to intracellular pathogens. IL-5 stimulated J774 A.1 cells (a murine macrophage-like cell line) induced 57% of the mRNA species observed in IL-10 stimulated cells, which supports that macrophages can respond to IL-5 [383] but does not demonstrate how IL-5 affects macrophage functionality. One study of individuals with chronic rhinosinusitis with nasal polyps (CRSwNP) demonstrated a correlation with presence of IL-5 and alternatively activated (M2) macrophages in nasal tissue, and a decreased ability to phagocytose *Staphlyococcus aureus* [384]. CRSwNP with high levels of IL-5 had less IFN-γ, IL-1β and TGF-β in nasal tissue homogenates compared to CRSwNP with low levels of IL-5. It was also determined that the addition of recombinant IL-5 to CD206+ macrophages increased the survival of *S. aureus in vitro*. This study demonstrates that IL-5 may decrease the ability of macrophages to kill intracellular pathogens and signal T cells by changing their activation status, because classically activated (M1) macrophages reduce *M. tuberculosis* growth more readily than alternatively activated (M2) macrophages [80].

IL-5, as a Th2 cytokine is generally categorized as anti-inflammatory [377, 380-382]. It is possible that an increase in IL-5 production may disrupt the Th1/Th2 balance needed to maintain proper functionality of *M. tuberculosis*-infected macrophages and *M. tuberculosis*-

specific T cells. More pathogenic strains of *M. tuberculosis* preferentially induce Th2 cytokine production (IL-4, IL-5, IL-10 and IL-13) while less virulent strains induce more Th1 cytokines (IFN-γ, TNF) by infected macrophages or monocytes [377, 385, 386]. For example, significantly more IL-5 mRNA was produced in mouse macrophages infected with virulent *M. tuberculosis* strain H37Rv compared to the avirulent strain H37Ra [377]. This upregulation of IL-5, along with IL-10 and IL-13, may play a role in the increased virulence of H37Rv compared to H37Ra. We showed that monkeys with latent TB that were co-infected with SIVmac251 had increased Th2 cytokine producing peripheral T cells *in vivo* during acute SIV infection [77], with the level correlating with time of reactivation. HIV can also lead to the production of Th2 cytokines ([387, 388] reviewed in [199]). Taken together, these studies demonstrate that a shift from Th1 to Th2 cytokines may cause a loss of immunologic control of TB. Whether IL-5 from monocytes, rather than T cells, can contribute to this shift remains unknown.

The ability of IL-5 to manipulate *M. tuberculosis*-specific T cells warrants more investigation and needs to be confirmed in human macrophages and T cells. If these results occur within human cells the next step should be to examine IL-5 neutralization *in vivo* because it may augment co-infection treatment. Humanized neutralizing antibodies to IL-5 are already in use to treat eosinophilia (reviewed in [389]), so the implementation of these antibodies is possible. This has the potential to be extremely beneficial because it would represent a completely novel treatment that could be used in parallel with chemotherapy.

7.5 ACKNOWLEDGEMENTS

The authors would like to thank Dr. Keith Reimann for supplying the SIVmac251. We are grateful for the assistance Edwin Klein and Chris Janssen for performing the necropsies that provided tissue homogenates. We are thankful for technical help from Olabisi Ojo and Philana Ling Lin. A set of experiments utilized SIVmac251 AT2 provided by Dr. Jeff Lifson from the AIDS and Cancer Virus Program, SAIC Frederick, Inc./National Cancer Institute, Frederick, supported by federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E.

8.0 SUMMARY OF THESIS

8.1 THE CO-INFECTION: DISEASE OF THE POOR

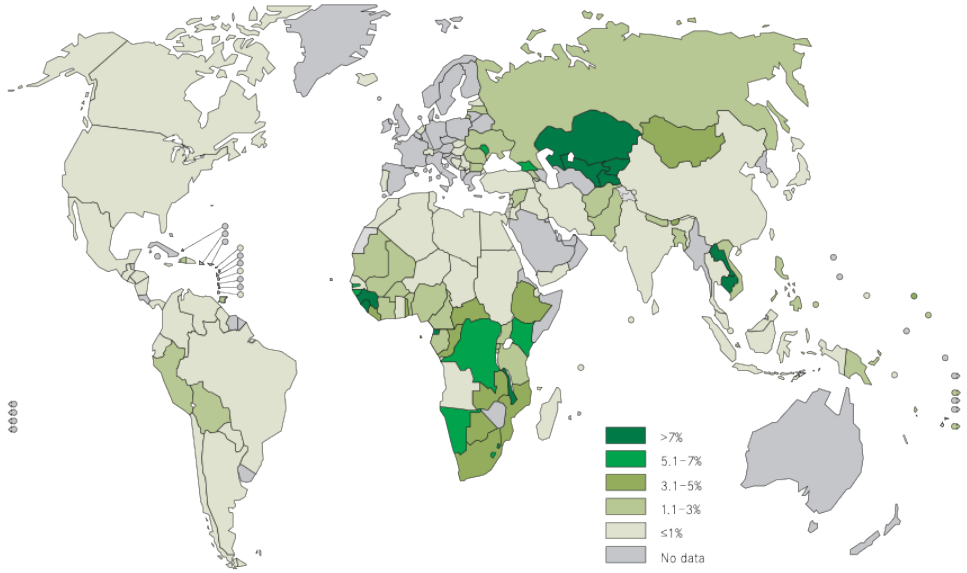

Figure 39. *The cost of TB control as a percentage of total health expenditures by public sector, 2009*. Figure was reprinted with permission from WHO 2011 Global Tuberculosis Control Report (11).

