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DEVELOPMENT OF A HUMANIZED MOUSE MODEL TO UNDERSTAND *M.TB* AND HIV/*M.TB* CO-INFECTION

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DEVELOPMENT OF A HUMANIZED MOUSE MODEL TO UNDERSTAND *M.TB* AND HIV/*M.TB* CO-INFECTION

by

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Dedication

To my husband Matt and kids - Natalia, Andrew, and Isabella –

for their continuous support and encouragement throughout these years.

We did it together.

Acknowledgements

I would like to begin by thanking my mentor, Dr. Janice Endsley, for her willingness to take me into her lab. She has continuously supported and guided me throughout these past few years both in and out of the lab. You have become more than my mentor, you have become a friend, someone I know I can continue to go for support, someone I can confide in, and someone I can go to just to talk. I would also like to thank the other members of my committee, Dr. Judith Aronson, Dr. Gustavo Valbuena, and Dr. Ray Waters for their continuous support, time, and effort in guiding me throughout these years to help me complete this work. I would like to give a special thank you to Dr. Alfredo Torres who has become someone I can confide in and who has helped me since the beginning. He has become more than just my committee member, he has become a friend. I would also like to acknowledge and thank the other members of the lab Sudhamathi Vijayakumar, Matt Huante, and Putri Sutjita for all your time and effort in helping me complete various aspects of this work. Each of you made my life a little easier and for that I am grateful.

DEVELOPMENT OF A HUMANIZED MOUSE MODEL TO UNDERSTAND *M.TB* AND HIV/*M.TB* CO-INFECTION

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Mycobacterium tuberculosis (*M.tb*) is the most common co-infection and leading cause of death among HIV^+ individuals. Several animal models are available to study TB infection although many do not reproduce disease pathology similar to humans. The human host tropism of HIV limits its study in animal models. The lack of a small animal model to study HIV/*M.tb* co-infection has limited our abilities to fully understand how HIV induces defects in the host immune response to increase susceptibility to *M.tb*. The goal of these studies is to develop the humanized BLT mouse as a small animal model to study HIV/*M.tb* co-infection and identify mechanisms of HIV-mediated immune dysfunction that alter the protective response against *M.tb* infection.

Humanized BLT mice are infected with *M.tb* and develop progressive bacterial infection in the lung that disseminates to other organs (liver, spleen). Pathology displays organized granulomas with cellular cuffing, bacilli localized to the periphery, and caseous necrosis in the center. Other important characteristics of human TB are also observed, including bronchial obstruction, foamy macrophages, lipid deposits, and cholesterol crystal formations. Human T cells ($CD3^+$) are organized at sites of infection

in the lung and liver, allowing for the study of mechanisms of immune protection and bacterial evasion.

HIV/*M.tb* co-infected mice develop productive viral infection which disseminates to various organs (lung, liver, spleen). Similar to humans, mice displayed reduced peripheral $CD4^+$ T cell numbers. Granulomatous lesions that vary in size and organization are observed in the lung from necrotic granulomas filled with bacilli surrounded by epitheliod-like macrophages and actively replicating virus, to caseous necrosis granulomas with bacilli localized to the periphery but minimal viral replication. Human T cells ($CD4^+$, $CD8^+$) are scattered throughout the lesions and tissues, and an array of cytokines (Th1, pro-inflammatory) and chemokines are also expressed by co-infected mice, demonstrating the potential to study how HIV alters cell-mediated immunity specifically at sites of infection.

These results provide evidence for use of the BLT mouse as a complementary animal model in the study of *M.tb* infection. Additionally, these results support its use as a small animal model to begin understanding the mechanisms of immune dysfunction to *M.tb* in HIV-positive populations.

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AICD	activation induced cell death
AIDS	acquired immune deficiency virus
APCs	antigen presenting cells
ARV	AIDS-associated retrovirus
ASK-1	apoptosis signal-regulating kinase-1
BAL	bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
CA	capsid
CFP-10	culture filtrate protein of 10 kDa
CIITA	class II transactivator
CLIP	class II-associated invariant chain peptide
CMI	Cell-mediated Immunity
CTD	c-terminal domain
CTL	cytotoxic T lymphocyte
DC	Dendritic Cell
Dos	dormancy
ESAT-6	early secreted antigenic target of 6 kDa
ESX	ESAT-6 secretion system
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
gp	glycoprotein
H_2O_2	hydrogen peroxide
HIV	Human Immunodeficiency Virus
HSC	hematopoietic stem cell
HTLV	human T cell leukemia virus
IFN-γ	interferon gamma
IL	interleukin
IL2Rγ	IL-2 receptor γ-chain
IN	integrase
i.n.	intranasal
i.p.	interperitoneal
i.v.	intravenous
kDa	kilo Dalton
KS	Kaposi sarcoma
LAV	lymphadenopathy-associated virus
LTBI	latent tuberculosis infection
LTR	long-terminal repeat
M	major
MA	matrix
mAGP	mycolylarabinogalacton peptidoglycan
MB	mega base pair
MDM	monocyte-derived macrophage
MGC	multi-nucleated giant cell

MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
M.tb	*Mycobacterium tuberculosis*
N	non-M or non-O
NC	nucleocapsid
Nef	negative effector
NF-κB	nuclear factor κB
NF-IL-6	IL-1-inducible nuclear factor
NHP	non-human primate
NK	natural killer
NO	nitric oxide
NOD	non-obese diabetic
NSG	NOD/SCID/γ_c^{null}
NSI	non-syncytium inducing
O	outlier
OI	opportunistic infection
P	putative
p-TEFb	positive transcription elongation factor 1
PCP	*Pneumocystis carinii* pneumonia
PPD	protein purified derivative
PR	protease
RD	region of difference
Rev	regulator of virion gene expression
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
rpf	resuscitation promoting factors
RT	reverse transcriptase
SCID	severe combined immunodeficiency virus
SI	syncytium-inducing
SIV	simian immunodeficiency virus
SU	surface
T-reg	T-regulatory
Tat	transcriptional transactivator
TB	Tuberculosis
TGF-β	transforming growth factor beta
Th	T helper
TM	transmembrane
TNF-α	tumor necrosis factor – alpha
TRAIL	tumor necrosis factor-related apoptosis induced ligand
Vif	viral infectivity factor
Vpr	viral protein r
Vpu	viral protein u
WHO	World Health Organization

CHAPTER 1 - INTRODUCTION

Mycobacterium tuberculosis

"The captain of all these men of death that came against him to take him away, was the consumption, for it was that that brought him down to the grave"

John Bunyan, 1680

EPIDEMIOLOGY

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M.tb*), is a major global health threat [1]. The World Health Organization (WHO) recognized TB as a 'global public health emergency' in 1993 [1]. Worldwide, TB is the second leading cause of death among infectious diseases with approximately 1.4 million deaths reported in 2011 [1, 2]. One third of the world's population (about two billion people) are estimated to be infected with latent TB and in 2011 there were approximately 9 million newly infected cases as shown in Illustration 1 [1, 2]. Following *M.tb* exposure, 90% of individuals will develop latent TB infection (LTBI), while 10% will develop active TB disease [3-6]. Of those who develop active disease, half will develop primary TB disease within 18 months of exposure and the other half will develop post-primary TB infection, or reactivation, within their lifetime [4, 5, 7]. Given these statistics, new vaccines and therapeutics are urgently needed to reduce and prevent the global burden of infections, and eventually eliminate TB from the world.

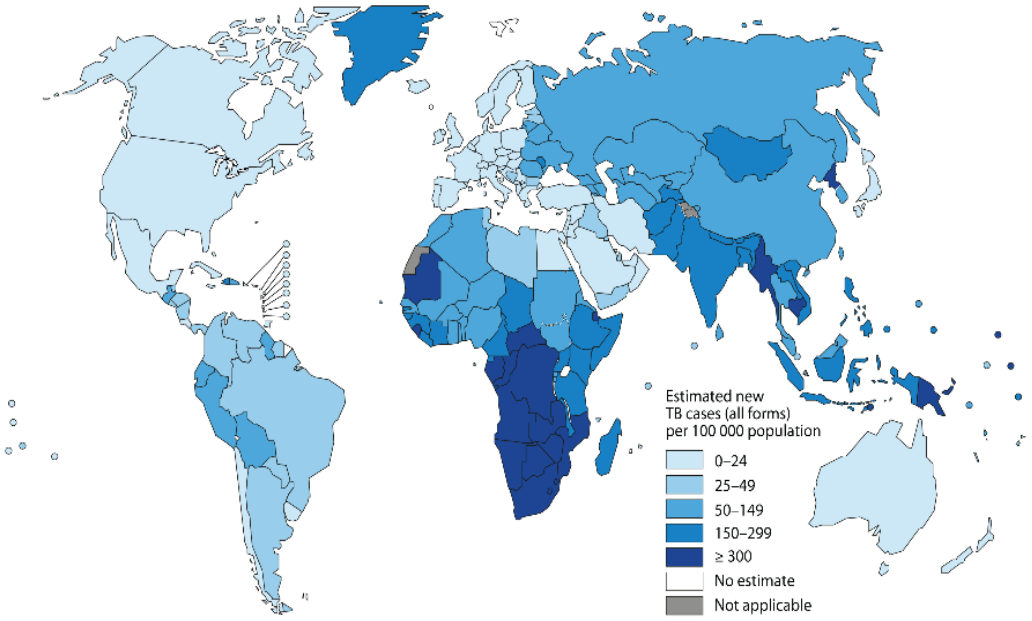


Illustration 1: Estimated TB incidence rates, 2011 [1]

HISTORY OF TUBERCULOSIS

TB has been present in humans for thousands of years. The earliest evidence of TB was found in the skeletal remains of prehistoric humans from a Neolithic settlement in the Eastern Mediterranean (7000-4000 BC) [8-12], in the spines of Egyptian mummies (3000-2400 BC) [8, 13, 14], and during the pre-Columbian era in the New World [8, 15, 16]. Around 460 BC, Hippocrates described TB as phthisis, the Greek word for consumption or "wasting away", because the disease appeared to consume people from within [17, 18]. TB also became known as the white plague because of the loss of skin color seen in patients [8, 19]. In 1680, English Christian writer and preacher John Bunyan (1628-1688) described TB as "the captain of all these men of death" when annual rates in London were 1000 per 100,000 in the population [17, 19-21]. In 1689, English doctor Richard Morton described *M.tb*-infected individuals as having "consumption" [17, 22, 23].

Our understanding of the pathology and pathogenesis of TB began in the late 17th century (1680) when Franciscus Sylvius first identified characteristic pulmonary nodules he called "tubercula" or small knots in consumptive individuals that evolved into lung ulcers or cavities [17, 18, 24]. In 1819, French physician René Théophile Hyacinthe Laennec described pulmonary lesions in individuals that had died of TB [17, 18, 25, 26]. Person to person transmission of TB was proposed in 1722 by a British doctor named Benjamin Marten [17, 18, 27]. By 1865, French physician Jean-Antoine Villemin demonstrated that TB could be transmitted from host to host in rabbits [17, 18, 25, 28]. Unfortunately, the causative agent responsible for this disease remained unknown.

By 1882, German physician Robert Koch isolated and identified tubercle bacillus as the causative agent of TB [17, 18, 25, 29]. Koch also contributed to the development of staining techniques to detect for *M.tb*, development of culture media to grow the bacteria, mode of transmission, and recommended the isolation of TB infected individuals [25, 29]. Additionally, while attempting to find a treatment for TB, Koch accidently discovered tuberculin [25]. Koch described a substance that came from the culture media used to grow the bacteria, he thought would cure or at least reduce disease severity; unfortunately it was not a cure [25]. The substance, named tuberculin and later known as protein purified derivative (PPD), became a diagnostic tool to identify animals and human infected with *M.tb* through the development of an immune reaction; this is now known as the tuberculin skin test [17, 18, 25, 30]. The continued need for a treatment against TB lead to the establishment of sanitariums by Edward Livingston Trudeau with treatment based on fresh air, rest, and a healthy diet [17].

In 1896, an advancement was made in the development of a vaccine against *M.tb* when Theobald Smith, an American microbiologist, discovered that *Mycobacterium bovis* (*M. bovis*) was the causative agent of bovine TB and not *M.tb* [17]. In 1908, Albert Calmette and Camille Guérin isolated *M. bovis* and grew it media until eventually the bacterium became avirulent and demonstrated protective effects in several *M.tb*-infected

animal models [17]. In 1921, the live attenuated bacilli became known as the Bacille Calmette-Guérin (BCG) vaccine that is widely used throughout the world today [17, 31]. It is most protective in infants from more severe TB forms, such as TB meningitis and miliary TB, although its protective effects wane over time [1]. Efficacy rates of the BCG vaccine varying from 0 to 80%, although approximately 50% protection has been reported as demonstrated by a meta-analysis in 2000 [32, 33]. However, in infants with HIV infection, BCG vaccination is not recommended given the risk of disseminated BCG disease [1]. Thus new vaccines are urgently needed with improved effectiveness for adults, children, and HIV-positive populations.

MYCOBACTERIUM GENUS

The *Mycobacterium* genus belongs to the *Mycobacteriaceae* family, part of the *Corynebacterium-Mycobacterium Nocardia* branch of Gram-positive bacteria [34]. It was originally thought that *M.tb* evolved from *M. bovis*, essentially human TB infection was derived from cattle [35-37]. Following a comparative analysis of the genome structure of *M.tb* and *M. bovis*, it was determined that this theory was unlikely given the deletion of several regions within *M. bovis*. Since the genome of *M.tb* is larger than the *M. bovis* genome, the insertion of DNA segments within *M.tb* would not be possible [35, 38, 39]. As a result, it was concluded that *M. bovis* and other animal strains of mycobacteria that are designated as *M.tb* complex, evolved from the human *M.tb* strain [39].

The *Mycobacterium* genus has over 120 species which differ in their host tropism, genomic composition, and pathogenicity [35, 40]. *M.tb* and *M. leprae* (the etiological agent of leprosy) are known to cause significant burden in humans [35, 41, 42]. The *M.tb* complex is composed of a group of human and zoonotic species and subspecies [35]. This complex includes *M.tb*, *M. bovis*, *M. bovis* subsp. *caprae* comb. nov., *M. africanum* subtype 1 (clade 1 and clade 2), *M. canetti*, *M. microti*, and *M. pinipedii* [35, 43]. *M.tb*, as previously described, is the etiological agent of human disease; though is can also infect

non-human primates (NHPs) and many other species including goats [44], dogs [45, 46], cats [46], birds [47], and elephants [48]. *M. bovis*, primarily the etiological agent of TB in cattle, also infects domestic and wild animals such as badgers [49], white-tail deer [50], goats, possums, and sheep [51, 52]. *M. africanum* is the causative agent of disease in humans, primates [53], cattle [54], and pigs [54]. *M. microti* is primarily found in small rodents [55], although has been seen in cats, pigs [56], and llamas [57]. *M. pinnipedii* can be found in marine mammals such as seals [58] and sea lions [59], and terrestrial mammals such as tapirs, camels, and porcupines [60]. There is also a group of mycobacteria known as the 'atypical mycobacteria' or non-tuberculous mycobacteria (NTM) that commonly cause opportunistic infections, such as those seen in immunocompromised patients; they include *M. avium*, *M. marinum*, *M. fortuitum*, *M. kansasii*, *M. smegmatis*, and *M. ulcerans* to name a few [34, 41]. NTM can cause localized or disseminated TB disease. *M. avium* and *M. kansasii* are known to cause pulmonary disease [41]. *M. fortuitum* can cause infections in the soft tissue, bone, and joints; while *M. marinum* causes bone, joint, and tendon infections along with pulmonary and disseminated TB disease [41]. *M. ulcerans* is the causative agent of buruli ulcer [41].

BACTERIAL STRUCTURE, GENOME, AND REPLICATION

M.tb is an obligate intracellular, aerobic bacteria that preferentially grows in the lungs with high oxygen levels [20]. This acid-fast, rod-shaped bacillus lacks flagella, and does not form a spore, have a capsule, or produce toxins [17, 20].

The cell wall of mycobacteria consists of a plasma membrane surrounded by an inner and outer layer [34, 61, 62]. About 60% of its cellular wall is composed of lipids, glycolipids, and mycolic acids [17, 34, 42]. The inner layer is composed of peptidoglycan covalently bonded to arabinogalactan then mycolic acids; this section is called the mycolylarabinogalactan-peptidoglycan (mAGP) complex [62]. The outer layer is composed of free lipids and proteins [61, 62]. During bacterial staining by the Ziehl-

Neelson technique, this high lipid cell wall content allows for the retention of the carbol-fushsin staining dye even after treatment with acidic alcohol, thus the name acid-fast bacilli [17, 20].

The *M.tb* complex contain 90 to 100% DNA homology, while the nucleotide sequence is 99.95% identical [35, 44, 63, 64]. The genome of virulent mycobacteria strains has been shown to be smaller than those of less virulent or avirulent strains [35]. The *M.tb* complex is about 4.4 mega base pair (Mbp) and the genome of *M. leprae* is about 3.3 Mbp [35, 42]. In contrast, the genome of less virulent strains such as *M. smegmatis* is about 7 Mbp [35, 42]. The *M.tb* genome contains approximately 4000 genes which code for bacterial survival within the host (lipolysis) and cellular envelope synthesis (lipogenesis) enzymes [17].

In addition to genome size, rate of growth plays a role in mycobacterial virulence. The *M.tb* species are subdivided into fast-growers and slow-growers; fast-growers are usually non-pathogenic while slow-growers are pathogenic and primarily cause disease in humans [35, 42]. *M.tb*, *M. bovis*, *M. leprae*, *M. ulcerans*, and *M. avium* are slow growing bacteria, taking approximately 24 hours to divide [17, 20]. Within the laboratory, growth requires 3 to 4 weeks for visual observation on solid media; colony appearance is dry and wrinkled [17]. Bacterial growth is one of the ways to determine if an individual is infected and the primary method for diagnosis of drug-resistant TB. This slow growth rate of *M.tb* can limit the initiation of treatment, especially in cases with drug-resistant TB where early initiation of drug therapy is important. Thus, there is currently a need to develop new diagnostic methods for the rapid detection of *M.tb* and especially drug-resistant *M.tb*.

MYCOBACTERIAL REGIONS OF DIFFERENCE

The mycobacterial genome has been shown to consist of 16 regions of difference (RD), or open reading frames. *M.tb*, *M. bovis*, and *M. africanum* along with NTM (*M.*

kansasii, *M. marium*, *M. flavescens*, *M. szulgai*, and *M. smegmatis*) genomes contain the RD1 which is essential for bacterial virulence [65-72]. Other regions of difference that have been identified include the RD2 to RD16 [68, 73]. Deletions of various RD regions have been established in various mycobacterial species. As previously stated, mycobacterial transmission occurred from human to animals; it has been elucidated that these animal-adapted strains initially resulted from the deletion of the RD9 in *M. africanum* subtype 1 (clade 1) [35, 42, 74]. RD deletions from other mycobacterial strains include: *M. africanum* subtype 1 (clade 2), *M. microti*, and *M. pinnipedii* with deletions of RD7-10; and *M. bovis* with deletions in RD4, RD5, RD7-10, RD12, and RD13 [35, 42, 74]. *M. bovis* BCG attenuation results from the deletion of the RD1-15 regions [68, 69, 75, 76], the most important is RD1.

RD1 encodes 9 genes including the early secreted antigenic target of 6 kDa (ESAT-6) and culture filtrate protein of 10 kDa (CFP-10) [65, 68, 77-79]. ESAT-6 and CFP-10 form a tight dimer, known as the ESAT-6:CFP-10 complex, which is important for stability within and secretion from the bacteria [65, 80, 81]. The genomic region surrounding the ESAT-6 and CFP-10 regions encode for the ESAT-6 secretion system (ESX), which is important for secretion of the ESAT-6:CFP-10 complex [65, 67, 82-84]. There are five ESX secretion systems (ESX-1 to ESX-5), though ESX-1 is involved in mycobacterial virulence and cellular evasion [85]. When ESAT-6 and CFP-10 form a dimer, the well-conserved C-terminal residues of CFP-10 are not involved and are left unstructured [65, 81]. Additional factors within the RD1 regions are responsible for binding to the unstructured C-terminal residues of CFP-10 and delivering the complex to the bacterial membrane for secretion [65, 80]. In the cell's cytoplasm, the ESAT-6:CFP-10 complex alters the host immune response to benefit the bacteria and allows the bacteria to persist [86]. Further discussion of the ESAT-6-CFP-10 complex and how it modifies the host immune response will be discussed later.

MYCOBACTERIAL PERSISTENCE & RESUSCITATION

Within the *M.tb* genome, are a group of genes that code for the dormancy (Dos) regulon [61]. These proteins, known as DosR, DosS, and DosT, are responsible for responding to environmental stresses that cause a reduction in oxygen and an increase in nitric oxide (NO) and carbon monoxide levels [61, 87, 88]. DosS has been shown to be a redox sensor, while DosT senses hypoxia; DosR and DosS are induced by NO [61, 89]. DosR and DosS reduce bacterial growth and transition from an aerobic to anaerobic state ensuring bacterial survival by inducing a dormant or persistent state [61, 88, 90]. DosR has also been shown to be critical for bacterial growth after resuscitation from dormancy, allowing the bacteria to rapidly transition from anaerobic to aerobic conditions without compromising viability [88, 91, 92].

Reactivation is typically seen when the host's immune system has been weakened or suppressed; this is commonly seen during HIV infection [91]. Reactivation has been shown to be dependent upon a group of proteins known as the resuscitation promoting factors (rpf) A through E [61, 91, 93, 94]. Though not essential for survival or pathogenesis, rpf proteins are important for the revival of dormant *M.tb* and growth [61, 91, 95, 96]. It is assumed that *M.tb* tightly cross-links the peptidoglycan strands within its cell wall to reduce permeability; it is further thought that rpf proteins hydrolyze these tightly packed peptidoglycan strands, allowing for bacterial growth [61, 91].

PATHOGENESIS

M.tb is an intracellular pathogen, spread from person to person in airborne droplets of 1-5 microns in size [5, 6, 8]. The most common form of TB is pulmonary; although disease can occur in any organ [91, 97, 98]. The bacteria will enter through the respiratory tract and lodge in the alveolar sac where it is initially engulfed by antigen-presenting cells (APCs), alveolar and blood-derived macrophages and dendritic cells (DCs) [4-6, 98-100]. There are three possible outcomes following *M.tb* infection: (i)

spontaneous elimination of the bacteria by the host's immune system, (ii) progression to active disease, commonly seen with immuncompromised individuals, or (iii) containment by the host immune response, known as LTBI (Illustration 2) [97-99, 101].

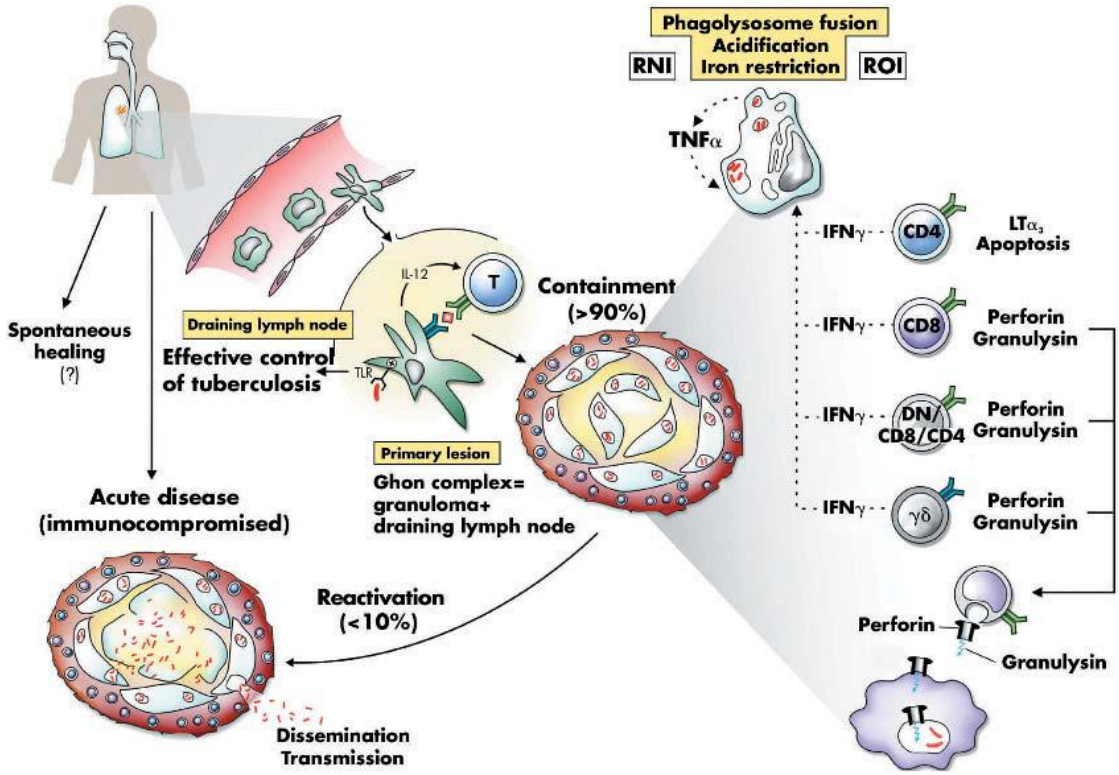


Illustration 2: *Mycobacterium tuberculosis* infection and immune mechanisms of infection [98]

IMMUNITY TO *M.TB*

INNATE IMMUNITY

M.tb primarily resides in macrophages, although it can grow extracellularly [102]. During infection, the activated macrophage has two potential roles: control or killing of the bacteria or providing an environment that allows for bacterial growth [20]. Macrophage activation can occur at two stages of TB infection, during the innate immune

response following initial infection and during the adaptive immune response from interferon-gamma (IFN-γ) produced by *M.tb*-specific T-cell [103, 104].

Various effector functions have been proposed to have antimicrobial activity against *M.tb*, including reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), and phagolysosome fusion. The first effector molecule to be identified was hydrogen peroxide (H_2O_2), a macrophage generated ROI [97, 105, 106]. The role of ROI during *M.tb* infection remains controversial [97, 107-109]. The production of RNI has also been shown as a mechanism of host defense during *M.tb* infection. Though this protective role has primarily been observed in mouse models [107, 110-112], evidence is mounting to show a role in *M.tb*-infected humans [97, 113-115]. Recent studies have elucidated the mechanism of the host defense by RNI may not be elimination of the bacteria but inhibition of its replication [116]. This may inhibit the bacteria enough to allow for other host defense mechanisms to develop and clear the bacteria.

Along with ROI and RNI, antimicrobial activity has been demonstrated with the fusion of the phagosome and lysosome [97]. Mycobacteria undergo phagocytosis to enter macrophages by way of phagosomes [108, 117]. Lysosomes, highly acid vacuoles with hydrolytic enzymes capable of degrading bacteria, fuse with the phagosome forming a phagolysosome [97, 118]. The phagolysosome eliminates the bacteria using these acidic hydrolytic enzymes which degrade the bacteria [97, 119]. *M.tb* has evolved to circumvent phagolysosome fusion, thus apoptosis is believed to be alternative host defense responses under these circumstances [120]. Virulent mycobacteria can inhibit apoptosis and induce necrosis to allow for bacterial spread [120, 121]. However, autophagy has been shown to induce phagolysosomes and inhibit *M.tb* viability [122, 123]. Since several of these host defense mechanisms were performed *in vivo* or *ex vivo* and the role of some host defense mechanisms remain controversial, it is important to validate these mechanisms *in vivo*.

CELL-MEDIATED (ADAPTIVE) IMMUNITY

Following infection in mice, there is a 9 to 10 day delay in T cell activation [124, 125]. At which point some infected APCs - primarily DCs - will migrate to the draining lymph nodes and activate naïve T cells [98, 124-126]. These activated T cells will proliferate and differentiate into effector T cells based on the cytokines secreted [124]. Effector T cells, along with APCs, will migrate from the lymph nodes back to the primary site of infection in the lung 18 to 20 days after infection [98, 124, 126]. Cytokines and chemokines secreted by active cells will recruit additional immune cells to the infection site [97, 117]. Cell-mediated immunity (CMI) is initiated two-to-eight weeks after infection following antigen presentation to T-lymphocytes [102, 117]. At the primary site of infection, antigen-specific $CD4^+$ T cells will continue to proliferate and differentiate into T helper (Th) 1 or Th2 cells [108, 127]. Th1 cells are important for a pro-inflammatory or protective immunity against *M.tb* infection [98, 108]. Th2 cells direct anti-inflammatory responses and during *M.tb* infection can antagonize or inhibit the production or effects of the pro-inflammatory response [108]. The development and expansion of Th1 cells is directed by macrophage-derived interleukin (IL)-12 which is secreted after *M.tb* phagocytosis [97, 108, 128, 129]. Th1 cells produce a variety of cytokines including IFN-γ and tumor necrosis factor-alpha (TNF-α) [99, 108]. IFN-γ, which is primarily produced by $CD4^+$ T cells, but is also secreted by $CD8^+$ T cells and Natural Killer (NK) cells, plays a protective role in *M.tb* infection by activating macrophages to inhibit *M.tb* replication and ultimately eliminate the bacteria [97, 99, 104, 130, 131]. Its protective abilities during *M.tb* infection are evident by IFN-γ knockout mice [108, 130, 132, 133] and individuals with IFN-γ receptor deficiency that are susceptible to mycobacterial infections [108, 134, 135]. IFN-γ also enhances major histocompatibility complex (MHC) class II and co-stimulatory molecule expression on macrophages that allows for improved antigen presentation to T cells [117, 136]. TNF-α,

which is produced by macrophages and DCs, is essential for the control of acute TB infection by activating macrophages, maintaining formation of the granuloma which contains the bacteria and inhibits its replication, and preventing disease reactivation [100, 108, 117, 137-141]. TNF-α is ineffective alone, but when combined with IFN-γ, it can induce antimycobacterial activity through the production of NO and RNI [97, 109, 142].

Th17 cells have also been described in *M.tb* infection; they develop under the influence of IL-6 or IL-21 and low levels of transforming growth factor beta (TGF-β) [143-145]. These cells produce IL-17, which stimulates fibroblasts, epithelial cells, and keratinocytes to secrete additional IL-6 along with IL-8, granulocyte-colony stimulating factor (G-CSF), and granulocyte macrophage-colony stimulating factor (GM-CSF) [143, 146-148]. Additionally, IL-17 is involved in the recruitment of neutrophils and mediating inflammation [143, 147]. The role of IL-17 in *M.tb* infection has not been fully elucidated; although studies have shown that IL-17 deficient mice have a reduced number of neutrophils, do not develop mature granulomas, and fail to control infection [143, 149, 150]. During *M.tb* infection, γδ T cells are the primary producers of IL-17 [143, 151]. It is thought that IL-17 may be involved in early granuloma formation by assisting in neutrophil recruitment [143, 152]. In neutrophil-depleted mice, delayed granuloma formation was observed following *M.tb* infection [143, 152]. It is further thought that this neutrophil accumulation can promote IL-12 induced Th1 differentiation since neutrophil degranulation promotes IL-12 production [143, 153]. On the other hand, the role of neutrophils during later stages of infection remains unclear considering bacterial growth is not controlled in the presence of neutrophils [154, 155]

$CD8^+$ T cells assist in controlling mycobacteria through cytotoxic T lymphocyte (CTL)-mediated killing [156]. This includes lysis of infected macrophages to release intracellular bacteria that can be endocytosed and killed by activated macrophage, direct killing of intracellular mycobacteria, or production of IFN-γ for macrophage activation [97, 100, 156-160]. Lysis of *M.tb*-infected cells occurs through the secretion of perforin,

granzyme B, and granulysin [97, 156, 161]. Perforin causes the formation of pores in the cellular membrane of infected APCs that allows for the influx of granule proteins such as granzyme B and granulysin, a cytotoxic granule-associated protein involved in antimicrobial activity against *M.tb* [97, 100, 156, 159, 160]. Although granulysin-mediated lysis can occur through a perforin-independent mechanism by directly killing extracellular bacteria; killing of intracellular bacteria occurs through perforin-mediated pore formation that allow for the passage of large molecules such as granulysin [156, 160, 162]. In *M.tb* infection, reduced bacterial load was observed with the lysis of infected APCs [97, 136, 159, 163].

It is important to note that a complete understanding of the host immune response to TB is limited by the inability to perform studies within humans, as immune responses may differ from traditional mouse models. The immune response within current mouse models does not allow for the development of LTBI, reactivation, or TB disease as observed in humans. As a result, studies in a human immune system are needed to confirm many of these findings.

GRANULOMA, THE PATHOLOGIC HALLMARK OF HUMAN TUBERCULOSIS

The granuloma is the pathologic hallmark of human TB infection [99]. The pathologic hallmark of an individual to elicit a CMI response able to control and prevent progression of infection is known as a "Ghon complex" [91, 164]. Development of the granuloma has been described as a three-step process that involves: (i) monocytic infiltration, (ii) aggregation, maturation, and mononuclear cell organization, and (iii) epithelioid granuloma development [4, 165]. The granuloma is primarily composed of neutrophils, epithelioid or activated macrophages, DCs, $CD4^+$ and $CD8^+$ T cells, B cells, multi-nucleated giant cells (MGC, Langhans giant cells), and fibroblasts, and serves to isolate the bacteria and prevents its replication [166, 167]. Neutrophils and macrophages are responsible for initial granuloma formation [166, 168] which has been suggested to be

mediated by IL-17 from γδ T cells and possibly Th17 cells [150, 167]. Early in infection, neutrophils may also be involved in controlling infection through the recruitment of leukocytes and possibly by neutrophil extracellular traps [91, 166, 169, 170]. Macrophages recruited to the site of infection are activated to control *M.tb* through mechanisms previously described [165, 166, 171]. Activated macrophages develop elongated nuclei and tightly bind their cell membranes together to form a barrier to prevent bacterial dissemination [91]. Macrophages can also fuse to form MGC, which is characteristic of granulomas in TB infection [165, 166]. To maintain control of infection, the granuloma is not believed to be inactive but instead in a continuous state of mononuclear cell death, recruitment, and replacement [20, 165].

Granulomas are found in individuals with LTBI and active TB disease. Various types of granulomas with different microenvironments exist to either restrict or promote bacterial growth. The granuloma's ability to promote *M.tb* growth is evident in individuals with LTBI that are able to reactivate after several years. The most common granuloma type is a caseous granuloma, named so because of its "cheese-like" appearance in the center [166, 171]. Caseous granulomas consist of an acellular necrotic center surrounded by epithelioid macrophages and a lymphocytic cuff containing T and B cells sometimes enclosed by a fibrotic layer [171, 172]. Caseous granulomas also contain neutrophils [171]. During LTBI, caseous granulomas can become calcified, also known as solid granulomas, consisting of few immune cells and relatively few bacteria thought to be in a dormant stage with low metabolic activity and little to no replication; these granulomas represent successful control of TB [92, 171, 172]. During active TB disease, caseous granulomas can have centers that become liquefied leading to bronchial erosion and the formation of a cavity [92, 101]. These types of granulomas can contain high numbers of bacilli and the caseous material appears to provide the bacteria with nutrients required for growth; ultimately, disintegration of the granuloma structure allows for release of the bacteria, permitting dissemination and transmission [92, 172, 173]. Caseous

granulomas are thought to arise from necrotic granulomas which are seen early during active TB disease [92]. Necrosis is regarded as a "crossroad" whereby the granuloma can resolve itself by becoming fibrotic and calcify to prevent bacterial dissemination and transmission, or alternatively expand and break into the bronchus to produce a cavity allowing for bacterial dissemination [102]. Necrotic granulomas are well-defined structures with a necrotic center that is made up of cell debris and this debris can provide the oxygen needed for the mycobacterial resuscitation and replication [92].

Various other types of granulomas have also been described during TB infection such as non-necrotic granulomas, necrotic neutrophilic granulomas, and completely fibrotic granulomas [171]. Non-necrotic granulomas, commonly seen in active TB disease, mostly consist of macrophages and a few lymphocytes [171, 174]. Fibrotic granulomas, mainly found during LTBI but can occur in active disease, primarily consist of fibroblasts and a few macrophages [171, 174]. Localization of the bacteria varies depending on the type of granuloma. Necrotic granulomas have bacteria localized within the lymphocytic cuff [175, 176], while bacteria in non-necrotic granulomas are found throughout the lesion [176].

The various types of granulomas and microenvironments that have been identified, to date, demonstrate the possibility that unidentified forms of granulomas may exist. With the development of antibiotics which alters the immune response to protect the host and eliminate *M.tb*, information regarding the natural progression of *M.tb* disease and granuloma structure will be lost. This is demonstrated in current studies that are performed using either autopsy samples from individuals whose response may be confounded by treatment or mouse models who response may not adequately replicate the human response.

MYCOBACTERIAL VIRULENCE & EVASION

As previously stated, RD1 encoding the ESX system is involved in mycobacterial virulence. Similar to CFP-10, ESAT-6 has an unstructured region on the C-terminus that can bind the toll-like receptor (TLR)-2 on macrophages and inhibit TLR-signaling; this subsequently inhibits pro-inflammatory factors and nuclear factor κB (NF-κB) [80, 86, 177]. Macrophages will secrete anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β [136, 178-183]. IL-4 suppresses the production of IFN-γ and reduces macrophage activation [108, 184, 185]. IL-10 down-regulates pro-inflammatory cytokines, specifically IFN-γ, TNF-α, and IL-12 [108, 186]. TGF-β reduces CMI by inhibiting antigen presentation, pro-inflammatory cytokine production, and activation in macrophages; it also inhibits T-cell proliferation and IFN-γ production [108, 187]. TGF-β also induces IL-10 which together suppresses the production of IFN-γ [108, 188]. In addition, binding of ESAT-6 can also induce apoptosis through the formation of pores on macrophages and *M.tb*-specific T cells [86, 136, 189, 190]. CFP-10 can also bind the macrophage surface and modulate the cell's behavior by reducing ROS levels through the breakdown of H_2O_2 to produce water [81, 86, 191]. Within the macrophage, ROS production is decreased by ESAT-6, CFP-10, and the ESAT-6:CFP-10 complex, although ROS was reduced to a greater extent by the ESAT-6:CFP-10 complex, this inhibits NF-κB activation [86, 192].

The ESAT-6-CFP-10 complex has been shown to inhibit phagosome-lysosome maturation within the macrophage [85, 193, 194]. Under acidic conditions, which are typically found in the phagosome, ESAT-6 separates from CFP-10 and interacts with and lyses the lysosome [86, 195]. This allows the bacteria to escape possible degradation within the phagolysosome and persist within the host. Given the conserved region of the RD1 region within virulent *M.tb* strains, ESAT-6 and CFP-10 have been potential targets

for vaccine development; they are also targets for diagnostic tools in identifying individuals infected with virulent mycobacterial strains.

M.tb also interferes with macrophage MHC class II antigen processing and presentation molecules [91, 136, 196]. For classical antigen presentation, MHC class II molecules are synthesized in the endoplasmic reticulum (ER) and then targeted to the endocytic pathway for processing into a MHC class molecule that is associated to a class II-associated invariant chain peptide (CLIP) [196, 197]. CLIP is subsequently removed by a MHC-encoded molecule known as HLA-DM and replaced by a processed antigen which is transferred to the cell surface for presentation to $CD4^+$ T cells [196, 198, 199]. Antigen processing can also occur through phagosomes via proteolytic mechanisms as shown by the processing of antigens from *Escherichia coli* and *Salmonella* Typhimurium [196, 200, 201]. During *M.tb* infection, MHC class II molecules have been found within phagosomes [196, 202]. With the ability of *M.tb* to prevent phagosome-lysosome fusion, it is thought that antigen processing and loading onto MHC class II molecules may be inhibited subsequently preventing presentation to $CD4^+$ T cells [196]. Antigen processing may be inhibited by the reduced acidic environment or inhibited lysosomal proteases [196]. It has been shown that *M.tb* inhibits antigen processing by decreasing MHC class II mRNA and mouse HLA-DM (H2-DM) expression [203]. Antigen presentation can be reduced by *M.tb* 19-kDa lipoprotein which is involved in inhibiting the expression of MHC class II and HLA-DR [91, 136, 204-206]. Furthermore, the 19-kDa lipoprotein can inhibit IFN-γ induced MHC class II and class II transactivator (CIITA) expression, a transcription regulator needed for the production of MHC class II genes [91, 207, 208]. Given the ability of *M.tb* to inhibit these IFN-γ-driven macrophage responses, it may be possible that other IFN-γ-mediated host defenses are also being altered during infection. Understanding these and other mechanisms of *M.tb* evasion will ultimately advance the development of vaccines and therapeutics by identifying and targeting critical virulence mechanisms.