Tuberculosis and HIV/AIDS are two major independent health concerns [11, 22]. Together, they cause much more mortality and morbidity than either pathogen alone [16, 244, 249, 285]. A major obstacle in controlling tuberculosis has been the emergence of HIV. HIV-infection is the single greatest risk factor for tuberculosis [11]. The highest rates of HIV and tuberculosis are in sub-Saharan Africa [11, 22], which is made up of resource-poor nations [390]. In some of these

nations, TB control accounts for more than 7% of total health expenditures, which is far more than the United States and other developed world countries (Figure 39) [11]. The high cost of tuberculosis control has not correlated with a significant reduction of tuberculosis in countries with the most disease [391]. Prevention, treatment and early tuberculosis detection have led to a gradual decline in incidence, prevalence and mortality of tuberculosis globally over the last decade (Figure 40). Unfortunately, this decline has been less prevalent in Africa (Figure 41), the area where tuberculosis and HIV are most detrimental.

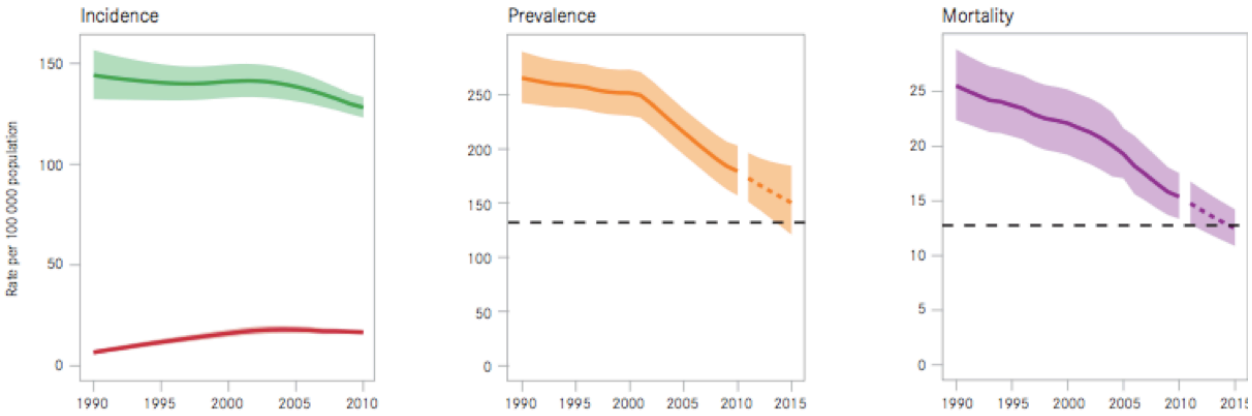

Figure 40. *Global trends in estimated rates of TB incidence, prevalence and mortality*. Left: Global trends in estimated incidence rate including HIV-positive TB (green) and estimated incidence rate of HIV-positive TB (red). Center and right: trends in estimated TB prevalence and mortality rates 1990-2010 and forecast TB prevalence and mortality rates 2011-2015. The horizontal dashed lines represent the Stop TB partnership targets of a 50% reduction in prevalence and mortality rates by 2015 compared with 1990. Shaded areas represent uncertainty bands. Mortality excludes TB deaths among HIV-positive people. Figure was reprinted with permission from WHO 2011 Global Tuberculosis Control Report (11).

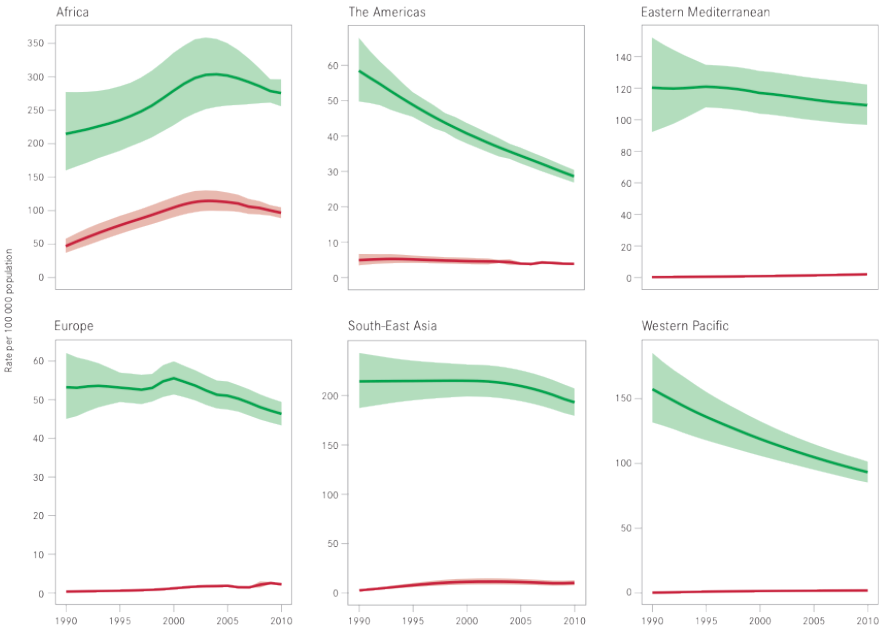

Figure 41. *Estimated TB incidence rates by WHO region, 1990 to 2010.* Regional trends in estimated TB incidence rates (green) and estimated incidence rates of HIV-infected TB (red). Shaded areas represent uncertainty bands. Figure was reprinted with permission from WHO 2011 Global Tuberculosis Control Report (11).