Human Immunodeficiency Virus

EPIDEMIOLOGY

In 2011, there were approximately 34 million people living with human immunodeficiency virus (HIV) and about 2.5 million new cases worldwide [209, 210]. Approximately 69% of individuals living with HIV are located in sub-Saharan Africa [210]. If untreated, HIV can lead to acquired immune deficiency syndrome (AIDS); it can take 10-15 years for an HIV-positive individual to develop AIDS [209]. Worldwide, AIDS is one of the world's most serious health problems and the leading cause of death from an infectious disease [1]. In 2011 there were approximately 1.7 million AIDS-related deaths [1, 209, 210]. AIDS associated immune deficiency increases the risk of acquiring opportunistic infections (OIs), which are the primary cause of morbidity and mortality in immunosuppressed populations [211]. It is evident that HIV is a major health problem and these statistics demonstrate that new antivirals and vaccines are rapidly needed to reduce HIV infection and prevent AIDS development. Moreover, the risk of OIs has become a serious concern in HIV-infected individuals, especially when considering treatment options.

HISTORY OF HIV

In the United States, HIV was first recognized in 1981 when five previously healthy homosexual men in California presented with *Pneumocystis carinii* pneumonia (PCP), of which two died [212]. Since PCP is primarily found in immunosuppressed individuals, and the patients in California did not have an underlying immunodeficiency, the cases were considered unusual [212, 213]. Subsequent reports described cases of individuals with other OIs and Kaposi sarcoma (KS), which is life-threatening [213, 214]. Severe immune system suppression was the main factor for the unknown disease and as a consequence individuals were predisposed to OIs such as KS [213]. A case-control study

in early 1982 revealed that gay men with KS or OIs were more sexually active and more likely to have other sexually transmitted diseases than control gay men; thus the disease was initially considered to be associated with a homosexual lifestyle [213, 215, 216]. Additional studies reported a sexual link between PCP patients and another KS or PCP patient within 5 years of symptom onset [213, 217, 218]. These studies strongly suggested that the unknown disease was a sexually transmitted infectious disease [213]. Within months, immunodeficiency and OIs were also identified in heterosexual individuals that were infected through unprotected sexual intercourse [219, 220], through the transfusion of contaminated blood [221, 222], by sharing contaminated needles, and from mother to child – either during pregnancy, birth, or breastfeeding [213, 223, 224].

Illustration 3: Adults and children estimated to be living with HIV, 2011 [210]

In 1983, Luc Montagnier at the Pasteur Institute in Paris isolated a retrovirus that belonged to the human T cell leukemia viruses (HTLV) family, from an asymptomatic patient [225, 226]. The retrovirus was named lymphadenopathy-associated virus (LAV) and, unlike other leukemia viruses, was cytopathic and displayed a specific characteristic

to kill $CD4^+$ T cells in PBMCs [225, 227]. Robert Gallo at the National Institutes of Health subsequently called the retrovirus HTLV-III and demonstrated that the virus was linked to immunodeficiency in high-risk individuals [225, 228]. Jay Levy at the University of California in San Francisco referred to the retrovirus as AIDS-associated retrovirus (ARV) [225, 229]. ARV was isolated from high-risk AIDS patients and high-risk health individuals; it was determined that infection could be symptomatic and asymptomatic [225, 229]. The virus was subsequently named human immunodeficiency virus because of its similar morphology and genetic characteristics to the *Lentivirus genus* [225, 230].

HIV TYPES AND CLASSIFICATION

HIV-1

There are two types of HIV: HIV-1 and HIV-2 [231, 232]. HIV-1 is found throughout the world and is primarily responsible for AIDS [232]. It has been suggested through retrospective studies and serological evidence that the AIDS epidemic began in west-central Africa in the 1970's [230, 231, 233]. It is thought that HIV-1 evolved from the transmission of simian immunodeficiency virus (SIV) that infects chimpanzees (SIV_{cpz}) [234, 235] and western gorillas (SIV_{gor}) [225, 231, 236].

There are various distinct HIV-1 subtypes which have been classified into the major (M), outlier (O), or non-M or non-O (N) group [231, 234, 237-239]. Group M contains eight separate clades or subtypes (A, B, C, D, F, G, H, and J) and contains over 95% of the HIV-1 isolates around the world [225, 231, 240, 241]. All HIV-1 subtypes are found in sub-Saharan Africa [225]. Globally, subtype C is the most prevalent HIV-1, accounting for 48% of infections, it is commonly found in southern Africa and India; subtype A accounts for 12% of infections and is primarily found in eastern Europe; subtype G accounts for 5% of infections and subtype D accounts for 2% of infections; 22% of infections are from recombinant strains of HIV-1 [225, 231]. Subtype B, which is

commonly found in North and South America and Western Europe, causes 11% of HIV-1 cases [225, 231]. HIV-1 isolates from group O are rarely found and have been isolated from patients in Cameroon, Gabon and Equatorial Guinea [225, 231, 237, 238]. Group N HIV-1 was identified in a few patients [225, 231, 239]. More recently, a putative (P) group was also isolated from patients in Cameroon [231, 242, 243]. The genetic variation and continued identification of new subtypes among HIV-1 remains a major obstacle in the development of a new vaccine, thus an HIV vaccine would need to be effective enough to target all or, at least, several HIV-1 subtypes.

Several HIV-1 isolates exist and are classified according to their tropisms, replicative, and cytopathic properties as shown in Table 1 [225]. The cellular tropism of HIV-1 determines its host cell; T-tropic isolates replicate and establish infection within $CD4^+$ T cells, M-tropic isolates replicate in macrophages, and dual tropic isolates are able to infect macrophages and $CD4^+$ T cells [225, 244, 245]. M-tropic viruses are more commonly isolated from patients early during infection and during asymptomatic periods; while a shift to T-tropic viruses during late infection is associated with $CD4^+$ T cells decline and progression to AIDS [246]. HIV-1 isolates are described as either "slow/low" or "rapid/high" [225, 247]. Based on their *in vitro* replicative ability, isolates that replicate faster and generate high levels of viral particles are "rapid/high", while "slow/low" isolates replicate slowly and generate lower levels of viral particles; most HIV-1 isolates are designated "slow/low" [225, 247]. HIV-1 isolates are also classified according to their ability to induce syncytia formation (SI, syncytium-inducing) [225, 248]. Syncytia formation involves the fusion of infected cells with infected and uninfected cells [249] and has been shown to be a predictor of disease progression to AIDS [250, 251]. Typically, T-tropic isolates are characterized by SI with "rapid/high" replication while M-tropic isolates are non-syncytium-inducing (NSI) with "slow/low" replication; dual tropic isolates are commonly SI and have "rapid/high" replication [225, 252].

Cellular Target	Co-receptor	Replication	Syncytium formation
T cell	CXCR4	Rapid/High	Inducing
Macrophage	CCR5	Slow/Low	Non-inducing
Dual	CXCR4/CCR5	Rapid/High	Inducing

Table 1: HIV Classification

Since the discovery of co-receptors CXCR4 [253] or CCR5 [254] for viral entry into host cells, HIV-1 is commonly classified based on receptor ligation [225]. T-tropic HIV-1 isolates that are SI forming and "rapid/high" replicating use the CXCR4 co-receptor, they are also designating X4 viruses; M-tropic HIV-1 isolates that are NSI forming and "slow-low" replicating use the CCR5 co-receptor, these are also designating R5 viruses [225, 255]. Dual tropic HIV-1 isolates that are able to infect T-cell and monocytes can use either the CXCR4 or CCR5 co-receptor for entry into the host cell [225, 256]. However, M-tropic strains are able to use CXCR4, though less common, on macrophage for entry [257].

HIV-2

HIV-2 was initially found in Western Africa and infections are predominantly observed in patients that reside in West African countries such as Guinea-Bissau, The Gambia, Senegal, Cape Verde, and Cote d'Ivoire [225, 258]. However, over time HIV-2 has spread throughout Africa, into Europe, India, and the United States [232]. It is believed that HIV-2 evolved from a SIV that infects the sooty mangabey monkey (SIV_{sm}) [231, 259, 260]. HIV-2 also has distinct subtypes that have been assigned to groups A-H [261-263]. The most prevalent and only pathogenic subtypes are A and B [261]. HIV-2 is less pathogenic than HIV-1 [232, 261], infected patients remain asymptomatic for longer

periods of time [232, 264], and have lower viral load than patients with HIV-1 [232, 265]. Overall, the rising incidence of, and prolonged infection of, HIV-2 emphasizes the need for new diagnostics able to provide early identification of HIV-2 infected individuals. The slower progression to AIDS compared to HIV-1 may benefit the host by allowing for the initiation of antiretroviral therapy (ART) before disease progression and immune dysregulation. Overall, the development of a vaccine able to induce immune memory against conserved epitopes of both HIV-1 and HIV-2 genomes would prove highly beneficial in reducing or eliminating the continuously increasing burden of HIV infections.

GENOMIC STRUCTURE

HIV belongs to the *Lentivirus* genus of the *Retroviridae* family [249, 266, 267]. It consists of a truncated cone shaped core with two copies of the single-stranded RNA genome and the reverse transcriptase enzyme [249]. Reverse transcriptase, a characteristic of retroviruses, transcribes viral RNA into DNA [266]. The core is enclosed by a protein matrix which is surrounded by a lipid envelope that contains the envelope glycoprotein 160 (gp160); gp160 includes external surface protein (SU, gp120) and transmembrane protein (TM, gp41) [249]. The HIV viral genome and structure is shown in Illustration 4.

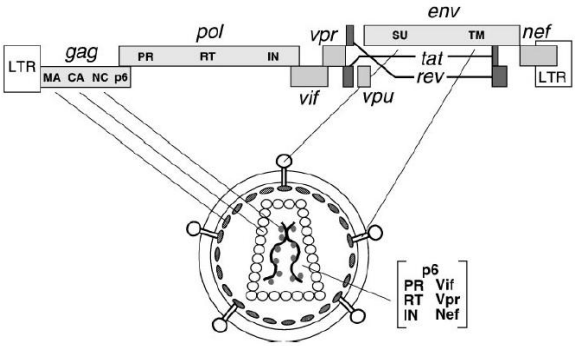


Illustration 4: HIV-1 Viral Genome and Structure [268]

HIV has a 10 kilobase genome that encodes 15 proteins grouped into structural and regulatory classes of proteins [268]. The viral core and outer membrane are composed of structural proteins Gag, Env, and Pol [267, 268]. These proteins are further proteolyzed to make individual proteins: Gag produces matrix (MA), capsid (CA), nucleocapsid (NC), and p6, while Env produces surface (SU, gp120) and transmembrane (TM, gp41) proteins that make up the outer membrane envelope [268]. Structural protein Pol produces protease (PR), reverse transcriptase (RT), and integrase (IN) which are encapsulated within the virus particle and provide enzymatic functions [268]. In addition, there are six accessory proteins that include transcriptional transactivator (Tat), regulator of virion gene expression (Rev), negative effector (Nef), viral infectivity factor (Vif), viral protein r (Vpr), and viral protein u (Vpu) [267, 268]. Proteins Tat and Rev are regulatory genes; while Nef, Vif, Vpr, are located within the viral particle [268]. The precise role of each protein in viral replication and pathogenesis will be discusses in detail below.

VIRAL REPLICATION

HIV enters the host cell via the CD4 receptor and a secondary receptor, either CXCR4 or CCR5 as previously described [225, 249, 269]. HIV primarily targets human $CD4^+$ T cells, although the CD4 receptor is also found on activated monocytes, macrophages, and DCs; thus these cell types can also be infected [225, 249]. HIV infection begins with binding of the viral surface protein gp120 to the CD4 receptor and co-receptor on the target cell [225, 268]. A conformational change occurs with TM gp41 causing the viral membrane and cellular membrane to fuse, this allows for the virion core to enter the cellular cytoplasm [225, 249, 268]. The core of the virion undergoes partial uncoating releasing subviral particles MA, RT, IN, Vpr, and the viral RNA into the cytoplasm [225, 249, 268]. Reverse transcription of the viral RNA genome by RT is initiated resulting in double-stranded DNA that is transported into the nucleus of the host

cell where it is integrated by IN into the host chromosomal DNA [225, 249, 268]. Subsequent transcription of viral DNA by DNA-dependent RNA polymerase II (Pol II) leads to viral mRNA production. HIV transcription begins at the promoter site located on the long terminal repeat (LTR), transcription is enhanced by the Tat protein which recruits human positive transcription elongation factor b (p-TEFb) to phosphorylate the RNA polymerase II C-terminal domain CTD; phosphorylation of CTD is essential for the activation of elongation during transcription [225, 268, 270]. Viral protein Rev transports viral mRNA to the cytoplasm where it is translated into viral proteins and prepared for release [225, 268]. Within the ER, Env mRNA is translated then transported to the cell membrane [225, 268]. Polyproteins Gag and Gag-Pol are transported to the plasma membrane to assemble the viral core along with Vif, Vpr, Nef, and the viral RNA [225, 268]. The immature virus buds from the host cell membrane and maturation occurs, this includes processing of Gag and Gag-Pol into MA, CA, NC, p6, PR, RT, and IN by PR which produces the cone shaped core [225, 268]. This process generates a mature virion that is able to infect other cells as shown in Illustration 5 [225, 268].

HIV has high genetic variability given its low proof-reading abilities and high mutations rates [271, 272]. Nonetheless, it does contain highly conserved regions that encode for essential pathogenic functions [272] such as host cell binding, protein synthesis, replication, viral assembly, and release from the host membrane. The field of vaccine development may benefit from the various proteins by providing multiple antigen targets for an effective immune response. However, the constant error in replication is a major hurdle in the development of a HIV vaccine because the genetic sequence of the viral genome changes preventing the host from developing an effective antigen-specific response.

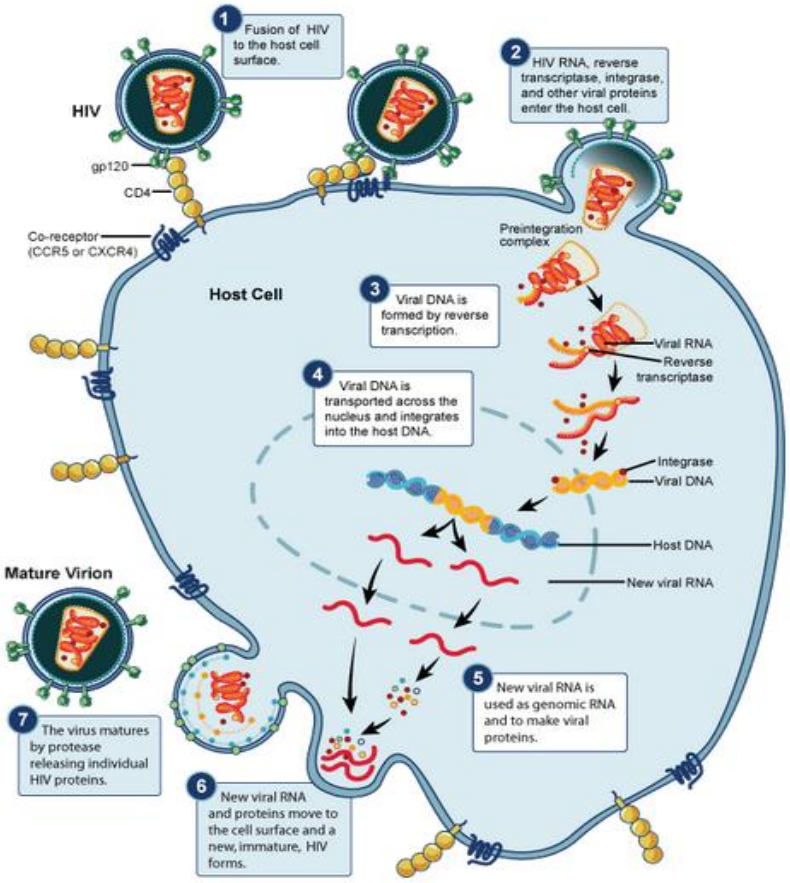


Illustration 5: HIV Replication [273]

HIV Pathogenesis and Immune Dysregulation

The hallmark of HIV infection is progressive depletion of $CD4^+$ T cells; as a result, immune dysregulation and opportunistic infections occur [225, 227]. Since its emergence in the early 1980, HIV infection was characterized clinically as a persistent decrease in helper/inducer T lymphocyte subset number and function, and suppressor/cytotoxic subset activation [274, 275]. Prior to this reduction and early during HIV infection, $CD4^+$ T cell immune responses are affected including reduced proliferation and altered cytokine production [274, 276]. Furthermore, since HIV can infect macrophages innate immune responses are also dysregulated [274].

HIV DISRUPTION OF CD4$^+$ T-LYMPHOCYTES

Though HIV infection is characterized by CD4$^+$ T cell depletion, functional impairment of T-lymphocytes may occur prior to this decline resulting in immune evasion and pathogenesis [4, 277, 278]. As HIV progresses, Th1 cytokine production decreases and there is a shift in CD4$^+$ T cells to express a Th2 immune response that includes production of IL-4 and IL-10 [279, 280]. Produced by monocytes and CD4$^+$ T regulatory cells (T-regs), IL-10 promotes a Th2 response by down-regulating Th1 cytokines [279, 280]. Similarly, IL-4, produced by activated CD4$^+$ T cells and NK cells [257] has been implicated in suppressing the production of Th1 cytokines including IL-2 [280], TNF-α, and IL-1β [279]. The effects of IL-4 and IL-10 are discussed in regard to their effects on monocytes/macrophages later.

This Th1 to Th2 switch has been reported to be mediated by several HIV viral proteins. HIV viral proteins Tat and Vpr are able to inhibit IL-12 production [249, 267, 281, 282]. Tat along with Env is also able to stimulate the secretion of IL-10 [249, 274, 280]. Tat along with Env also inhibits IL-2 production and IL-2 surface receptor expression which can affect T cell activation and proliferation, respectively [4, 280, 283, 284]. On the other hand, Vpr can induce pro-inflammatory cytokine TNF-α which was shown to increase HIV replication [267, 285]. In addition, Vpr activates transcription factors NF-kappaB and IL-1-inducible nuclear factor (NF-IL-6) in T cells and macrophages to induce the expression of IL-8 [267, 286]. Activation of NF-κB and NF-IL-6 may promote HIV transcription while IL-8 production may contribute to HIV dissemination by attracting and infecting APCs at the site; these newly infected cells would then migrate to the draining lymph node and enter the peripheral blood to spread to other organs [286].

HIV viral proteins can also inhibit the expression of molecules involved in eliciting a protective immune response. Viral proteins Vpu and Nef down-regulate

expression of the CD4 T cell receptor [267, 268]. Similar to CD4 molecules, HIV Env is synthesized in the ER, CD4 and Env bind to form a CD4-Env complex that is unable to transport to the cell surface, in this way Env also inhibits surface expression of the CD4 molecule [267, 287, 288]. Vpu prevents CD4-Env formation by binding to the cytoplasmic CTD of CD4 [268, 289], targeting CD4 for ubiquitin-mediated proteasomal degradation [267, 290], and allowing Env to traffic to the cell surface [267, 291]. Nef down-regulates CD4 surface expression by interacting with the cytoplasmic end of CD4 then promoting endocytosis [267, 292]. CD4 is targeted for degradation via the lysosomal pathway [267, 293, 294]. Nef also down-regulates the CD4 molecule by inhibiting its exit from the Golgi complex [267, 292, 295] and possibly targeting it for degradation. In these ways, Vpu and Nef enhance viral release and subsequent cell surface binding on uninfected cells since the CD4 receptor within the virus would inhibit its release and infectivity [267, 296-298]. HIV protein Tat causes $CD4^+$ T cell depletion by reducing $CD4^+$ T cell proliferation which is mediated by the up-regulatation of immunosuppressive cytokine TGF-β [267, 299]. Considering down-regulation of the CD4 molecule by viral proteins requires a conserved site for binding, drug compounds could be aimed at binding to the conserved region of the viral proteins (Vpu, Nef) which would prevent its binding to the CD4 molecule. This would allow CD4 to translocate to the cell surface allowing for antigen presentation; in the presence of Env, it could inhibit release of the virion or prevent the released virion from binding to uninfected cells. Similarly, conserved binding sites on the viral proteins (Vpu, Nef, Env) could provide target epitopes for the development of antigen-specific T cells when developing a HIV vaccine.