A more aggressive strategy is needed for identifying and treating the co-infection to make the largest impact in countries with the highest disease burden [391]. To help reduce tuberculosis and HIV burden we need to identify infected individuals earlier so they can get appropriate treatments, along with researching more effective chemotherapies and vaccines. Development of new cures and prevention strategies is dependent on a major increase in our understanding of how HIV and *M. tuberculosis* interact within the host. *The main goal of this thesis is to expand our basic understanding of how HIV manipulates M. tuberculosis-specific immunologic responses to increase TB susceptibility.*

8.2 OVERALL IMPACT OF THESIS

As mentioned in Chapter 4 there are intelligent hypotheses suggesting possible causes for the increased susceptibly of tuberculosis in HIV-infected individuals, based on peripheral sample evidence in humans, that need to be confirmed in tissue-based experiments (Table 4, [238]). Although these hypotheses are probably correct, the development of an HIV/TB animal model (Chapter 6), based on cynomolgus macaques with latent TB that are then co-infected with SIVmac251, helped address these hypotheses in a way that is not ethically possible in human studies. This model provided a platform for examining immunological and microbiological changes over the entire course of disease that included both tissue- and peripherally-based experiments over time and at necropsy. We also developed *in vitro* and *ex vivo* systems to address how HIV reduces *M. tuberculosis*-specific effector T cell responses by manipulating antigen-presenting cells (Chapter 7). The overall goal of my thesis work, to provide insight into the mechanisms by which SIV increases the risk of tuberculosis, has been achieved through the integration of data from the *in vivo* and *in vitro* systems, along with comparisons to available data from human studies.

Table 4. How does HIV increase TB risk?
Adapted from Diedrich C R , Flynn J L Infect. Immun. 2011 (238); with permission.

Hypothesis	Figs/Table	References
HIV replication increases at sites of M. tuberculosis infection, which leads to an increase in TB pathology		
SIV replication occurs within granulomas and thoracic lymph nodes, albeit it is not ideal	T3, F20	271
Tissue from co-infected monkeys have a trend for more virus than tissue of SIV-only infected monkeys	F20	§
Increase in HIV p24 and viral load in BAL cells from TB involved vs. uninvolved lung		274
Increase in HIV load in pleural fluid than plasma in individuals with pleural TB		264
Increase in HIV replication in stimulated macrophages infected with Mtb and HIV compared macrophages infected with HIV alone		256, 257
HIV induces active or reactivated TB through decreasing CD4 T cell counts within granulomas		
Decrease in CD4 and CD8 T cell counts within granulomas from co-infected monkeys compared to macaques with active TB	F19	271
AIDS patients with tuberculosis adenitis have few CD4 T cells in lymph node granulomas		252
HIV-infected individuals with fewer peripheral CD4 T cells are more prone to TB than HIV-infected individuals with more CD4 T cells.		270
Co-infected individuals have fewer BAL fluid CD4 T cells than individuals with TB alone		281, 286
HIV reduces Mtb-specific T cell responsiveness at sites of disease		
Co-infected animals have fewer IFN-γ releasing *M. tuberculosis*-specific T cells within thoracic lymph nodes than macaques with active TB	F18	§
Exogenously added SIV to thoracic lymph nodes stimulated with CFP10 reduces TNF production by CD4 and CD8 T cells within 12hrs	F24	§
Fewer IFN-γ releasing Mtb-specific memory CD4 T cells after HIV infection in individuals with latent TB		261,283
Fewer IFN-γ-TNF-IL-2 polyfunctional BCG-specific CD4 T cells in airways of HIV-infected individuals than in of HIV-uninfected individuals		286
Lower IFN-γ mRNA production and cellular proliferation in airways of patients with AIDS and TB than individuals with TB alone		304,305
Lower IFN-γ, TNF and IL-2 production and cellular proliferation in *M. tuberculosis*-specific peripheral T cells in HIV-infected individuals than in HIV-uninfected individuals with active TB		143,282,283,303
The ability of HIV to manipulate macrophage function may reduce T cell signaling and M. tuberculosis killing		
Exogenously added SIV to PBMC, CD3- PBMC, and *M. tuberculosis*-infected monocytes cause a reduction of TNF and IFN-γ production by *M. tuberculosis*-specific CD4 T cells in less than 1 day by manipulating monocytes directly	F21, 25-27	§
Decrease in TNF production in CD4 T cells is caused by SIV-induced monocyte dysfunction that involves SIV-induced production of IL-5 after a 24hr incubation	F25, 33, 37, 38	§
HIV and *M. tuberculosis* co-infected macrophages induce less TNF-dependent apoptosis than macrophages infected with only *M. tuberculosis*		212,289,293
Co-infected macrophages release less TNF than macrophages infected with only *M. tuberuclosis*		289,293
HIV decreases the ability of *M. tuberculosis*-infected macrophages to acidify vesicles		290,297
Acute phase of HIV dictates TB outcome within individuals with latent TB		
Correlate acute peripheral T cell counts with reactivation	F10	271
During acute SIV the depletion of peripheral T cells is followed by an increase in peripheral effector T cell responses and activation, which suggest a spike in bacterial load due to early disruption in granuloma function	F12-14,16, T2	271
Late reactivators contain more fibrotic granulomas than early reactivators, active, and latent monkeys, which demonstrate healing from an initial disruption	F16, T2	271
Early reactivators have fewer BAL CD4 T cells than late reactivors 10 weeks post SIV inoculation	F10	271
Exogenously added SIV to PBMC, CD3- PBMC, Mtb-infected monocytes causes a reduction of TNF and IFN-γ production by Mtb-specific CD4 T cells in less than 1 day	F21, 25-27	§
Decrease in TNF production in CD4 T cells is caused by SIV-induced monocyte dysfunction that involves SIV-induced production of IL-5 shortly after incubation with SIV	F25, 33, 37, 38	§