HIV-INDUCED $CD4^+$ T-DEPLETION

HIV viral proteins can induce $CD4^+$ T cells death in infected cells through several mechanisms such as syncytium formation, membrane permeability, and apoptosis. HIV-

infected cells can undergo syncytium formation which has been associated with increased $CD4^+$ T cell death and disease progression [251, 300]. HIV viral protein Vpu can cause membrane permeability through the formation of ion channels in the lipid bilayer that can lead to cell death [248, 301]. Vpr induces apoptosis by permeabilizing the mitochondrial membrane which activates the cysteine-dependent aspartate-specific protease (caspase) pathway [267, 302-304]. Alternatively, Vpr has been shown to inhibit apoptosis which is possibly dependent on its level of expression and the stage of infection [267, 302, 305]. Apoptosis can also occur following prolonged Vpr-mediated cell cycle arrest [302, 306] resulting from the depletion of the cell cycle regulatory kinase, Wee1 [267, 307].

Similar to Vpr, HIV viral proteins Vpu, Nef, Tat, and Env can induce apoptosis. Vpu can induce apoptosis of $CD4^+$ T cells by inhibiting nuclear factor-kappa B (NF-κB) which down-regulates anti-apoptotic molecules and activates pro-apoptotic molecules, specifically caspase 3 [267, 308]. Nef, Tat, and Env can induce Fas-mediated apoptosis [248, 267]. Nef up-regulates surface expression of Fas (CD95) and FasL (CD95L) on the surface of infected cells which promotes uninfected bystander cell apoptosis [267, 309, 310]; simultaneously, Nef prevents apoptosis in the infected cell by inhibiting apoptosis signal-regulating kinase 1 (ASK1), an intermediate molecule in Fas and TNF-alpha-mediated apoptosis [267, 311]. Viral protein Tat also up-regulates CD95L in HIV-infected T cells [267, 312] and tumor necrosis factor-related apoptosis-induced ligand (TRAIL) in HIV-infected and uninfected macrophages [267, 313] to stimulate apoptosis in uninfected bystander T cells. As previously mentioned, CD4 and Env are able to bind and form a complex in the ER. At the cell surface, Env can induce CD4 molecule cross-linking that up-regulates CD95 expression on uninfected T cells inducing apoptosis [248, 314].

Viral proteins released into the extracellular environment can also trigger uninfected cells to undergo apoptosis or activation induced cell death (AICD) [248]. Following release from the infected cell Env, Nef, Tat, and Vpr are able to enter

uninfected cells and induce apoptosis [248]. Secreted Env induces apoptosis through AICD by cross-linking the CD4 receptor and inducing partial T cell activation [248, 315]. Nef can induce cell death of uninfected cells by inserting into the plasma membrane causing cytolysis [248, 316]. Tat can be endocytosed by uninfected cells to induce apoptosis through mechanisms previously described [248, 317]. While Vpr can induce apoptosis by penetrating uninfected cells, localizing to the nucleus, and causing DNA fragmentation [248, 318].

CYTOTOXIC CD8$^+$ T CELLS CONTROL HIV INFECTION

With the dysregulation and depletion of CD4$^+$ T cells as just described, CD8$^+$ T cells are seen as a form of protective immunity during HIV infection. During acute infection, the Th1 immune response produces IFN-γ which activates CD8$^+$ T cells [279]. CD8$^+$ T cells are important for the control of viremia through CTL-mediated lysis of HIV-infected cells [274, 319-321]. CD8$^+$ T cells kill infected cells through perforin-mediated or FasL-mediated cell death [279]. In seropositive asymptomatic individuals with high CD4$^+$ T cell levels (termed long-term non-progressors) [321-324] or seropositive individuals able to maintain low (virus controllers) [321, 325] or undetectable (elite controllers) [321, 326] viral replication, CD8$^+$ T cells have been shown to elicit an effective immune response early in infection to control viral replication [321]. The HIV-specific CD8$^+$ T cell response coincides with viremia peak which is followed by viral control [320]. During chronic infection, this CTL response is decreased but measurable suggesting its continuous role in protection [320].

Epitope-specific CD8$^+$ T cell responses can direct antiviral responses. Several studies have demonstrated low viremia is associated with Gag-specific responses during chronic HIV and SIV infection [321, 327-329]. Epitopes within Pol [321, 330], Vpr [321, 330, 331], and Rev [321, 331] have also been detected and elicit an antiviral response immediately after infection [321]. Gag-specific CD8$^+$ T cells have the strongest anti-

retroviral response and viral load was the lowest [321, 329]. It is also thought that Nef-specific $CD8^+$ T cells are produced since mutations can develop in Nef epitopes, mutations can render the viral protein able to reduce or eliminate recognition [332, 333]. Mutations have also been identified in Gag epitopes recognized by $CD8^+$ T cells [321, 334].

A vast array of effector responses are elicited by antigen-specific $CD8^+$ T cells during HIV infection to inhibit viral replication [321]. $CD8^+$ T cells in virus controllers and individuals vaccinated against Env and Gag-Pol expressed macrophage-inflammatory protein 1 beta (MIP-1β) and CD107 [321, 335, 336]. Virus controllers also produced higher MIP-1α and IFN-γ than elite controllers [321, 337]. MIP-1β is involved in antiviral responses, through a mechanism yet to be determined; while CD107 is used to measure cytotoxic degranulation [321, 335]. Increased perforin was observed in elite controllers with a correlation between perforin-producing cells and viral load [321, 337]. Furthermore, elite and virus controllers expressed higher IL-2, TNF-α, and IFN-γ than progressive patients [321, 337].

The ability to develop antigen-specific CD8+ T cells lends to the possibility of generating a vaccine against the various viral epitopes. Antigen-specific $CD8^+$ T cell development could provide an immediate CTL-mediate immune response that hinders HIV disease progression until ART can be initiated, and subsequently these responses can aid in eliminating the virus.

MONOCYTES/MACROPHAGES

A protective immune response against infection requires the activation of T cells by APCs, such as macrophages [249]. Unlike T cells, macrophages are able to become infected when they are not activated; although replication of HIV occurs slower in macrophages [249, 338].

HIV viral proteins have been identified in modifying the host's immune response to infection. It has been shown that $CD8^+$ cytotoxic T cells are able to kill HIV-infected macrophages [339, 340]. Since HIV does not appear to target $CD8^+$ T cells directly, CTL responses may be inhibited by other mechanisms such as preventing antigen presentation. Vpu and Nef down-regulate MHC class I surface expression to inhibit antigen presentation to $CD8^+$ T cells [267, 268]. Vpu retains the MHC class I molecules in the ER [267, 341], while Nef can either retain these molecules in the Golgi complex or bind to the cytoplasmic end and internalize the MHC molecule, targeting it to endosomes for degradation [267, 342, 343]. Similarly, Tat can prevent antigen presentation to $CD4^+$ T cell by inhibiting the expression of MHC class II molecules [4, 344]. Ultimately, the inability of MHC class I molecules to present antigen to $CD8^+$ T cells prevents the CTL immune response from developing; it is possible that this response is important in protecting the host to ward off the detrimental effects of HIV infection.

Macrophages are able to promote viral infection by recruiting target T cells to sites of infection [339, 345]. It has been demonstrated that the virus can be transmitted from infected macrophages to uninfected $CD4^+$ T cells through a "virological synapse" during cell-to-cell contact [339, 346, 347]. HIV proteins Gag and Env on the macrophage surface attach to the T cell via the CD4 receptor, allowing for HIV transmission and infection of the $CD4^+$ T cell [346, 348]. In effect, macrophages may be involved in viral dissemination. Considering HIV is typically transmitted via the genital mucosal barrier, migration to the lymph nodes is not common for macrophages residing at the mucosal site [339]. Circulating monocytes are recruited to the site of infection and differentiate into macrophages [339]. Following infection, macrophages will migrate to the mesenteric lymph nodes, possibly infecting T cells and entering the peripheral blood to disseminate throughout the body [339]. Similarly, a compromised epithelial barrier can allow virus to enter the bloodstream, both of which lead to increased viral load in the plasma [339, 349, 350].

The Th1 to Th2 switch, generally observed during HIV infection, can affect the functions of macrophages and other APCs. Early in infection, viral replication is suppressed by IL-10 secreted from macrophages [351-353] which is also associated with the inhibition of IL-6 and TNF-α, both of which are known to increase viral replication [257, 354]. However, disease progression is still promoted by its ability to up-regulate CCR5 co-receptors that mediates HIV entry into the host macrophage [249, 351, 355, 356]. At the latest stage of HIV infection, IL-10 deactivates the macrophage which is proposed to result in failure of the immune response [351]. Alternatively, IL-4 is a multifunctional cytokine able to stimulate or inhibit viral replication in macrophages [257, 351]. This effect is dependent upon the stage of macrophage differentiation with up-regulated replication in primary human monocytes [257, 351, 357, 358], while replication was inhibited in differentiated macrophages [257, 351, 359]. Similar to IL-10, IL-4 can suppress TNF-α along with IL-1β which can up-regulate viral replication [279, 352]. The effects of IL-4 may coincide with disease stage; during HIV disease progression, IL-4 stimulates viral replication, although during chronic HIV infection, IL-4 inhibits viral replication. Late in HIV disease, IL-4 levels decline [280], this may possibly coincide with IL-10-mediated macrophage deactivation and immune collapse associated with AIDS development. Understanding how HIV skews the cytokine immune response can aid in the development of therapeutics to support the host's T cell response and possibly limit the virus' pathogenic effect.

HIV/M.tb Co-infection

EPIDEMIOLOGY

TB is the most common co-infection and leading cause of death among HIV-positive individuals [360]. Worldwide, approximately 1.1 million people with TB were

HIV-positive and there was an estimated 400,000 HIV-associated TB deaths in 2011 [1]. Considered to have a 'deadly synergy', HIV infection promotes TB disease while TB promotes HIV progression [5]. Whereas HIV-negative individuals have a 10% risk of developing active TB disease within their lifetime, HIV-positive individuals have a 5-15% annual risk of reactivation of latent TB infection [4, 5, 7]. HIV-positive individuals are also at a 20% greater risk of developing new *M.tb* infections or developing active disease following *M.tb* exposure compared to HIV-negative individuals [4]. Furthermore, HIV-positive individuals are more likely to develop extrapulmonary and disseminated TB disease [4, 361].

The misuse of antibiotics and mismanagement of therapy has resulted in the rising incidence of multi-, extensively, and totally drug-resistant TB [1] which will be exacerbated the HIV/*M.tb* epidemic. In combination with HIV infection, the availability of TB treatment measures becomes limited and death is inevitable [362-364]. When considering the development of new drug compounds for co-infection treatment, it is important to understand the limitations of current regimens, specifically non-compliance associated with adverse side effects, high pill burden, drug interactions, and immune reconstitution inflammatory syndrome (IRIS) [365]. It is evident that vaccines and therapeutics are urgently needed to counter the rising burden of HIV/*M.tb* co-infection.

HIV/*M.TB* CO-INFECTION PATHOGENESIS

In general, the risk of TB infection or reactivation is greater in HIV-positive individuals than HIV-negative individuals [366, 367] Several studies have established a role for the interference of HIV in the immune response against *M.tb* including depletion of antigen-specific lymphocytes, reduced phagocytosis, and decreased Th1 immune response [368-376]. TB susceptibility is inversely correlated with $CD4^+$ T cell levels. Compared with HIV positive patients that have >500 CD4 T cells/μl of blood, HIV-positive patients with CD4 T cell counts <200 CD4 T cells/μl of blood (clinically

regarded as having AIDS) are at an increased risk of *M.tb* infection or reactivation [366, 377]. Furthermore, HIV-positive individuals are at an increased risk of death following TB co-infection [366, 378-382].

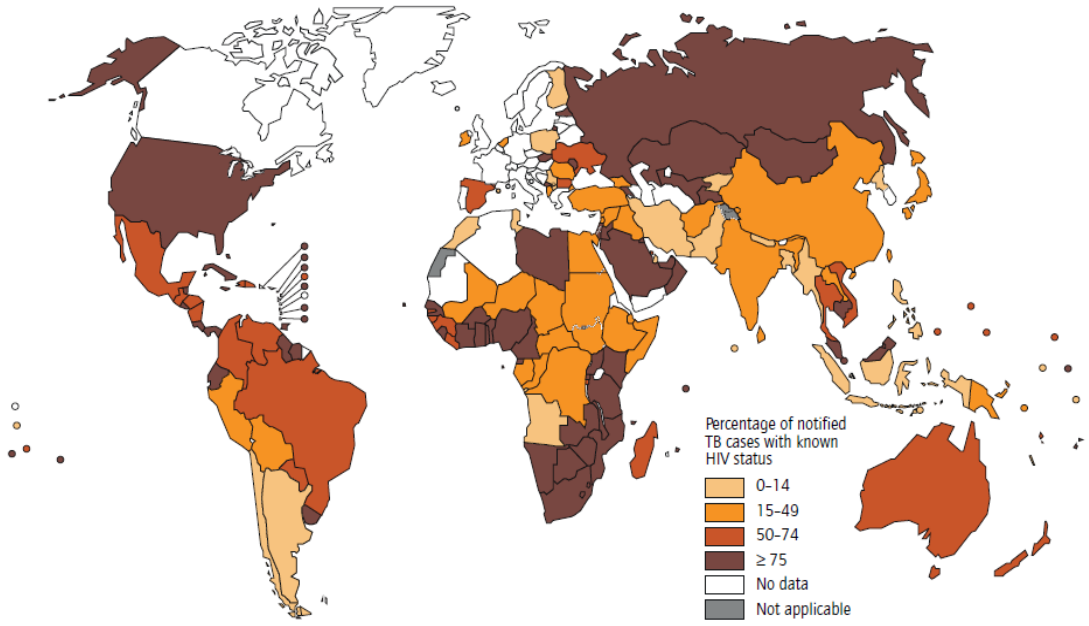


Illustration 6: Percent of TB patients with HIV infection, 2011 [1]

IMMUNE RESPONSE IMPAIRMENT

HIV infection can impair various macrophage-mediate defenses [4]. These impaired functions can contribute to immune dysregulation during *M.tb* infection. Direct evidence is not readily available to demonstrate innate immune impairment against HIV/*M.tb* co-infection, although several studies have shown HIV to impair innate immune defenses against various other intracellular organisms, including *Candida albicans* [383], *Candida pseudotropicalis* [384], *Pneumocystis carinii* [385], *Toxoplasma gondii* [386, 387], *Cryptococcus neoformans* [388], and *Mycobacterium avium* [4, 389, 390]. Impaired macrophage functions include: decreased phagocytosis [383-385, 387,

389]; decreased microbicidal activity [388]; decreased antigen processing and presentation through the down-regulation of the CD4 receptor, MHC class II and MHC class I molecules [267]; and decreased Th1 cytokine production, such as IL-2, IL-12, and IFN-γ [279, 280]. Subsequently, the ability of HIV to hinder various components of the immune response results in uncontrolled mycobacterial growth [390, 391].

While HIV can impair various immune responses associated with *M.tb* control, *M.tb* can promote aggressive HIV progression due to its effects on viral replication. *M.tb* infection drives activation of $CD4^+$ T cells and macrophages, the target cells of HIV replication [366]. During active TB infection, viral load is high because HIV-infected cells – T cells and macrophages – are activated and viral replication is increased, particularly at sites of *M.tb* infection such as the lung [4, 366, 392-396]. HIV replication is stimulated by the ability of *M.tb* to increase CCR5 co-receptor expression [394] by a mechanisms yet to be determined. Various cytokines expressed during *M.tb* infection can also stimulate HIV replication. *M.tb* infection activates the transcription of TNF-α, IL-1β, and IL-6 via binding of NF-κB and NF-IL6 proteins to sequences on the host DNA [393]. In turn, TNF-α can activate binding of NF-κB and NF-IL6 to the long terminal repeat (LTR) promoter of HIV, which contains similar NF-κB and NF-IL6 binding sequences, thus promoting viral replication [393, 397]. Moreover, HIV replication can be increased at sites of *M.tb* infection such as the bronchoalveolar lavage (BAL) fluid. Studies have demonstrated increased viral load and p24 capsid protein in the BAL of HIV/*M.tb* co-infected patients with active TB disease compared with HIV-positive patients without TB disease [393].

While HIV is known to deplete $CD4^+$ T cells, during *M.tb* co-infection it is thought that HIV specifically depletes *M.tb*-specific $CD4^+$ T cells [366, 398-400]. This data is primarily based on individuals with latent TB infection, decreases in *M.tb*-specific $CD4^+$ T cells occurred rapidly within the first year of HIV seroconversion [376]. It is thought that *M.tb*-specific $CD4^+$ T cells are reduced as a result of direct HIV infection

[401]. HIV infection can also lead to the functional impairment of *M.tb*-specific memory $CD4^+$ T cells [4]. *M.tb*-specific $CD4^+$ T cells from HIV-positive individuals are shown to proliferate less and produced less IFN-γ, IL-12, and IL-2 compared to *M.tb*-infected individuals without HIV [366, 368, 370, 375]. Overall, understanding the mechanisms used by the host defense or pathogen immune evasion can support in the development of therapeutics, antiretrovirals, and antibiotics to either assist the host in protection or circumvent the detrimental effects of co-infection.

ALTERED T_H1/T_H2 CYTOKINE BALANCE

As previously stated, a Th1 cytokine response is required for effective control of *M.tb*; HIV skews this response to express Th2 cytokines thus affecting the ability of the host to generate an effective immune response against *M.tb* [4, 108]. Both *M.tb* and HIV inhibit Th1 cytokines while promoting a Th2 immune response [80, 179, 181, 279, 280]. Production of IL-4 down-regulates IFN-γ and macrophage activation; while IL-10 further inhibits the Th1 responses and reduces the effector function of macrophages by down-regulating MHC class II molecules [108, 184-186, 279, 402]. These mechanisms promote *M.tb* and HIV disease progression. Furthermore, Th1 cytokine TNF-α has been shown to stimulate apoptosis during co-infection at disease sites [4, 403].

GRANULOMA DYSREGULATION

The ability of HIV to dysregulate the host response to *M.tb* infection leads to alteration in the granuloma. Three histological stages of the granuloma have been characterized in co-infected individuals: (1) granulomas from immunocompetent individuals have an abundant amount of epithelioid macrophage cells, MGCs, $CD4^+$ T cells located around the periphery, and few bacteria; (2) granulomas from moderately immunodeficient individuals do not have Langhans giant cells, epithelioid differentiation, or macrophage activation, $CD4^+$ T cells are reduced and more bacteria are seen; and (3)

granulomas from advanced immunosuppressed or AIDS patients (<200 CD4 T cells/μl) lack formation and have reduced cellular recruitment, fewer $CD4^+$ T cells, and a greater number of bacteria [4]. HIV can dysregulate the granuloma functions through many of the mechanisms previously described, such as disruption of mononuclear cell functions, depletion of mononuclear cells, and impairment of the granuloma structure and function by trafficking of HIV-infected cells into the granuloma [4, 366]. Access to activated immune cells within the granuloma may promote viral replication and dissemination along with mycobacterial replication and dissemination resulting in reactivation of TB disease [4, 366].

Increased viral replication in granuloma lesions can lead to reduced $CD4^+$ T cells and directly disrupt *M.tb* containment [366, 374]. Within these $CD4^+$ T cell-depleted granulomas, $CD8^+$ T cells were distributed throughout rather than in the periphery of the granuloma suggesting that $CD4^+$ T cells are responsible for maintaining the granuloma structure [404]. Furthermore, AIDS patients with <100 CD4 T cells/μl were more likely to have bacteria in the granuloma compared to patients with >100 CD4 T cells/μl [366, 405].

Granuloma dysfunction can also occur when HIV reduces *M.tb*-mediated apoptosis, though this is not completely understood [366, 369, 406]. Alternatively, increased apoptosis can occur in HIV/*M.tb* co-infected individuals compared to individuals infected with *M.tb* only which may benefit both pathogens by allowing for their release and dissemination [366, 407]. Such inconsistencies could be improved with the development of a suitable model to study HIV/*M.tb* co-infection by providing the precise mechanism of immune protection or dysfunction.

It is important to understand that several of the studies aimed at understand the pathogenesis of co-infection are based on indirect evidence from *in vitro* and *ex vivo* studies along with autopsy samples. These types of studies may not be representative of human immune responses; in addition, antiretroviral and antimycobacterial treatment

may be interfering with the displayed response. Deciphering how immune dysregulation occurs during HIV/*M.tb* co-infection is needed to begin to develop vaccines and therapeutics to prevent disease and aid in the host's survival. The establishment of a small animal model would greatly aid in understanding the mechanism by which HIV alters the host's immune response to prevent the control of *M.tb*.