Blue- indicates our contribution to co-infection literature

Black- Current HIV/TB literature

F- Figure; T- Table

§- Paper in preparation

8.2.1 A Novel HIV/*M. tuberculosis* co-infection animal model

The need to better understand how HIV induces the reactivation of latent TB led us to designing an HIV/*M. tuberculosis* co-infection animal model [77, 271]. This model is critical because it allows researchers to use known and characterized strains and doses of SIV and *M. tuberculosis*, and provides easy and repeated access to PBMC, peripheral lymph nodes, and bronchial alveolar lavage (BAL) cells over the entire course of disease. Of great importance, an animal model of co-infection allows one to measure immunologic responses along with viral loads and bacterial growth within granulomatous and non-granulomatous tissue at necropsy. Human studies generally take a metaphorical snapshot of peripheral immunological responses between co-infected and singly infected individuals [16, 252, 261, 270, 283, 286, 392-395]. Although these studies are extremely useful in increasing our basic understanding of HIV and TB immunology, they may not demonstrate the whole picture, because immunologic responses in TB wax and wane over time [72, 77, 113, 271, 396], and the peripheral responses do not adequately reflect the tissue based responses [77].

8.2.1.1 *In vivo* firsts: Acute phase of SIV infection dictates tuberculosis reactivation time

We demonstrated, for the first time, that the acute depletion of peripheral CD4 and CD8 T cells during the first 8 weeks of infection significantly correlated to reactivation time [271]. We also observed a transient spike in IFN-γ releasing *M. tuberculosis*- and SIV-specific peripheral T cells along with an increase in activated (CD29+) T cells between 6 and 10 weeks post SIV inoculation. A spike in plasma IFN-γ, TNF and IL-10 concentrations between 2 and 4 weeks post SIV inoculation preceded the increase in the number of effector T cells. We believe these transient increases in cytokine levels and effector T cells are a result of SIV disrupting the

homeostatic balance within granulomas, causing a rapid increase in bacterial growth and viral replication, resulting in more available antigen that activates effector T cells and primes naïve T cells. We generally observe higher peripheral effector T cell responses in monkeys with active TB than monkeys with latent disease (both without SIV) [113]. These data led us to our current hypothesis that the acute phase of HIV directly dictates the longer-term effects on control of latent tuberculosis (Table 4). This idea was supported by not only the increased depletion of CD4 and CD8 T cells within co-infected monkeys compared to monkeys with TB alone but the increased number of fibrotic granulomas in late reactivating co-infected monkeys, which suggest a healing response (Flynn and Lin unpublished data, [271]). Taken together, these data suggest that the acute peripheral T cell depletion may also be occurring within granulomas. Animals that cannot contain disease reactivate early after SIV inoculation (<17 weeks) with late reactivators (>26 weeks) temporarily regaining or maintaining control of the *M. tuberculosis* infection.

If the early events after HIV infection influence the risk of reactivation in humans, it demonstrates the need to start retroviral therapy for co-infected individuals as soon as possible. Starting antiretroviral therapy early on has been proposed to prevent CD4 T cell depletion in co-infected individuals to lessen their risk of TB by maintaining a higher level of peripheral CD4 T cells [270]. However, the acute depletion of CD4 T cells did not correlate to peripheral T cell counts at time of necropsy or reactivation; in fact some of the monkeys in our study regained and maintained "normal" peripheral CD4 T cell levels, even when they reactivated. This suggests that peripheral T cell counts are not the only factor that influences the risk of TB in co-infected individuals.

8.2.1.2 *In vivo* firsts: SIV decreases T cell counts within *M. tuberculosis* granulomas

One hypothesis is that HIV increases TB susceptibility by causing a reduction of CD4 T cells within the granuloma (reviewed in [238, 249, 363]), although there are no human data to support this. The non-human primate study detailed in this thesis was the first time a retrovirus has been shown to cause a decrease in T cells in pulmonary granulomas. One previous study demonstrated that patients with tuberculosis adenitis with AIDS had reduced levels of CD4 T cells within their affected lymph nodes [252], which is not surprising because AIDS patients have a significant reduction in CD4 T cells in blood and tissues. Since most co-infected patients do not have AIDS it is important to understand the pathology of HIV-infected individuals before they are in a 'worst case scenario.' Our study demonstrated that a 'normal' level of T cells within PBMC, axillary and inguinal lymph nodes did not correlate to T cell composition within lung granulomas at time of necropsy. This means that peripheral T cell levels may not be representative of granulomatous T cells after the acute phase of infection. This further demonstrates that peripheral responses probably do not accurately reflect T cell responses at sites of disease. Our study provides the first evidence that retroviruses actually reduce T cell levels in granulomas!

8.2.1.3 *In vivo* firsts: HIV infects *M. tuberculosis* lung granulomas

Granulomas have also been proposed as ideal sites for HIV replication (reviewed in [238, 249, 363]). Surprisingly, no published studies have demonstrated that HIV infects granulomas. This aforementioned hypothesis is based on co-infection macrophage data demonstrating more HIV replication than macrophages infected with HIV alone [257, 288], a study that demonstrated BAL cells extracted from areas of lungs with *M. tuberculosis*-infiltrate determined by x-ray contained higher viral replication and p24 levels than normal lung [274] and pleural fluid in co-infected individuals with pleural TB have a higher viral load than their plasma [264, 276]. It is

important to remember that TB is not an airway disease and these samples may not represent what is occurring within lung granulomas.

We provide the first evidence that a retrovirus infects lung and thoracic lymph node *M. tuberculosis* granulomas in a highly variable manner at necropsy [271]. Reactivation and pathology did not correlate to viral load, which suggests that viral load within granulomas does not directly correlate to TB susceptibility. We believe that there is a low threshold amount of HIV (or SIV) that is needed to disrupt granuloma function. This study demonstrated, for the first time that TB granulomas contain SIV, although they are not necessarily ideal for viral replication!

8.2.1.4 *In vivo* firsts: SIV decreases granulomatous *M. tuberculosis*-specific T cell responses

Another hypothesis is that SIV manipulates *M. tuberculosis*-specific T cell responses within granulomas to increase susceptibility to active TB (reviewed in [238, 249, 363]). This hypothesis is based on an extensive amount of work examining changes in *M. tuberculosis*-specific T cell responses in PBMC [16, 261, 282, 283, 302] and BAL cells [286] in co-infected individuals. Comparing T cell responses in PBMC, BAL cells, lung and lymph node granulomas at the same time demonstrate a dramatic difference in responses [77, 112, 113], which may mean that the studies demonstrating that HIV decreases peripheral T cell responses may not correlate to changes in the granuloma. Although our study demonstrated no significant difference in the number of IFN-γ releasing T cells within lung granulomas [271] we did demonstrate a significant decrease in IFN-γ releasing T cells within thoracic draining lymph nodes between co-infected monkeys and monkeys with active TB (Diedrich, paper in preparation). This adds the first

evidence that granulomatous *M. tuberculosis*-specific T cells within thoracic lymph nodes may be affected by a retrovirus!

8.2.1.5 No experiment is perfect: Issues with our model.

I believe our model can make a lasting impact on understanding HIV/*M. tuberculosis* co-infection immunology, however there are some issues that should be addressed prior to using this model again to examine different parameters. One issue with our co-infection model is that we inoculated the macaques with 10^6 to 10^7 TCID50 units of SIVmac251 [271]. This amount of virus is higher than an individual would contract during intravenous drug use or sexual contact. Despite this high level of inoculum, the animal's peripheral viral loads did peak within a normal level (plasma: 10^7 /ml and PBMC: 10^6/ml) and viral loads were generally lower after the acute phase of infection than in the plasma and PBMC than what is normally observed in humans [397]. This demonstrates that viral load may not be as important to TB reactivation during the chronic phase of HIV as previously thought. However, cynomolgus macaques are not as susceptible to SIV as rhesus macaques. We could not use rhesus macaques for these studies, because they rarely develop latent *M. tuberculosis* infection. So the lower viral load set points are likely due in part to the choice of macaque species. This may be remedied by studying co-infection in cynomolgus macaques from the island of Mauritius, as some of these animals have been shown to maintain higher viral burdens, similar to rhesus macaques.

We also may want to use different strains of SIV in future experiments. SIVmac251 was chosen because it would infect tissues readily and it does not cause a significant and sustained depletion of peripheral CD4 T cells. However, our *in vitro* data suggests that SIVmac251 infects macrophages and monocytes more readily than it infects T cells (Mattila, unpublished data).

Since HIV generally infects both T cells and macrophages to different degrees over the course of disease [118], using a different M- and T-tropic strain may prove useful.

One issue that cannot ethically or cost-effectively be dealt with in any nonhuman primate model is the low number of animals used in each study. We used 7 co-infected and 4 SIV-only infected animals, which is a reasonable number of nonhuman primates in any study. One issue that may come up is that we included historical TB-only controls to compare our findings too. Although, the TB-only controls underwent the same immunological procedures, individual reviewers have presented reservations to comparing data to these controls.