Animal Models

Several animal models are available to study *M.tb* infection, although there are no animal models to study HIV/*M.tb* co-infection. Considering the high prevalence of co-infection, a suitable animal model to understand the mechanisms of disease are urgently needed. Understanding the mechanisms of disease within this animal model will assist in the development of vaccines, therapeutics, antivirals, and antibiotics to alleviate the detriment of HIV/*M.tb* co-infection worldwide.

M.tb infection is studied in the mouse, guinea pig, rabbit, and non-human primates (NHP); though each model has advantages and disadvantages. The most widely used model is mice because they are easy to handle, relatively cheap, available in a variety of mutants and genetically altered strains, and immunological reagents are readily available [408]. Unfortunately the mouse model is limited by its inability to develop a granuloma similar to human infection [408]. Whereas human granulomas present as well organized formations composed of macrophages surrounded by lymphocytes with caseous necrosis in the center, mouse granuloma are unorganized structures of macrophages and lymphocytes and only display necrosis with high bacterial load [408-411]. Furthermore, mouse granulomas do not develop caseation, fibrosis, calcification, or cavitation which is commonly seen in human *M.tb* infection [408-411]. The development of LTBI is also a major limitation within the mouse. In humans, reversion to latency

controls infection and reduces bacterial load; in the mouse, while infection can also be controlled, bacterial load will remain high [408]. The Cornell mouse model, developed at Cornell University by McCune and colleagues, has been used as a model of TB dormancy [412]. However, this model is limited by its inability to replicate human TB latency and lacks a standard protocol for the establishment of dormancy [408, 413]. In addition, problems arise after cessation of treatment including spontaneous reactivation in groups not receiving an immunosuppressive intervention or the inability to achieve reactivation in groups receiving the intervention [412, 413] Overall, these limitations make it difficult to understand the mechanisms of human TB dormancy, latency, and reactivation in the mouse model.

Another model of *M.tb* infection, the guinea pigs, are also relatively easy to use and inexpensive to house [408]. A major advantage of guinea pigs is the development of granulomas that are well organized and necrotic similar to humans; unfortunately bacterial load is not adequately contained [408, 411, 414, 415]. Guinea pigs are considered to be the most susceptible animal model to *M.tb* [408, 411, 416]. As a result, they are typically used for vaccine testing since it is thought that a good vaccine candidate would protect against infection in the most susceptible model [408]. Though they are a good animal model of human TB granuloma formation and vaccine efficacy, use of the guinea pig is limited by the scarce availability of immunologic reagents [408].

Similar to guinea pigs, rabbits develop organized granulomas with caseous necrosis or cavity formation similar to humans [408, 411, 417, 418]. However, rabbits are resistant to *M.tb* so studies are performed using *M. bovis* [408, 418, 419]. Additional limitations include the expense and difficulty of housing rabbits and the scarce availability of immunologic reagents [408].

Currently, the optimal animal model for understanding TB infection is the NHP. Using the NHP provides the ability to study various forms of TB infection, including active TB disease and LTBI [408, 411, 420]. Further, TB infection within the NHP

resembles that of a human with granulomas displaying caseous necrosis and cavitary lesions [408, 421]. Other advantages of using the NHP include the availability of immunologic and pathologic reagents (compared to the guinea pig and rabbit), tissue availability, and the ability to conduct co-infection with an HIV-like virus, SIV [408, 422]. Though the NHP appears to be an ideal model to study *M.tb* infection, several limitations exist. The cost of purchasing and maintaining animals can be expensive since it includes employing the necessary amount of trained staff and housing within approved facilities [408, 411]. In addition, the limited availability of these animals results in studies with small sample sizes [408, 411]. A major concern in using NHPs is the possibility of TB outbreaks which could eliminate entire colonies [422].

Regardless of the disadvantages of using a NHP, it remains the preferred model for *M.tb* infection and the only available model that simulates HIV/*M.tb* co-infection [423]. Development of an alternative animal model to use for understanding the mechanisms of *M.tb* infection and HIV/*M.tb* co-infection should include a low cost model with readily available reagents and capable of displaying a similar immune response to human infection. This ideal animal model would greatly benefit the further development of vaccines and therapeutics to reduce the global burden of *M.tb* and HIV/*M.tb* co-infection.

DEVELOPMENT OF THE HUMANIZED MOUSE

Studies with immune deficient mice began with the discovery of the severe combined immunodeficiency (SCID) mouse by Bosma *et al*. [424, 425]. SCID mice were found to be deficient in the ability of stem cells to differentiate into mature lymphocytes and deficient in the ability to reject antigenically distinct grafts [425]. With the emergence of AIDS in the 1980s, the SCID mouse was looked at as a possible model to study the human immune system in response to infection. McCune *et al*. lead the way by reconstituting SCID mice with human lymphocytes [426]. To do this mice were

implanted with human fetal thymus and injected with human fetal liver cells; these were known as SCID-hu mice [426]. However, lymphocyte rates and duration were variable and mice lacked functional T cells [426]. It was later determined that SCID mice, though defective in T and B cells, did express functional NK cells and APCs which may by affecting lymphocyte production and function [427-429]. To compensate for the possible effects from NK cells and APCs, SCID mice were backcrossed with non-obese diabetic (NOD/Lt) mice to produce NOD/SCID mice which results in defects of the innate and adaptive immune cells and high reconstitution rates when compared to SCID mice [427]. Unfortunately, limitations existed within this model including slight NK cell function and shortened life span of mice [430]. Studies by Sugamura *et al*. revealed that mutation of the IL-2 receptor γ-chain (IL2Rγ) was shown to cause deficiencies in NK cell development and activity [431, 432]. The IL2 γ-chain is crucial for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 binding and signaling [432, 433]. This subsequently lead to the development of nonobese diabetic-severe combined immune deficient/gamma chain deficient (NOD/SCID/γ_c^{null}, NSG) that were generated from NOD/SCID mice with the IL2Rγ deficiency [430]. These mice are deficient in T, B, and NK cells, and have low levels of macrophages and DCs [424, 430, 434].

BONE MARROW, LIVER, THYMUS (BLT) MOUSE

The development of the humanized BLT mouse has greatly circumvented the limitations of previous animal models. BLT mice are generated by the implantation of fetal liver and thymus, and then supplemented with human $CD34^+$ hematopoietic stem cells (HSCs) into NSG mice as described in previous studies [435-438]. The humanized mouse has provided opportunities for the study of various important infectious diseases including Dengue virus [439, 440] *Plasmodium falciparum* [441], Epstein-Barr virus [435], and *Salmonella* [442]. The humanized BLT model has become especially important in the study of HIV infection since its human host tropism limits its use in

other animal models [436, 438, 443, 444]. Currently there is not a small animal model to study HIV/*M.tb* co-infection and the humanized BLT mouse has not been developed to study *M.tb* infection. The humanized BLT could aid in expanding knowledge of the human immune response to *M.tb* and HIV/*M.tb* co-infection; it could further be used as a model for the development of vaccines and therapeutics of *M.tb* in HIV-positive populations.

SPECIFIC AIMS & HYPOTHESIS

The objective of this proposal is to develop a small animal model and identify mechanisms whereby HIV affects the host's immunity during *M.tb* infection. The long term objective is to develop a small animal model to advance HIV/*M.tb* co-infection knowledge and use the model to test prophylactics, therapeutics, and vaccines for *M.tb* in HIV-positive populations. It is hypothesized that *M.tb* pathogenicity will increase following HIV infection and protective immune responses to *M.tb* will be compromised during co-infection in the humanized mouse model. The following aims will be used:

Specific Aim #1. Evaluate the development of *M.tb* in the BLT humanized mouse model. HYPOTHESIS: *M.tb* infection in the humanized mouse will display similar pathogenesis to *M.tb* infection in the human. Specific aim 1a. Establish optimal *in vivo* imaging techniques for visualization of *M.tb* within the humanized mouse. Specific aim 1b. Define *M.tb* infection parameters and disease outcome in the humanized mouse.

Specific Aim #2. Determine the course of *M.tb* infection following HIV infection in the BLT humanized mouse. HYPOTHESIS: *M.tb* disease course will be accelerated and increased in severity upon HIV co-infection in the humanized mouse. Specific aim 2a. Evaluate the kinetics of *M.tb* infection in HIV-infected humanized mice. Specific aim 2b. Evaluate the pathology of HIV/*M.tb* co-infection compared to the pathology of *M.tb*-infected humanized mice.

Specific Aim #3. Determine how the host defense to *M.tb* infection is altered during HIV infection. HYPOTHESIS: HIV infection will suppress the protective immune response to *M.tb* infection. Specific aim 3a. Determine how the host immune responses

are altered during HIV/*M.tb* co-infection in the humanized mouse. Specific aim 3b. Determine how the pathological immune responses during *M.tb* infection are dysregulated during HIV infection in the humanized mouse.

CHAPTER 2[1]

Materials and Methods

ETHICS STATEMENT

All animal procedures were performed in accordance with the regulations of the NIH Office of Laboratory Animal Welfare and were approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee (IACUC). Discarded tissue from deceased human fetuses was obtained via a non-profit partner (Advanced Bioscience Resources, Alameda, CA) as approved under exemption 4 in the HHS regulations (45 CFR Part 46).

GENERATION OF HUMANIZED BLT MICE

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice, also known as NOD/SCID/γ_c^{null} or NSG mice (Jackson Laboratories), 3-5 weeks of age, were housed in a specific pathogen-free microisolator environment. Mice were engrafted with human fetal liver and thymus (Advanced Bioscience Resources, Alameda, CA) tissue after receiving 200 cGy of irradiation at a rate of 119 cGy/min (RS-200 Rad Source, Suwanee, GA) as previously described [435]. Following engraftment of tissue, mice were intravenously (i.v) injected with approximately 1×10^6 hematopoietic stem cells ($CD34^+$ cells) per mouse from the same human fetal tissue donor. For two weeks after implantation, mice received acidified drinking water (pH 3.0) with antibiotics. Twelve weeks post-engraftment, human leukocyte reconstitution was evaluated in peripheral blood.

[1] sections taken from Calderon *et al*, A Humanized Mouse Model of Tuberculosis, *PLOS* One, 8(5): e63331

ANIMAL *M.TB* INFECTIONS

M.tb (tdTomato H37Rv), a strain expressing a *tdTomato* fluorescent protein, was propagated by growth to log phase in Middlebrook 7H9 broth (Becton, Dickinson, and Company, Sparks, MD, USA) supplemented with 50% glycerol (Sigma, St. Louis, MO, USA), BBL Middlebrook albumin dextrose complex enrichment (ADC, BD), and 20% Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA) as previously described [445]. Mice were infected intranasally with 40 µl (20 µl/nare) of high dose (10^5–10^6, Figure 3,4,5) or low dose ($2.5x10^2$, Figure 6) *M.tb tdTomato* H37Rv diluted in Dulbecco's Phosphate-Buffered Saline (PBS, Cellgro, Manassas, VA, USA). The actual dose was further confirmed by CFU enumeration of inoculum suspension. At specified time points, mice were euthanized using isoflurane (Primal Critical Care, Inc., Bethlehem, PA) overdose. Cervical dislocation was subsequently performed to ensure death, as approved by the UTMB-IACUC. All animal experiments and work with *M.tb* were performed in CDC-approved animal biological safety level-3 (ABSL-3) and BSL3 facilities in the Galveston National Laboratory in accordance with biosafety procedures approved by the UTMB Environmental Health and Safety Division.

ANIMAL HIV AND HIV/*M.TB* CO-INFECTION

HIV-1 (JR-CSF), an M-tropic strain, was grown and provided by Dr. Monique Ferguson (Department of Internal Medicine-Infectious Disease Division, UTMB). Mice were infected intravenously (i.v.) with 2500 Tissue Culture Infective Dose $(TCID)_{50}$ JR-CSF HIV-1 in 50 µl PBS (Cellgro, Manassas, VA, USA). For co-infection, *M.tb (tdTomato* H37Rv) was propagated as previously described [445]. Three weeks post-infection (p.i.) with HIV or mock infection, mice were infected intranasally (i.n.) with 40 µl (20µl/nare) of low dose $2.5x10^2$ *M.tb tdTomato* H37Rv diluted in PBS (Cellgro, Manassas, VA, USA). At specified time points, mice were euthanasized using isoflurane (Primal Critical Care, Inc., Bethlehem, PA) overdose and tissues harvested.

IN VIVO IMAGING

Mice were anesthetized with 3% isoflurane (Piramal Critical Care, Inc. Bethlehem, PA, USA) in an oxygen-filled induction chamber, transferred to an isolation chamber, and placed in the imaging chamber which contains an integrated anesthesia system. Anesthesia was continued to be administered at 1-2% isoflurane. *In vivo* fluorescent images were acquired using the *In Vivo* Imaging System (IVIS) Spectrum (Caliper Corporation, Alameda, CA, USA). In some experiments, animals were euthanized immediately following *in vivo* imaging to evaluate fluorescent signal in excised organs relative to in vivo signal. Organs (liver, lung, and spleen) were aseptically removed, placed in a petri dish, sealed, and transferred to the imaging chamber. Images were acquired at each excitation and wavelength pair as previously described [446]. The fluorescent signal was obtained at a 535 nm and 605 nm excitation light and read at 580, 620, 640, 660, 680, 700 nm. Compensation was applied to correct for autofluorescence.

ISOLATION OF PBMCS AND LEUKOCYTE ANALYSIS

Pre- and p.i., reconstitution levels of lymphocytes and monocytes were determined by multi-variate flow cytometry. Peripheral blood was collected from the tail vein of humanized BLT mice and placed in 500 µl 3mM ethylenediaminetetraacetic acid (EDTA, Capitol Scientific, Austin, TX, USA) to prevent coagulation. Peripheral blood mononuclear cells (PBMCs) were isolated by incubation with Red Blood Cell Lysis Buffer (Sigma, St. Louis, MO, USA) as recommended by manufacturer. Subsequently, PBMC were incubated with CD16/CD32 Fc Block (BD Biosciences, San Jose, CA, USA) to reduce non-specific binding of antibodies. Cells were labeled with directly conjugated antibodies specific to human lymphocyte surface markers: PE-Cy7-CD45, Alexa Fluor 700-CD56, APC-Cy7-CD3, Pacific Blue-CD4, and PerCP-Cy5.5-CD8 (BD Biosciences) and is a separate experiment, γδTCR (eBioscience). In some experiments PBMC were labeled with antibody to human antigen presenting cell phenotype and

activation markers: APC-Cy7-CD14, PE-Cy7-CD40, PerCP-Cy5.5-HLA-DR, FITC-CD80, and PE-CD86 γδTCR (BD Bioscience). For determination of potential effector function, spleens from non-infected humanized mice were obtained and processed to single-cell suspensions by pressing through a 70 μm filter. Following incubation with Red Blood Cell Lysis Buffer, PBMCs were maintained in complete culture medium (cRPMI), RPMI 1640 supplemented with 10% fetal bovine serum (FSB), 2mM L-glutamine, 1mM sodium pyruvate, 0.1mM nonessential amino acids, and 1% penicillin-streptomycin (Life Technologies, Grand Island, NY, USA) and stimulated with IL-15 (15ng/ml) or antibody to CD3/28 (as recommended by the manufacturer Life Technologies), a T-cell stimulant, for 5 days. Cells were treated with GolgiStop (BD Bioscience) during the last 5 hours of culture, washed, and subsequently labeled with surface human lymphocyte antibodies. Cells were permeabilized using the BD Cytofix/Cytoperm kit (BD Bioscience), then labeled with antibodies specific to human APC-IFN-γ, FITC-granulysin, PE-Perforin, or a proliferation marker PE-Ki-67 (BD Bioscience). Samples were finally incubated for 48 hours in 4% formaldehyde (Polysciences Inc, Warrington, PA, USA) diluted in PBS prior to acquisition. A total of 50,000 gated events (based on expected leukocyte side scatter/forward scatter characteristics) were collected using a BD LSR II (Fortessa) flow cytometer (BD Biosciences). Analysis of data was performed by FCS Express (De Novo, Los Angeles, CA, USA) software. To control for background and to establish thresholds for gating positive cells, isotype matched antibodies labeled with the same fluorochromes were used. Cells were selected based on the human CD45 marker and further analyzed for human CD3, CD4, CD8, CD56, and CD14.

CFU ENUMERATION

At specified time points following aseptic removal of organs, half of the liver, lungs, and spleen were placed into 1 ml of PBS in 15 ml small tissue grinders (Kendall, Mansfield,

MA, USA). Following homogenization of tissues, serial dilutions of organ samples were prepared in PBS, and 6-5 µl droplets were placed on 7H11 agar plates (BD Biosciences) as previously described [447]. All studies were performed in a CDC-approved BSL-3 facility.

PATHOLOGY ASSESSMENT

At specified time points, following aseptic removal of organs, the remaining half of tissues were placed in 10% neutral buffered formalin (Statlab, McKinney, TX, USA) for 48 hours to inactivate infectious agent, changed after 24 hours, and finally stored in 70% ethanol. Tissues were embedded in paraffin then stained with hematoxylin and eosin (H&E). Additional sections were also stained using the Ziehl-Neelson method to visualize acid-fact bacteria (AFB) to detect *M.tb* bacilli. Processing and staining were performed at the University of Texas Medical Branch Research Histopathology Core. Paraffin embedded tissue samples were further stained with antibody to human CD3, CD4, and CD8 and detected using EnVision™ System-HRP (DAB) (Dako, Carpinteria, CA, USA) for CD3 and CD8 and EnVision™ G|2 System/AP, Rabbit/Mouse (Permanent Red) (Dako) for human CD4 and Myeloperoxidase (MPO) according to manufacture recommendations. Lung, liver, and spleen tissue was evaluated by a trained pathologist with expertise in TB disease progression, with confirmation done in a slide blinded manner by a collaborating pathologist at UT-Houston Medical School and UTMB.

HIV-1 P24 ANTIGEN ELISA

At specified time points, following aseptic removal of organs from HIV-infected and HIV/*M.tb* co-infected animals, tissues were homogenized. Supernatant was isolated and placed in 80^{o}C freezer. In addition, peripheral blood was collected from HIV-only infected animals and placed in 500 µl 3mM EDTA (Capitol Scientific, Austin, TX, USA). Plasma was isolated and placed in 80^{o}C freezer for storage. HIV-1 p24 levels were

measured using the HIV-1 p24 Antigen ELISA (Zeptometrix, Buffalo, NY, USA) as recommended by the manufacturer.

BIOPLEX ELISA

At specified time points, following aseptic removal of organs, tissues were homogenized. Supernatant was isolated and placed in 80^{o}C freezer. In addition, peripheral blood was collected and placed in 500 µl of 3mM EDTA (Capitol Scientific, Austin, TX, USA). Plasma was isolated and placed in 80^{o}C freezer for storage. Cytokine levels were measured using the 27-plex Bio-plex Pro Human Cytokine Assay (Bio-Rad, Hercules, CA, USA). Cytokines and chemokines include: interferon-gamma (IFN-γ), interleukin (IL)-12, IL-2, IL-4, IL-1β, IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), granulocyte-colony stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, MIP-1β, IFN-γ inducible protein 10 (IP-10).

RNA ISOLATION

At specified time points, following aseptic removal of organs, small samples of liver, lung, and spleen from HIV-infected or HIV/*M.tb* co-infected animals were placed in 500 µl of Total RNA Lysis Buffer (Bio-Rad, Hercules, CA, USA) and placed in a -80^{o}C freezer for storage. In addition, peripheral blood was collected and placed in 500 µl of 3mM EDTA (Capitol Scientific, Austin, TX, USA). Plasma was isolated and placed in -80^{o}C freezer. Total RNA was isolated from frozen samples using the RNeasy Mini Kit (Qiagen, Maryland, USA) as recommended by the manufacturer. All studies were performed in a CDC-approved BSL-3 facility.

REAL-TIME PCR

Prior to cDNA synthesis, RNA samples were concentrated by precipiation. Briefly, 100% ethanol (EtOH, info) was added to the sample followed by a 1/10 volume of 3M Sodium Acetate (NaAC) and 30 minute incubation at 70^{o}C. Supernatant was removed and 100 µl 80% ice cold EtOH was briefly added. Supernatant was removed and the sample was allowed to air dry after which 10 µl of RNase/DNase free water was added.