All of the animals with latent TB in our study that were co-infected with SIV and *M. tuberculosis* reactivated their latent TB [271]. There is a 10% annualized risk of reactivating latent TB in co-infected humans [11, 16, 285]. This rate is less than the 100% reactivation that we observed after 48 weeks post SIV inoculation, which may present an issue in correlating the model to humans. However, the objective of our study was to examine SIV-induced reactivation of latent TB so it is desirable that they all reactivate. We also monitor the monkeys far more intensely that humans with latent *M. tuberculosis* infection are monitored, so we catch very early signs of reactivation. Adjusting viral strains and inoculum dosage may address the issue of early reactivation in future studies that primarily want to examine specific immunological responses.

8.2.1.6 Future directions for the HIV/TB animal model

The animal model developed in this thesis work will provide an opportunity to examine basic immunological and microbiological questions along with the effectiveness of new drugs or vaccines on co-infected animals. Effective vaccines or drugs against tuberculosis need to work in HIV-infected individuals because of their increased susceptibility and the high prevalence of co-

infection. Determining the effectiveness of a vaccine in a co-infected nonhuman primate model would be an ideal step before moving on to humans.

Addressing whether there truly is a correlation between TB reactivation and acute granulomatous T cell depletion and dysfunction is an important continuation of this work. Our hypothesis could be tested by necropsying a group of co-infected monkeys with latent TB within the first 8 weeks post SIV inoculation and determining T cell counts, viral and bacterial loads and *M. tuberculosis*-specific T cell activity within granulomatous tissues. Although these experiments would not correlate to reactivation, they could determine specific SIV-induced changes that occur during the acute phase of infection that cannot ethically be examined in humans. Using serial PET-CT scanning, researchers could also determine whether the acute phase of SIV infection leads to reactivation by examining changes in the size, dissemination and metabolic activity of the granulomas in real time. PET-CT will provide a chance to determine how long it takes SIV to induce these changes and whether these changes are predictive of TB reactivation. PET-CT may also be used to determine anti-retroviral effectiveness at preventing *M. tuberculosis* dissemination. We could also determine anti-retroviral concentrations within granulomas to establish which type of drug penetrates granulomas most efficiently. Correlating anti-retroviral concentrations to viral loads and T cell counts within granulomas may help augment treatment.

Humans with HIV are more susceptible to primary tuberculosis, not just reactivation of latent infection. Inoculating SIV-infected monkeys with *M. tuberculosis* to determine how HIV changes initial tuberculosis susceptibility can be a simple and informative change to the animal model. These experiments may be used to predict TB susceptibility by correlating viral load and T cell counts prior to *M. tuberculosis* inoculation. This could also be used to determine the

effectiveness of a tuberculosis vaccine on an HIV-infected individual by vaccinating SIV-infected macaques prior to *M. tuberculosis* inoculation.

The co-infection animal model provides a chance for researchers to ask, "How HIV is manipulating host responses to *M. tuberculosis* during the entire course of disease?" Results obtained in this model could be translated into human studies. It is my hope that it will continue to increase our basic understanding of both of these pathogens.

8.2.2 *In vitro* examination of how SIV manipulates *M. tuberculosis*-specific T cell functions

It is well established that HIV manipulates *M. tuberculosis*-specific effector T cell function [16, 77, 238, 261, 282, 283, 286, 302]. It is not known how long it takes HIV to manipulate these cells or how HIV is causing this dysfunction. It has been hypothesized that HIV is exhausting and/or killing *M. tuberculosis*-specific T cells ([261], reviewed in [238, 249, 363]). Although *M. tuberculosis*-specific CD4 T cells may be preferentially infected by HIV, the majority of these cells are not infected [261] so another cause of this effector dysfunction needs to be determined. In order to determine a potential mechanism for this dysfunction we developed an *in vitro* T cell-monocyte model. These *in vitro* models were designed in part because they are more cost effective and more amenable to manipulation than co-infection animal studies.

8.2.2.1 *In vitro* firsts: SIV immediately disrupts *M. tuberculosis*-specific T cell effector functions

Since we hypothesize that the progression to TB reactivation is dictated during the acute phase of HIV we set out to determine whether SIV immediately causes a reduction in *M. tuberculosis*-

specific T cell effector function. In chapter 7 we demonstrate that exogenously added SIV overnight can decrease both CFP10-specific peripheral CD4 TNF-producing T cells and CFP10-specific TNF producing CD4 and CD8 T cells in thoracic lymph nodes from monkeys with TB (without SIV). The similar responses in both the involved tissues and PBMC demonstrate that SIV may manipulate these cells similarly, at least during the first day of *in vitro* infection. This effect also occurred in *M. tuberculosis*-specific peripheral T cells. Taken together, these data demonstrate that SIV decreases TNF production almost immediately. If this occurs in humans, when HIV enters a granuloma it may immediately cause a disruption in the homeostatic balance between immune function and bacterial growth (Figure 42). The literature and our study demonstrate that HIV/*M. tuberculosis*-infected macrophages release less TNF than *M. tuberculosis*-only infected macrophages [212, 289]. This is important because TNF is essential in granuloma function [72] and if HIV causes a decrease in TNF production by both CD4 T cells and macrophages granuloma integrity and function will probably be disturbed. This immediate disruption in TNF production supports the idea that co-infected individuals need to be treated with anti-retrovirals immediately.