Synthesis of cDNA was performed using Superscript III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) as recommended by the manufacturer. Gene expression differences were determined using specific primers and probes for HIV Gag: forward 5'-GAGCTAGAACGATCCGCAGTTAATC-3', reverse 5'-ATAATGATCTAAGTTCTTCTGATCCTG-3', and FAM-labeled probe 5'-CCTTTTAGAGACATCAGAAG-3' (Integrated DNA Technologies, Coralville, IA, USA). Template was normalized using the 18S ribosomal RNA control primers and VIC-labeled probe (Applied Biosciences, Foster City, CA, USA) which were added to each reaction. Determination of relative gene expression was determined using the Bio-Rad CFX96 Real-time System (Bio-Rad, Hercules, CA, USA). Target gene expression levels were determined by normalizing to the endogenous reference (18S) and relative to non-HIV infected samples.

CHAPTER 3[2]

Optimization of M.tb infection within the humanized BLT mouse.

TB continues to be the cause of massive morbidity and mortality throughout the world. BCG, the only available vaccine against TB, has variable efficacy and is not recommended for individuals with HIV [32, 448]. As a result, new vaccines and therapeutics are urgently needed to reduce the global TB burden. We anticipate that a new small animal model will greatly aid in advancing the development of preventative and prophylactic therapies against TB, specifically for use in HIV-positive populations.

The current method for determining treatment efficacy in *M.tb* infection is through bacterial growth on agar plates which involves sacrificing the animal and using target organs for cfu enumeration [449, 450]. Though reliable and accurate, this method is time-consuming taking four to six weeks to visualize mycobacterial growth [449, 450]. The method of bacterial detection and slow rate of growth entails many disadvantages including not knowing whether animals were successfully infected early in the study, inhibiting follow-up to determine possible long term effects or reactivation, slowing the speed of studies, and requiring large amounts of animals which increases study costs. Therefore new diagnostic methods in animal models are needed to promote the development of TB therapeutics and vaccines.

Development and evaluation of TB therapeutics and vaccines can be advanced by whole-body imaging using fluorescent proteins (FP). Whole body imaging is a non-

[2] sections taken from Calderon *et al*, A Humanized Mouse Model of Tuberculosis, *PLOS* One, 8(5): e63331

invasive real-time technique that allows for the monitoring of infection within live animals [451]. The fluorescent signal at the site of infection can also be quantified and used to determine bacterial load [449, 451]. This technology can eliminate the delay in determining cfu, allowing for more long-term studies with fewer mice and providing valuable information that might otherwise be lost following the animal's sacrifice [449, 452-454].

Our studies use an *M.tb* H37Rv strain with a plasmid expressing a *tdTomato* fluorescent protein for infection detection within deep tissues of live animals. Non-humanized NSG and humanized BLT mice were infected with various doses of *M.tb* (ranging from 10^6-10^2 cfu) and bacterial infection detected in the lung by *in vivo* imaging system (IVIS). Progressing infection is seen in the lung and disseminated bacterial infection is observed within the liver and spleen. A range of pathological characteristics are identified including granuloma formation with caseous necrosis centers, bronchial obstruction, foamy macrophage and lipid deposit, and cholesterol crystal formation. These studies begin to provide evidence of the BLT mouse as a model to simulate human TB disease. These and subsequent studies will significantly advance the way future animal studies are performed and will allow for a more effective way to test vaccines and therapeutics against TB.

RESULTS

In vivo imaging optimization of *tdTomato* H37Rv *M.tb* infection

To begin, NSG mice were infected i.n. with 10^6 CFU *tdTomato* H37Rv *M.tb*. An IVIS was used to visualize mycobacterial infection as previously described [451] in the lungs of live mice. At 6 days p.i., IVIS was performed although bacterial infection is not

visualized (Figure 1.1). Subsequent studies with IVIS performed at earlier time points displayed similar results. During these studies, autofluorescence can be seen and may be responsible for the lack of bacterial visualization. To reduce autofluorescence, animals are shaved prior to performing additional imaging. Similarly, bacterial infection is not detected and autofluorescence remains to be a problem (data not shown).

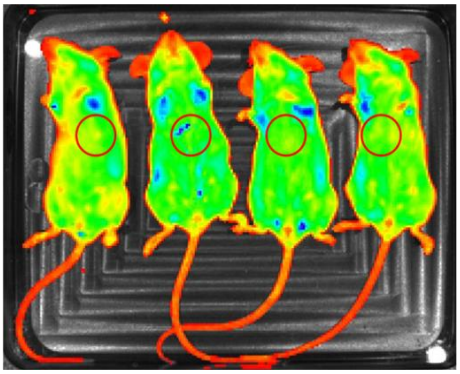


Figure 1.1: *M.tb* infection in the NSG mouse. Animals were infected i.n. with 10^5 CFU *tdTomato* H37RV *M.tb* and in vivo imaging (IVIS) performed at day 6 p.i. Shown is IVIS imaging of BLT mice: mock-infected, PBS, control mouse (n=1, left) and *M.tb*-infected NSG mice (n=3, right). Detection region (lung) is shown in the red circle.

With the inability to detect the *tdTomato M.tb* strain in live animals, we wanted to determine whether the *tdTomato* fluorescent protein could be observed with the imaging system. A 50 µl aliquot of 10^6 CFU *tdTomato* H37Rv *M.tb* was placed in a petri dish. Immediately following, the sample was placed in the imaging system and able to be visualized (Figure 1.2). This validated the use of the IVIS and proved that the *tdTomato* fluorescent strain could be used for detection of *M.tb* infection within humanized mice.

To optimize the IVIS detection technique within live animals, an NSG mouse was infected intraperitoneally (i.p.) with 10^8 CFU *tdTomato* H37Rv *M.tb*. IVIS was performed immediately after infection (day 0) and spectral unmixing was performed to detect the *M.tb* strain. Spectral unmixing is an analysis strategy used to reduce

autofluorescence [451]. As shown in Figure 1.3, bacterial infection was visualized at the injection site of the live animal (Figure 1.3). This validated that spectral unmixing needs to be performed to visualize the *tdTomato M.tb* strain in live mice and confirmed our ability to utilize the *tdTomato M.tb* strain during infection of BLT mice.

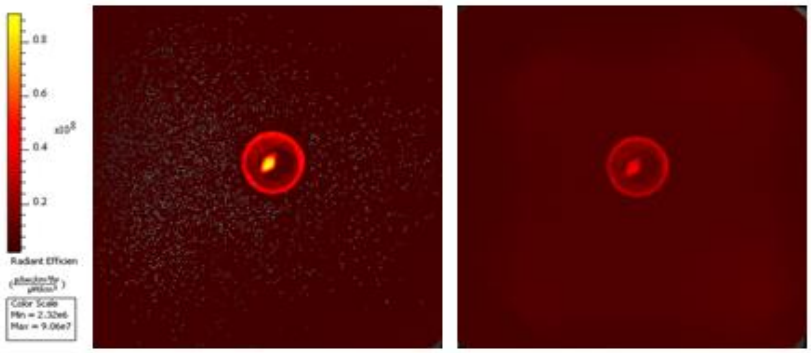


Figure 1.2: Inoculum of *tdTomato* H37Rv *M.tb*. A 50 µl aliquot of 10^6 CFU *tdTomato* H37RV *M.tb* was placed in a small petri dish. Shown is (left) fluorescent intensity signal represented with a pseudocolor scale ranging from yellow (most intense) to dark red (least intense); (right) imaging was performed at day 0 p.i

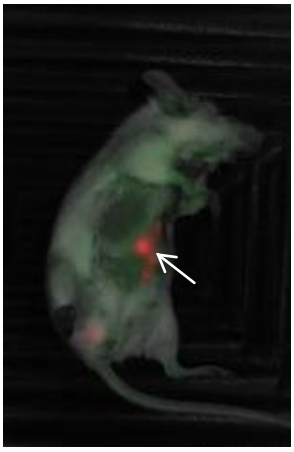


Figure 1.3: Spectal unmixing of *Mtb* infected NSG mouse. A mouse was infected i.p. with 10^8 CFU *tdTomato* H37RV *M.tb* and IVIS performed at day 0. Shown is IVIS imaging of a NSG mouse infected with *M.tb*.

Visualization of *M.tb* infection in humanized BLT mice.

Development of the humanized BLT mouse has previously been described [435-438]. A full description of humanized BLT mouse development by our group and subsequent *M.tb* infection will be discussed in Chapter 4. To visualize *M.tb*, BLT mice are infected i.n. with 10^6 CFU *tdTomato* H37Rv *M.tb*. Using IVIS, progressive bacterial infection was monitored within the lungs of live mice at weekly intervals (Figure 1.4B). Compared to control (non-infected, PBS) animals, bacterial infection could be observed in the lungs of *M.tb*-infected mice beginning at week 1. Organs were extracted following sacrifice and necropsy, beginning at 2 weeks p.i., and disseminated *M.tb* could be observed in the liver, but not the spleen, of infected mice (Figure 1.4B insets). Shown in Figure 1.4C, is a three-dimensional reconstruction of BLT mouse with localization of *M.tb* infection in the chest cavity, consistent with the lung.

Evaluation of *M.tb* infection in BLT mice with lower infection doses

Additional studies were performed in BLT mice infected with logarithmically decreasing infection doses ranging from 10^4 to 10^2 cfu (n=3/infectious dose). Progressive bacterial infection was monitored within the lungs of live animals by IVIS starting at week 2 and specified time points thereafter. Infection was allowed to progress until animals succumbed to death, after which organs were immediately obtained for bacterial load and pathology analysis.

Bacterial infection could be visualized in BLT mice infected with 10^4 cfu until week 3 p.i. compared to non-infected control animals (Figure 1.5B). At week 4 p.i., IVIS was not performed and all mice had succumbed to infection. CFU analysis, shown in Figure 1.5C, is representative of bacterial load at week 2 p.i. and demonstrates dissemination to various organs. At the time of necropsy, tissues were obtained as previously described. Briefly, left lung lobe and a lobe of liver, along with the entire

spleen, were used to determine cfu enumeration; the remaining lung and liver tissues were used for pathology analysis following hematoxylin and eosin (H&E) staining.

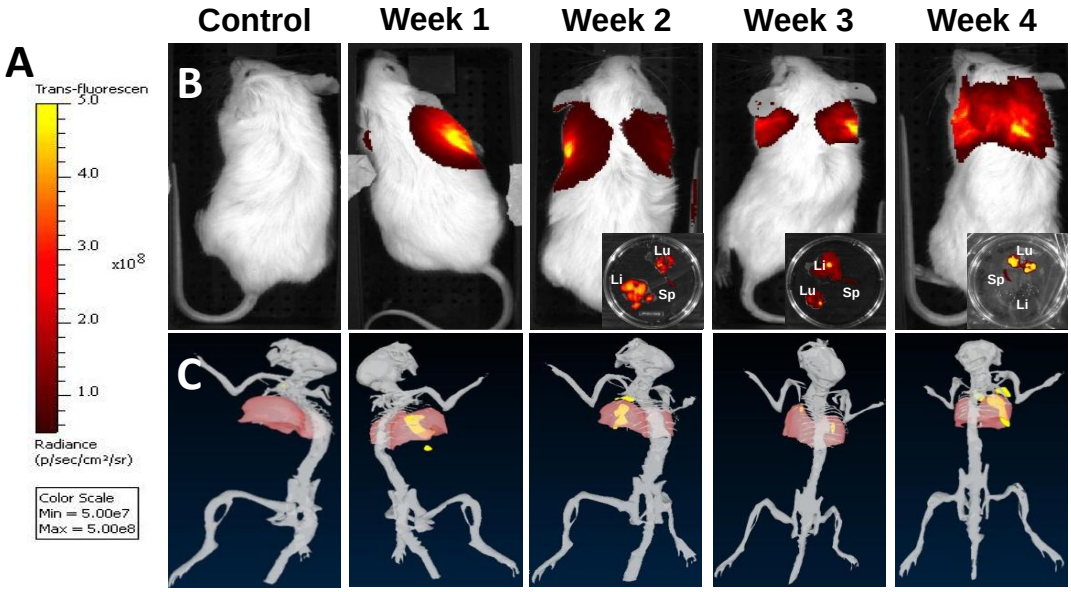


Figure 1.4: Visualization of high-dose *M.tb* infection in the humanized BLT mice. Following successful engraftment of human leukocytes, animals were infected i.n. with 10^6 CFU of *tdTomato* H37Rv *M.tb* and *in vivo* imaging (IVIS) performed at weekly time points. Shown in (A) is the fluorescent intensity signal represented with a pseudocolor scale ranging from yellow (most intense) to dark red (least intense). (B) IVIS images of BLT mice; across: non-infected control mouse and *M.tb* infected BLT mice at specified time points; (Inset) *M.tb* infected organs: Lu = Lung, Li = Liver, Sp = Spleen. (C) 3D reconstruction of *M.tb*-infected BTL mice at specified time points showing origin fluorescent signal.

Shown in Figure 1.5D, is lung at 2 weeks p.i. from the same animal represented in the cfu analysis. Interstitial thickening and cellular infiltration is observed throughout the lung with interspersed areas of healthy lung architecture (i.e. clear alveolar pockets lined with one to two epithelial cells). The liver is healthy with clearly visible hepatocytes and no cellular infiltration (Figure 1.5E).

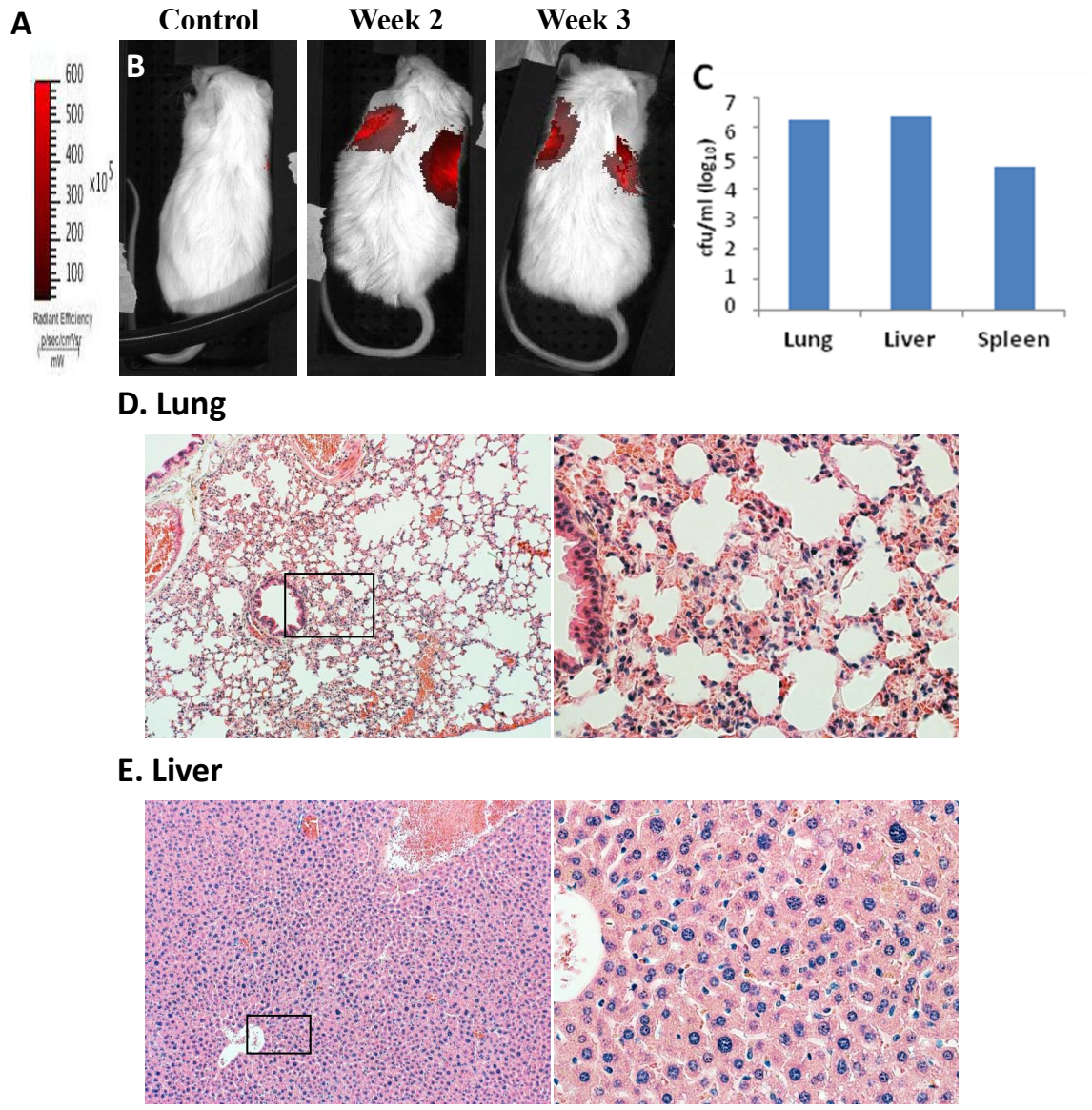


Figure 1.5: Humanized BLT mice infected with 10^4 cfu *tdTomato* H37Rv *M.tb*. Following successful engraftment of human leukocytes, animals were infected i.n. with 10^4 CFU of *tdTomato* H37Rv *M.tb* (n=3) and *in vivo* imaging (IVIS) performed at specified time points. Tissue pathology images (2 weeks p.i) are captured by brightfield microscopy following stain using hematoxylin and eosin (H&E). Shown in (A) is the fluorescent intensity signal represented with a pseudocolor scale ranging from bright red (most intense) to dark red (least intense). (B) IVIS images of BLT mice; across: non-infected control mouse and *M.tb*-infected BLT mice at specified time points. (C) colony-forming units (cfu; n=1/organ) of tissues at 2 weeks p.i. are shown per milliliter (ml). (D) lung tissue and (E) liver tissue visualized by H&E. Left panel, 10X; right panel, 40X of indicated region.

In BLT mice infected with 10^3 cfu, progressive bacterial infection is visualized in the lung until week 5 p.i. (Figure 1.6B); at which time the study was ended and animals were sacrificed and necropsy performed. Figure 1.6C shows bacterial load and demonstrates dissemination to various organs at week 5. Lung pathology shows caseous necrosis throughout the tissue and alveolar pockets filled with cellular debris (Figure 1.6D). Necrotic regions contain pyknotic nuclei, cellular fragmentation, and bacterial debris. Bronchial obstruction is also observed with cellular infiltrates. Lipid deposits and cholesterol crystallization is seen in and around necrotic areas along with foamy macrophages. Shown in Figure 1.5E is the liver pathology which contains foci of inflammation scattered throughout the tissue with infiltration around portal veins (Figure 1.5E).

In BLT mice infected with 10^2 cfu, bacterial infection is only visualized at week 2 p.i. (Figure 1.7B), after which bacteria could no longer be detected possibly due to the low number of bacteria expressing the fluorescent protein. CFU analysis shown in Figure 1.7C demonstrates dissemination to various organs at week 5. Interstitial thickening and cellular infiltration is seen throughout the lung along with interspersed areas of healthy lung structure. The liver appears health with a few inflammatory foci dispersed in the tissue and infiltration near portal veins (Figure 1.5E).

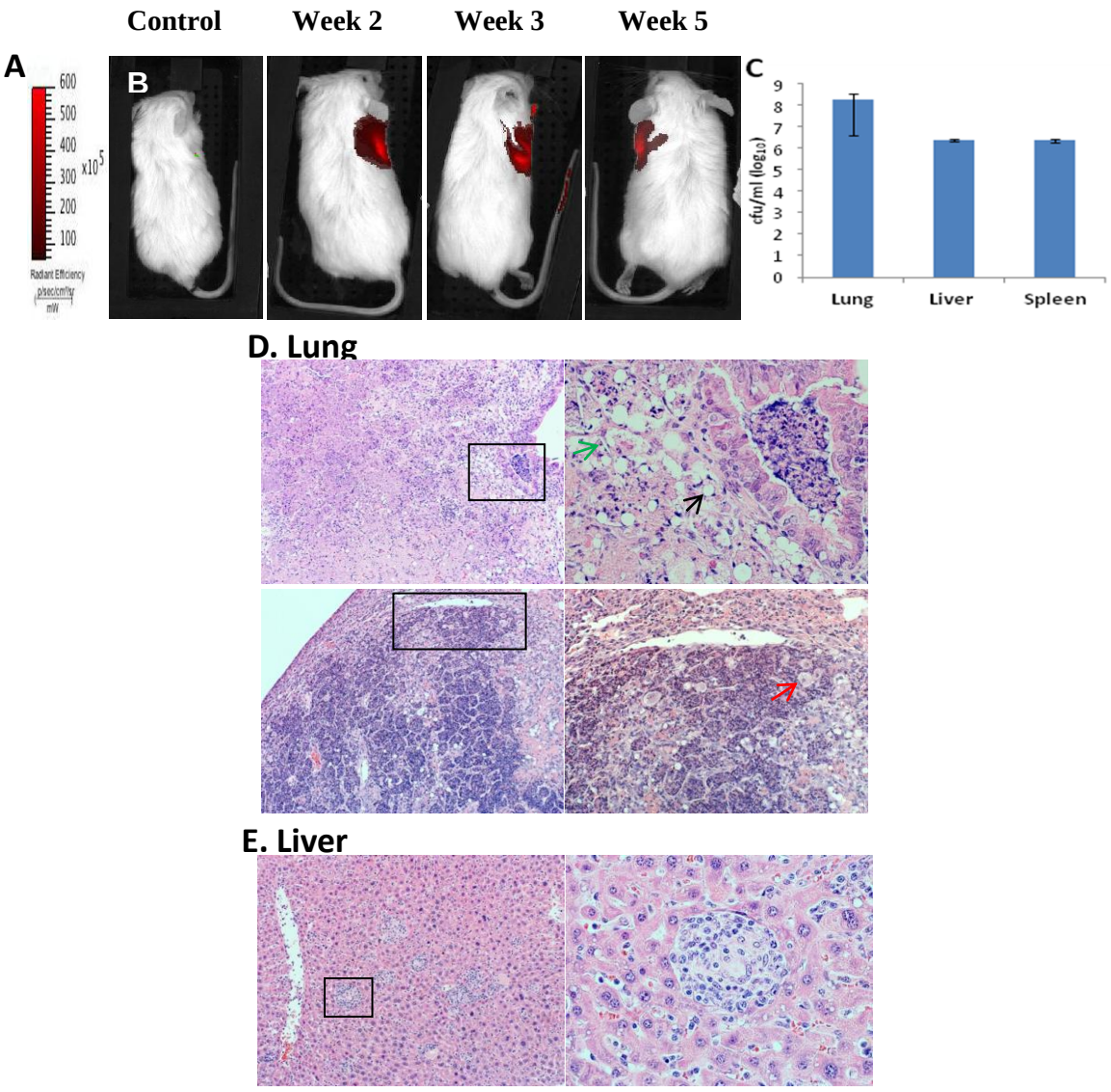


Figure 1.6: Humanized BLT mice infected with 10^3 cfu *tdTomato* H37Rv *M.tb*. Following successful engraftment of human leukocytes, animals were infected i.n. with 10^4 CFU of *tdTomato* H37Rv *M.tb* (n=3) and IVIS performed at specified time points. Tissue pathology images (5 weeks p.i) are captured by brightfield microscopy following stain using H&E. Shown in (A) is the fluorescent intensity signal represented with a pseudocolor scale ranging from bright red (most intense) to dark red (least intense). (B) IVIS images of BLT mice; across: non-infected control mouse and *M.tb*-infected BLT mice at specified time points. (C) cfu of tissues (n=2/organ) at 5 weeks p.i. shown per ml. (D) lung tissue visualized by H&E (10X) (top right, 40X: lipid deposits, black arrows and cholesterol crystals, green arrow; bottom right, 20X; foamy macrophage, red arrows). (E) liver tissue visualized by H&E. Left panels, 10X; right panels, 40X of indicated region.

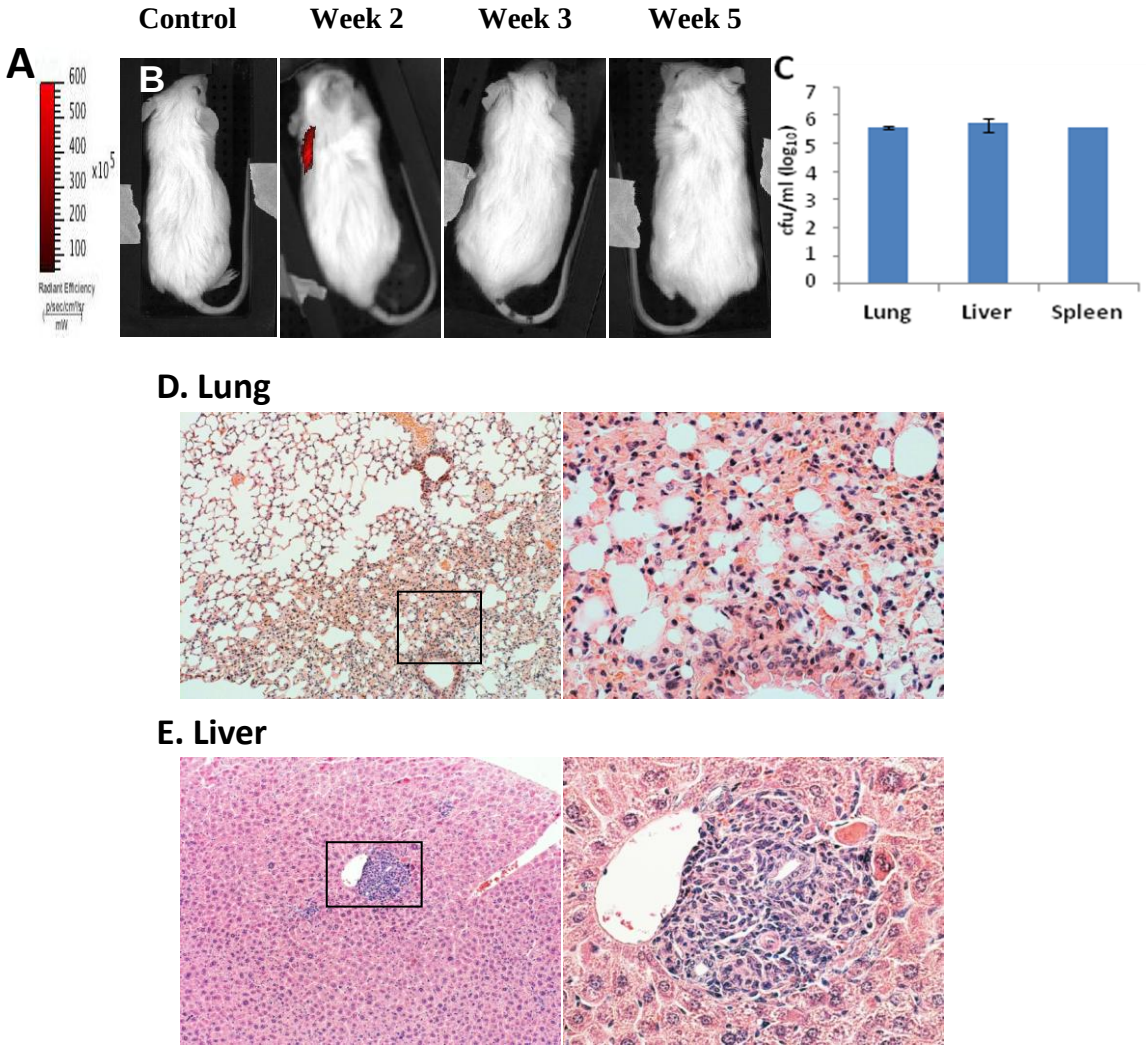
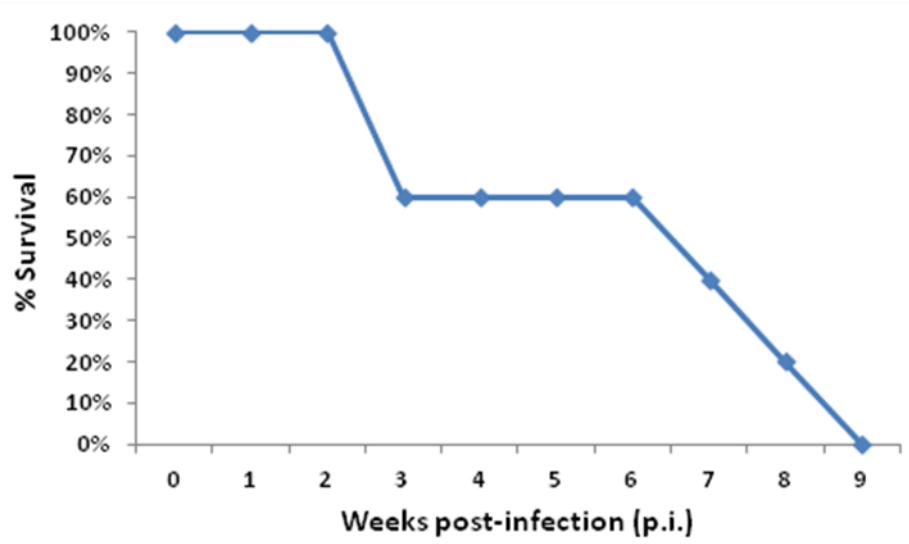


Figure 1.7: Humanized BLT mice infected with 10^2 cfu *tdTomato* H37Rv *M.tb*. Following successful engraftment of human leukocytes, animals were infected i.n. with 10^4 CFU of *tdTomato* H37Rv *M.tb* (n=3) and IVIS performed at specified time points. Tissue pathology images (5 weeks p.i) are captured by brightfield microscopy following stain using H&E and Ziehl-Neelson to detect for AFB. Shown in (A) is the fluorescent intensity signal represented with a pseudocolor scale ranging from bright red (most intense) to dark red (least intense). (B) IVIS images of BLT mice; across: non-infected control mouse and *M.tb*-infected BLT mice at specified time points. (C) cfu of tissues (n=2/organ) at 5 weeks p.i. shown per ml. (D) lung tissue visualized by H&E (left) and localization of bacilli by AFB (right). (E) liver tissue visualized by H&E (left) and AFB (right). Top images, 10X; bottom images, 40X of indicated region.

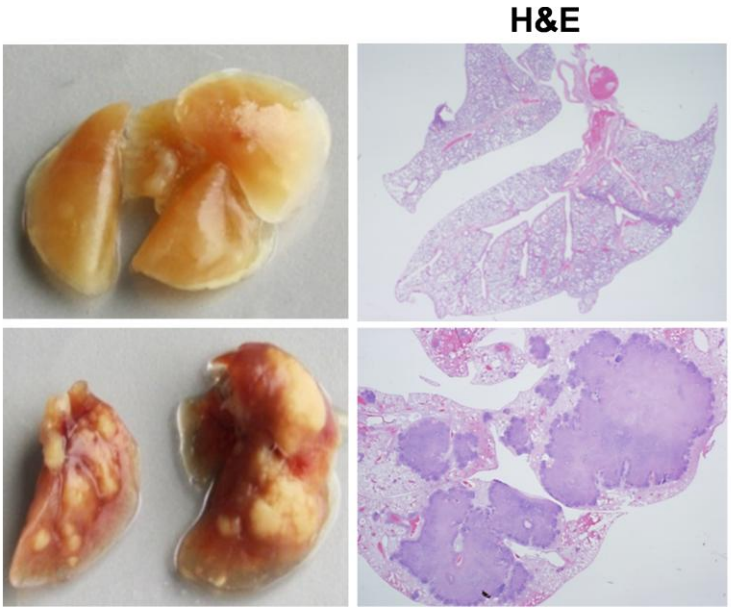
Development of TB in BLT mice infected with low-dose of *M.tb*

Though bacteria are not detectable by IVIS in mice infected with 10^2 cfu after week 2 p.i., we designated this infectious dose as optimal for our studies. An infection dose of 50 to 200 cfu is typically used for other animal models and is more relevant to human studies. A small group of humanized BLT mice (n=5) were infected with 250 cfu *M.tb*, termed "low-dose infection", and observed for an extended period of time to evaluate the development of TB disease. By week 3 p.i., approximately 40% (2/5) of mice had succumbed to infection; however, the remaining mice did not succumb to infection until 6 to 8 weeks p.i. (Figure 1.8A). Figure 1.8B shows the gross lung anatomy from a non-infected control BLT mouse (top) and from a *M.tb*-infected BLT mouse at 7 weeks p.i. (bottom). Large granuloma lesions with cellular cuffing and necrotic centers are seen throughout the tissue compared to the lung from the control mouse. Bacterial clustering is observed in the periphery of the lesion with bacterial and cellular debris in the center. Cholesterol crystal formation was seen within the lung granulomas. Bronchial obstructions are observed and contain massive amounts of bacteria (Figure 1.8C). Little pathological changes are observed in the lung among mice from 6 to 8 weeks (data not shown). Liver pathology displayed scattered foci of inflammation which contained bacteria (Figure 1.8D).

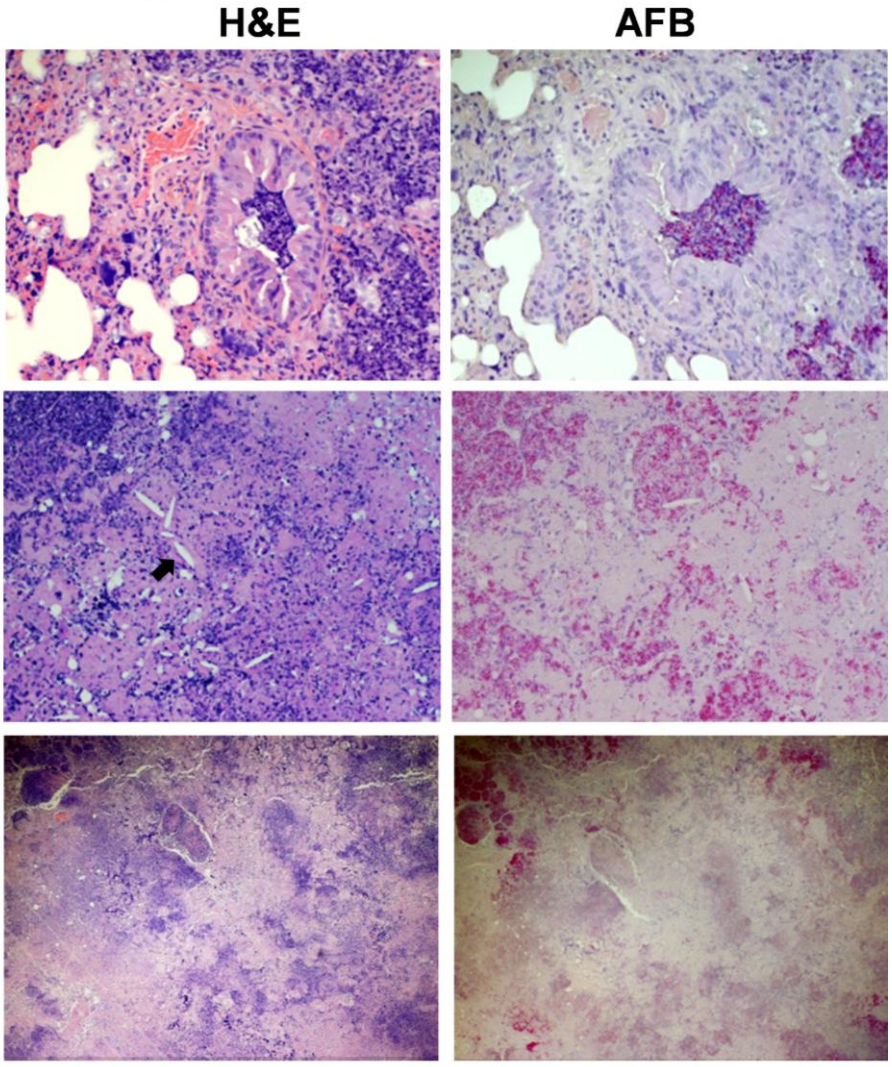
A


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Survival
0
1
2
3
4
5
6
7
8
9
Weeks post-infection (p.i.)

B. Lung


H&E

C. Lung



D. Liver

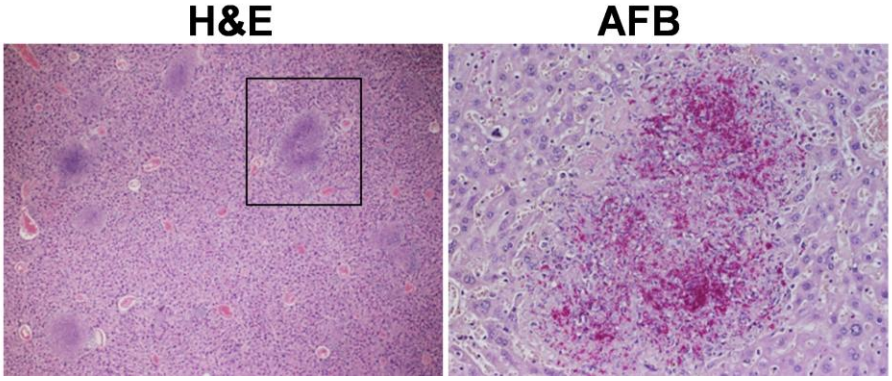


Figure 1.8: TB in BLT mice infected with a low-dose of *M.tb*. Following successful engraftment of human leukocytes, animals were infected i.n. with 250 CFU *tdTomato* H37RV *M.tb*. Survival was monitored and tissues (lung and liver) from representative animals were analyzed. Images demonstrate tissue damage and inflammation (H&E) and localization of *Mtb* bacilli (AFB) in formalin-fixed tissue sections from BLT mice 6-8 weeks p.i. Shown in (A) is survival curve of *M.tb*-infected mice with low-dose infection. (B) gross lung lobes (left panel) and sections of lung stained with H&E captured using a stereomicroscope (right panel). (C) lung tissue visualized by H&E staining (left panels) and AFB (right panels). Top panels (20X) demonstrate bronchial obstruction in the lung and bacteria within the obstruction. Middle panels (20X) show cholesterol deposits (black arrow) observed in large granulomas. Bottom panels (4X) shows center of large, coalescing granulomas characterized by necrosis and lack of AFB. (D) shows liver tissue (left, 4X) and AFB (right, 20X of the indicated region).

DISCUSSION

The development of a small animal model to study the mechanisms of human TB disease is essential to the advancement of TB therapeutic and vaccine development. These studies demonstrate *in vivo* imaging is a valuable technique in understanding the kinetics of *M.tb* infection in animal models, especially in humanized mice, preventing the burden of waiting for bacterial colonies to grow and unnecessarily sacrificing mice throughout studies. Humanized mice develop productive primary infection which can be monitored by IVIS and disseminates to organs beyond the lung, such as the liver and spleen. TB disease develops in the humanized mice with some characteristics similar to humans, including caseous necrosis granulomas, bronchial obstruction, foamy macrophages, lipid deposits, and cholesterol crystal formations. These studies begin to show our group's abilities to model human TB disease in BLT mice.

The use of a fluorescent isolate of *M.tb* allows us to follow the kinetics of infection as has previously been described for traditional animal models [449, 451]. Similar to these studies, we observed a progressive increase in bacterial burden over time and dissemination to organs beyond the lung, including the spleen and liver. An unexpected limitation of this technique is the inability to correlate fluorescence intensity with bacterial load. The lack of fluorescence detection and ability of animals to grow bacteria, as shown with BLT mice infected with 10^2 cfu, demonstrates that expression of the plasmid cannot be correlated with bacterial load. Initial studies using a high infectious dose of 10^6 cfu were meant as a 'proof of concept' to guarantee detection and infection. Ultimately, the optimization of *in vivo* imaging for use in humanized BLT mice allowed for disease progression to be followed and decisions to be made on necropsy time points. This is especially important in this model due to the expense and limited number of animals compared to the use of traditional, non-humanized mice. CFU enumeration of bacterial burden within tissues was utilized to confirm the *M.tb* growth that was indicated by *in vivo* imaging.

A surprising observation in *M.tb*-infected BLT mice was the development of granulomas with cellular cuffing and AFB clustering along the periphery of the lesion, similar to granulomas in human TB disease. More interesting, was the formation of various features distinctive of TB disease in humans. Bronchial obstruction, a characteristic not previously observed in murine models of *M.tb* infection, are filled with cellular infiltrates and AFB. A way of transmitting disease in humans by expelling bacteria upon coughing, bronchial obstruction is typically found early in post-primary TB [455]. Another interesting feature is the foamy macrophages and lipid deposits in necrotic

lung tissues. Though foamy macrophages usually develop from the build-up of lipids unable to transport out the cell; it is also thought that foamy macrophages and lipid deposits can result from bronchial obstruction preventing the release of surfactant which is subsequently taken up by macrophage [455, 456]. The lipids from foamy macrophages are thought to contribute to TB persistence by providing a nutrient source [457, 458]. Foamy macrophages are also involved in caseation through the release and accumulation of lipids in granulomas [457, 459]. Cholesterol crystal formation was also seen in granuloma lesions. Cholesterol is important for mycobacterial phagocytosis into macrophages, prevention of phagosome-lysosome fusion, and persistence with the host by providing a source of carbon [460, 461]. Based on these observations, it is thought that humanized mice have developed active TB disease as represented in Illustration 2 as acute disease that, if seen in humans, would be transmitting from person to person.