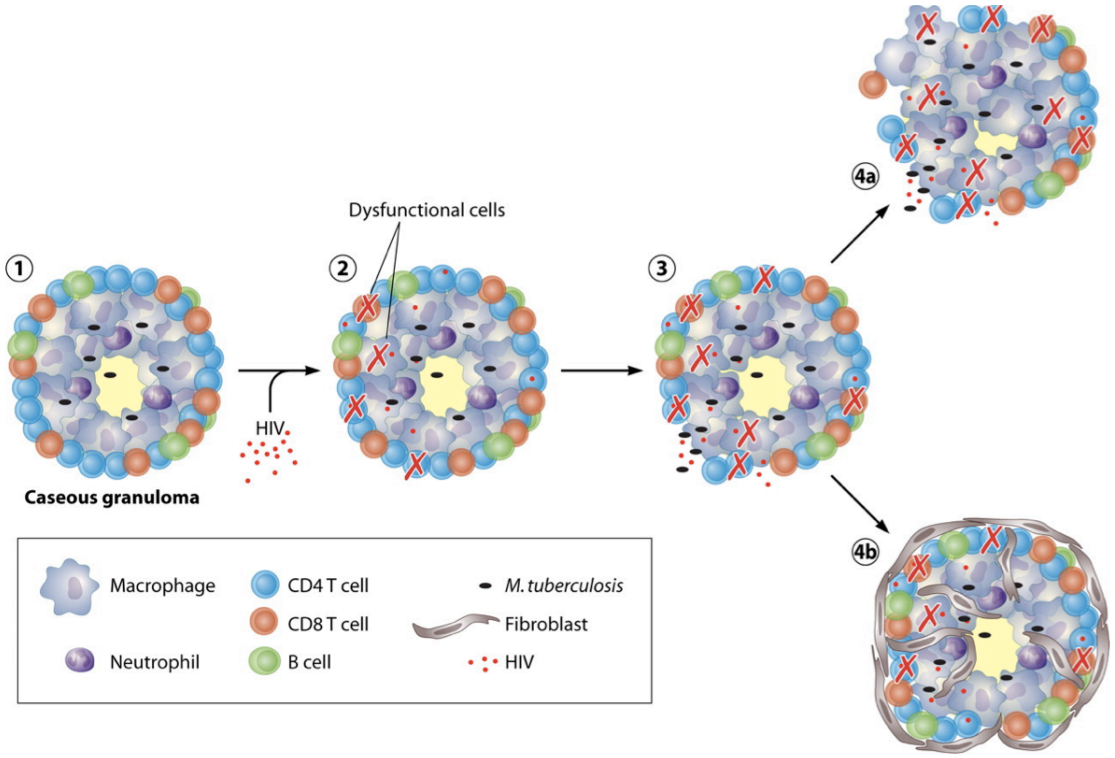

Figure 42. *Proposed mechanism of HIV-induced reactivation of latent TB*. (Stage 1) Necrotic granuloma functioning "normally" in an individual with latent TB. (Stage 2) HIV enters the granuloma and induces functional changes within T cells and macrophages. HIV also kills activated T cells. (Stage 3) The decrease in T cell number and increase in cellular dysfunction lead to a functional disruption of the granuloma. This may lead to increased dissemination. (Stage 4a) Granulomas functionally disrupted shortly after HIV infection leads to continued *M. tuberculosis* dissemination and early TB reactivation. (Stage 4b) Fibrotic granulomas temporarily re-establish granuloma containment, which prevents reactivation. Figure reprinted from Diedrich C R , Flynn J L Infect. Immun. 2011; doi:10.1128/IAI.01126-10 with permission (238).

We determined that incubating SIV with *M. tuberculosis*-infected monocytes indirectly caused a significant decrease in TNF and IFN-γ production in *M. tuberculosis*-specific T cells after a 24hr incubation period compared to T cells incubated with *M. tuberculosis*-only infected monocytes. The decrease in cytokine production by *M. tuberculosis*-specific T cells occurred when SIVmac251 was incubated with antigen-presenting cells and not when it was incubated with T cells overnight. This suggests that macrophages, the major component of granulomas, may be responsible for decreases in *M. tuberculosis*-specific T cell functions we observed in the thoracic lymph nodes of co-infected monkeys compared to active monkeys.

8.2.2.2 *In vitro* firsts: SIV induced monocyte production of IL-5 causes a decrease in TNF production in CD4 T cells

The observed decrease in TNF and IFN-γ production by *M. tuberculosis*-specific CD4 T cells results from a manipulation of the antigen-presenting cells. This manipulation was caused by SIV-induced production of IL-5 by monocytes. Neutralizing IL-5 during the incubation of SIV in *M. tuberculosis*-infected monocytes rescued autologous TNF production in CD4 T cells. The addition of recombinant IL-5 to *M. tuberculosis*-infected monocytes prior to the addition of autologous T cells caused a reduction in TNF. Neutralizing antibodies to IL-5 and recombinant IL-5 were only added to the infected monocytes so it appears that IL-5 is acting on the monocytes exclusively. Since IL-5 is considered a Th2 cytokine [374], it is possible that it is changing the activation status of infected monocytes from M1 to M2 [383-385], which have been shown to be less effective at killing *M. tuberculosis* [80]. However, since M2 macrophages may be needed to prevent too much inflammation within granulomas (reviewed in [28]), a treatment that prevents M2 activation would probably be detrimental. If these results are recapitulated in humans, neutralizing IL-5 in co-infected individuals may augment drug treatment.

8.2.2.3 No experiment is perfect: Issues with our *in vitro* model.

As with any *in vitro* experiment it is impossible to model exactly what is occurring *in vivo*. The monocyte-T cell model is less complex than the granuloma. However, this model does have its place because it allows us to examine changes in specific cells that could not be observed *in vivo*. This means that the *in vitro* results need to be recapitulated *in vivo*. Another issue is that monocytes are different from granulomatous macrophages. We used monocytes because of the relative ease of obtaining them and their availability; in addition, the experimental set-up then did not require two sequential blood draws from each monkey (one for monocytes to

differentiate into macrophages and another one for T cells). For a subset of monkeys, we repeated these experiments using monocyte-derived macrophages, and the TNF and IL-1β induction by M. tuberculosis was similar in our monocytes as compared to that in the literature for macrophages [212, 289, 365]. However, macrophages in the granuloma may be very different than monocyte or monocyte-derived macrophages, and these studies should be repeated using macrophages isolated from granulomas.

8.2.2.4 Future directions for the HIV/TB *in vitro* model

This model can be used to determine potential causes for *M. tuberculosis*-specific T cell dysfunction caused by antigen presenting cells. IL-5 production is probably not the only cause of CD4 T cell dysfunction so attempting to determine other factors that result in cytokine T cell dysfunction can be performed. This is especially important because IFN-γ production was not affected by monocyte production of IL-5. SIV (and HIV) may be disrupting MHC expression, co-stimulatory molecules, antigen presentation or processing within the antigen-presenting cell in a specific manner that affects the activation of *M. tuberculosis*-specific T cells.

These experiments also should be performed in human T cells and macrophages to validate the model. If HIV causes similar changes in TNF production by CD4 T cells then the possibility of using neutralizing antibodies to IL-5 in the SIV/*M. tuberculosis* co-infection model may be used to determine if it reduces TB reaction or susceptibility.

8.3 OVERALL CONCLUSION

This thesis developed both an HIV/*M. tuberculosis* co-infection animal model and an *in vitro* assay to examine how HIV increases TB susceptibility. These experiments provided the first tissue-based evidence that support current hypotheses of how HIV increases susceptibility (Table 4). Combining data from this thesis with HIV/tuberculosis literature, we developed a schematic of how HIV induces TB reactivation within a granuloma (Figure 42). These experiments also suggest novel hypotheses of how HIV manipulates *M. tuberculosis*-specific immunological functions. These experiments can and should be repeated with varying parameters to continue to build our basic understanding of the immunological ramifications of the co-infection. For example, both our animal model and *in vitro* data suggest that anti-retrovirals should be administered as soon as possible, regardless of CD4 T cell count in co-infected individuals. In addition, if the *in vitro* experiments hold true in human cells, neutralizing antibodies to IL-5 can also be used in conjunction with the SIV/TB animal model to determine if IL-5 is exacerbating TB in co-infected individuals. The more basic science we understand about the co-infection the better we can augment treatment and design vaccines.

APPENDIX A: PUBLICATION RECORD

Diedrich CR, Mattila JT, Klein E, Janssen C, Phuah J, Timothy JS, Montelaro RC, Lin PL, Flynn JL. Reactivation of Latent Tuberculosis in Cynomolgus Macaques Infected with SIV Is Associated with Early Peripheral T Cell Depletion and Not Virus Load. March 10, 2010 PLoS ONE 5(3): e9611,doi:10.1371/journal.pone.0009611. PMID: 20224771; PMCID: PMC2835744

- Chosen for Future Virology Priority Paper Evaluation: [397].

Mattila, JT, **Diedrich CR**, Phuah JY, Lin L, Flynn JL. SIV-induced changes in T cell phenotypes in macaques with latent *M. tuberculosis* infection are associated with reactivation. Journal of Immunology, 186:3527-3537 February 2011. PMID: 21317393.

Diedrich, CR & Flynn, J. HIV/*Mycobacterium tuberculosis* co-infection immunology: How does HIV exacerbate TB? Infection and Immunity. April 2011, p1407-1417, Vol. 79, No. 4. PMID: 21245275; PMCID: PMC3067569.

- Chosen as a Faculty of 1000 paper, http://f1000.com/13277035: [398].

Diedrich CR, Mattila JT, Rhode KH, O'Dee DM, Milk L, Russell DG, Flynn JL. SIV reduces TNF release by *M. tuberculosis*-specific T cells through a monocyte-derived IL-5 dependent mechanism. In preparation.

APPENDIX B: REPRINT PERMISSIONS

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