Overall these studies provide a method for detecting *M.tb* infection in live animals. In addition, valuable information on the optimal parameters for *M.tb* infection including infection efficiency, ideal infectious dose, and time points for sacrifice were determined which will be used for future studies. The ability of BLT to display pathological states of disease similar to human TB disease supports its use as a model of *M.tb* infection and subsequent HIV/*M.tb* co-infection.

CHAPTER 4[3]

A Humanized Mouse Model of Tuberculosis

M.tb infection is studied in various small animal models including mice, guinea pigs, and rabbits [408]. The ease and availability of mice make it the most used model for *M.tb* infection; although the inability of the model to develop granuloma lesions similar to human disease compromises the interpretation of experimental outcomes [462]. Guinea pigs, rabbits, and NHP are able to develop granulomas similar to humans TB disease, although the study of *M.tb* infection in the guinea pig and rabbit is limited by the unavailability of immunological reagents [408]. Moreover, these models cannot be used to study HIV/*M.tb* co-infection because of the host tropism of HIV.

We have shown in previous studies that BLT mice are able to develop pathology similar to human TB disease. These studies will further demonstrate the ability of the humanized BLT mouse to develop TB pathology similar to that observed in humans by following the course of infection and support its use to study immune mechanisms of disease. Humanized mice were successfully reconstituted with human leukocytes, including T cells, macrophages, NK cells, and APCs. Following *M.tb* infection humanized BLT mice displayed progressive and disseminated bacterial infection, and organized lesions with necrotic centers at sites of infection. Human T cells ($CD4^+$ as well as $CD8^+$) and monocytes/macrophages were distributed normally in tissues. Importantly, human T cells in our mouse model are functional, able to proliferate in response to

[3] sections taken from Calderon *et al*, A Humanized Mouse Model of Tuberculosis, *PLOS* One, 8(5): e63331

activation, express effector molecules (e.g. IFN-γ, granulysin), and are recruited to and organize at sites of infection. These studies represent a significant advance by providing a small animal model to understand the human immune response to *M.tb* and enable critical studies of HIV/*M.tb* co-infection.

RESULTS

Production of the humanized BLT mouse model to study TB

The BLT mouse has been previously described as a model where significant numbers of human leukocytes can be successfully reconstituted [435]. To demonstrate that our group can reproduce this model, NOD/SCID/γ_c^{null} (NSG) mice were engrafted with human fetal liver and thymus tissues and $CD34^+$ cells as previously described [435]. As shown in Figure 2.1 and 2.2, our group is able to successfully reconstitute human immune populations. Twelve weeks post-engraftment, BLT mice have high levels of human leukocytes ($CD45^+$ cells) in peripheral blood (average 27% of the total cells, n=44) (Figure 2.1A). Immediately prior to infection, human leukocytes were further evaluated in the peripheral blood of BLT mice to determine levels of human T cells, T cell subsets (CD4 and CD8), and APCs. Figure 2.1A and B demonstrate that a large proportion of the total human leukocytes were T cells (average 71% of the total human $CD45^+$ population), including both CD4 and CD8 T cells subsets. The circulating human leukocyte pool included small numbers of CD3-$CD56^+$ NK (Figure 2.1B) and NKT cells (~1% of the total human $CD45^+$ population, data not shown). Human monocyte/macrophage cells, identified by expression of the CD14 marker, were also observed (Figure 2.1B). To evaluate the potential for these cells to activate T cells, key surface markers on $CD14^+$ cells were evaluated; as shown in Figure 2.1D, the $CD14^+$

population expresses receptors required for antigen presentation (HLA-DR) and lymphocyte activation (CD40, CD80, and CD86).

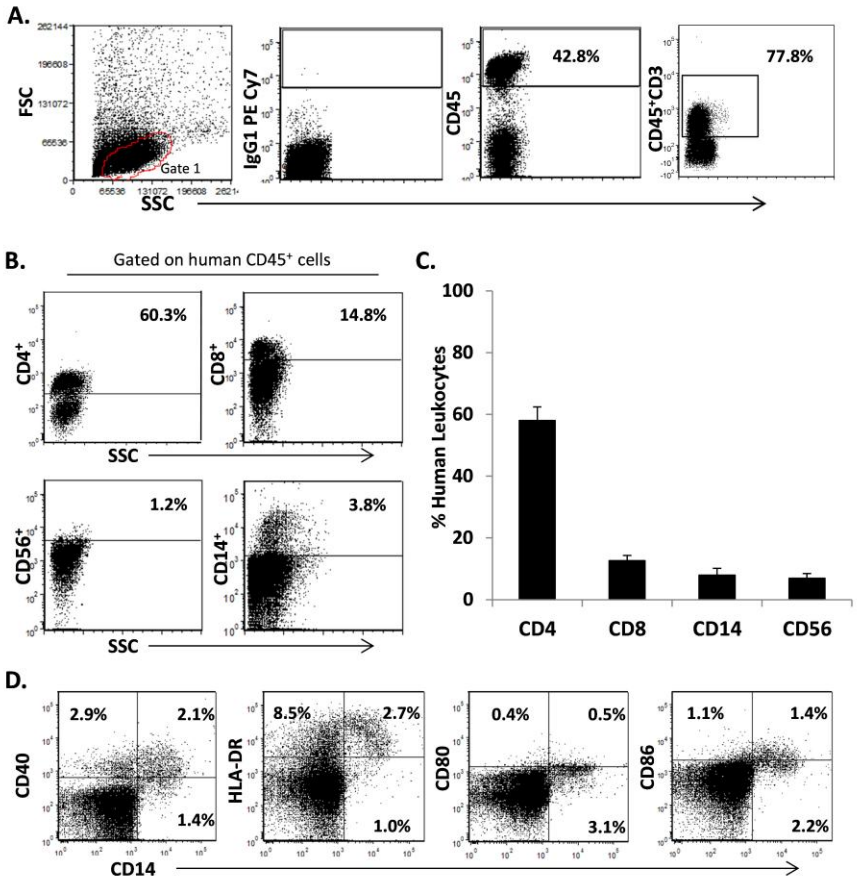


Figure 2.1: Production of the humanized BLT mouse to study TB. NOD/SCID/γ_c^{null} (NSG) mice were engrafted with human fetal liver and thymus, and supplemented with $CD34^+$ cells. Shown in A flow cytometry analysis displaying side scatter (SSC) and forward scatter (FSC) characteristics (Gate 1) of isolated peripheral blood from a representative BLT mouse twelve weeks post-engraftment. Plots 2-4 are the gating strategy for selection of cells expressing human CD45 pan leukocyte marker, the corresponding isotype control (IgG1 PE Cy7), and the $CD3^+$ population subgate. (B) percentage of the gated cells expressing markers for T cell subsets (CD4, CD8), NK cells ($CD3^-CD56^+$) and monocyte/macrophages ($CD14^+$). (C) average leukocyte % among gated CD45 cells in four groups of reconstituted BLT mice (n=44) used for the subsequent studies (Fig. 2-8). D shows the expression of antigen presenting cells (APC) markers relevant to antigen presentation (HLA-DR) and T cell activation (CD40, CD80, CD86) expressed by peripheral blood monocyte/macrophages.

Tissue distribution of human leukocytes in humanized BLT mice

TB is primarily a disease of the lung; however, the bacteria can additionally disseminate to and cause immune-mediated pathology in many organs. To assess the potential for human leukocytes to migrate to tissues, we evaluated leukocyte populations in the lung, spleen, and liver tissues of BLT humanized mice. Organs were harvested following reconstitution (≥12 wks), processed to single-cell suspensions, and labeled with human leukocyte surface phenotype markers (CD45, CD3, CD4, CD8, CD14, CD56, CD1a), or isotype-matched nonspecific antibody as a control, and analyzed by flow cytometry. Human leukocytes ($CD45^+$ cells) were abundant in the spleen (Figure 2.2) of non-infected BLT mice and could also be found in the lung and liver. T cells ($CD3^+$) were especially noted in the spleen and liver, while a large population of cells with a monocyte/macrophage phenotype ($CD14^+$) was noted in the lung. Cells with a tissue DC phenotype ($CD45^+CD14^-CD1a^+$) were also observed in the lung, liver, and spleen (data not shown) similar to reports by Melkus, *et al*, [435].

Both $CD4^+$ and $CD8^+$ T cell subsets were represented in all 3 tissues, while NK cells ($CD56^+$), though low, were most abundant in the spleen (Figure 2.2). In samples from the blood and spleen, the $CD4^+$ and $CD8^+$ T cell subsets generally accounted for >95% of the total $CD3^+$ population. A preliminary experiment with samples from 1 animal demonstrated that human $CD3^+$ cells expressing the gamma delta T cell receptor (γδ-TCR) can also develop in our BLT mouse (data not shown). In both blood and tissue, the NK cells and APC populations generally accounted for ~50% of the CD3- (non-T cell) $CD45^+$ cells. This suggests that other human leukocytes, possibly B cells and DCs, also comprise a large part of the non-T cell population. Overall, the distribution of human

leukocyte populations that we observed in our model is fairly consistent with those reported by Melkus, *et al*., [435] though we noted generally less human leukocytes in the liver in our mice (Figure 2.2).

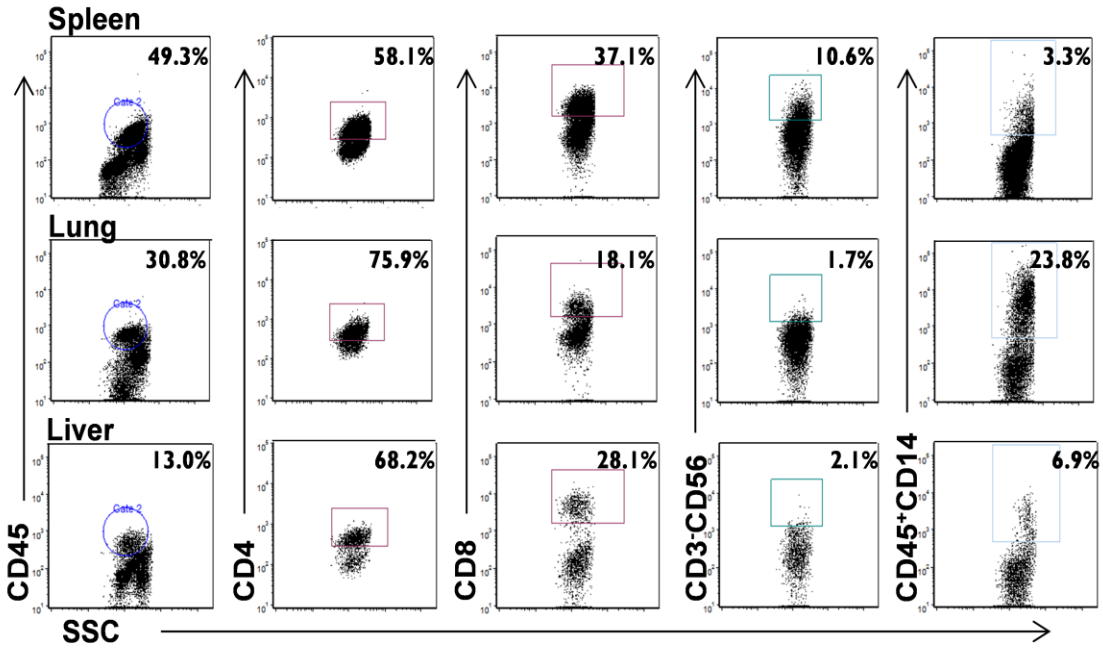


Figure 2.2: Functional potential of splenic T cell populations in the BLT mouse. Spleens were disrupted to single-cell suspensions and activated with control (media), antibodies to human CD3/CD28, or rIL-15 (15 ng/ml) for 3 days. Following activation, the surface expression of cellular phenotype markers (CD45, CD3, CD4, and CD8) and intracellular proteins (Ki67, granulysin, perforin, and IFN-γ) was detected using flow cytometry. Shown in A are side scatter and forward scatter characteristics of isolated splenocytes (Gate 1), gating strategy to enable analysis of human CD45 $^{+}$ and CD3^{+} cell populations and individual T cell (CD4 and CD8) subset gating. (B) increase in % of human CD45^{+} cells within the isolated splenocytes following 5 d activation with recombinant IL-15 (15 ng/ml) (upper plots) and activation-induced increase in expression of the proliferation marker Ki67 (lower plots) by treatment displayed as a color dot plot overlay. (C) expression of effector molecules (granulysin, perforin, IFN-γ) shown by dot plot overlays demonstrating inducible expression upon activation with CD3/CD28 (red) and rIL-15 (blue) compared to non-activated cells (black). Data shown in A, B, and C is from a representative mouse (n=3).

Functional potential of splenic T cell populations in the BLT mouse

Though human T cells are generally well reconstituted in various humanized mouse models, in other reports these cells have been shown to have functional defects [463, 464]. In the BLT model, studies have shown the ability of humanized T cells to recognize antigen, proliferate, and express human cytokines upon activation [435, 438, 465]. To assess the functional potential of cell-mediated immune populations from the humanized mice generated in our laboratory, we activated splenocytes using anti-CD3/CD28 or recombinant IL-15 (rIL-15) as positive stimuli. A strong proliferative response was observed by both $CD4^+$ and $CD8^+$ T cells to positive stimuli, as measured by intracellular levels of the Ki67 proliferation marker (Figure 2.3B, lower plots). This effect was also evident from the change in % of total cells in the human $CD45^+$ cell gate following 5 d of activation with rIL-15 (Figure 2.3B, upper panels). Importantly, both T cell populations expressed increased levels of the effector molecules IFN-γ, perforin, and granulysin in response to activation as shown in Figure 2.3C. Similar to human peripheral blood T cells [467], $CD4^+$ T cells constitutively express IFN-γ while $CD8^+$ T cells constitutively express perforin and granulysin, and can also greatly increase expression of these cytotoxic/antibacterial molecules upon activation. We additionally show that splenic T cells from BLT mice are able to proliferate and increase effector protein expression in response to recombinant human IL-15 (Figure 2.3C), an important molecule for T cell survival and effector activity.

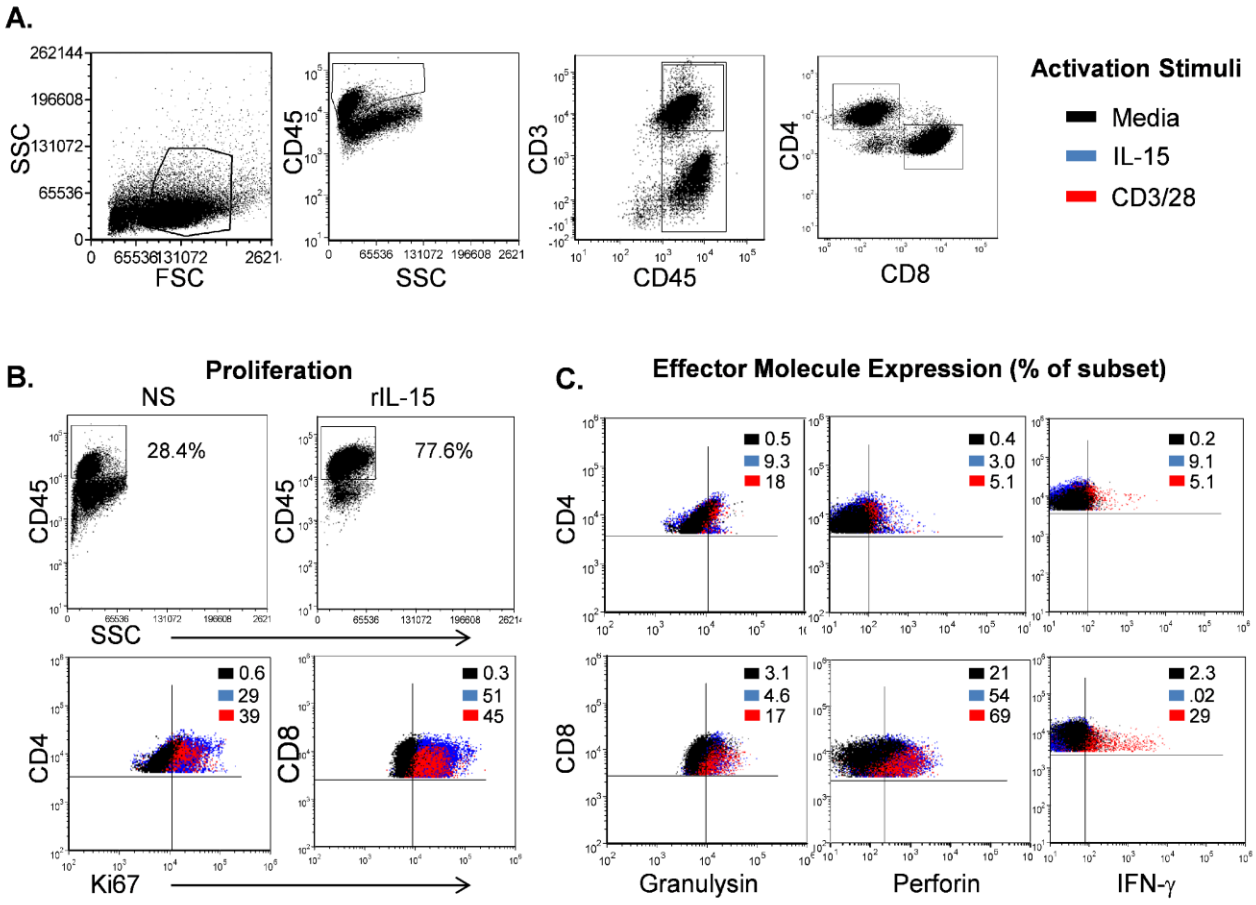

A.
SSC
FSC
CD45
SSC
CD3
CD45
CD4
CD8
Activation Stimuli
Media
IL-15
CD3/28
B.
Proliferation
NS
rIL-15
28.4%
77.6%
CD45
SSC
CD4
Ki67
CD8
0.6
29
39
0.3
51
45
C.
Effector Molecule Expression (% of subset)
CD4
CD8
0.5
9.3
18
0.4
3.0
5.1
0.2
9.1
5.1
3.1
4.6
17
21
54
69
2.3
.02
29
Granulysin
Perforin
IFN-γ

Figure 2.3: Functional potential of splenic T cells demonstrating expanded effector biosignature. Spleens were disrupted to single-cell suspensions and activated with control (media), antibodies to human CD3/CD28, or rIL-15 (15 ng/ml) for 3 days. Following activation the surface expression of cellular phenotype markers (CD45, CD3, CD4, and CD8) and intracellular proteins (Ki67, granulysin, perforin, and IFN-γ) was detected using flow cytometry. Shown in A is side scatter and forward scatter characteristics of isolated splenocytes (Gate 1), gating strategy to enable analysis of $CD45^+$ and $CD3^+$ cell populations and individual T cell (CD4 and CD8) subset gating. (B) increase in % of human CD45+ cells within the isolated splenocytes following 5 d activation with antibody to CD3/CD28 (top panels) and activation-induced increase in expression of the proliferation marker Ki67 due to activation. (C) expression of effector molecules (granulysin, perforin, IFN-γ) upon activation with CD3/CD28 and rIL-15. Shown in the bottom panels in B and in C are color dot plot overlays with expression of intracellular proteins for non-activated (black), and activated (CD3/CD28; red, IL-15; blue) CD4 and CD8 T cells from a representative mouse (n=3).

M.tb infection in the BLT mouse

Currently, there are no published studies, to our knowledge, describing *M.tb* infection in the BLT, or other humanized mouse models. To begin to evaluate the development of *M.tb* infection in BLT model, mice were infected with i.n. with a "high-dose" of 10^6 CFU *tdTomato* H37Rv *M.tb*. CFU analysis at weekly time points (Figure 2.4) demonstrates the ability of *M.tb* to progress in the lungs and disseminate to various organs within BLT mice.

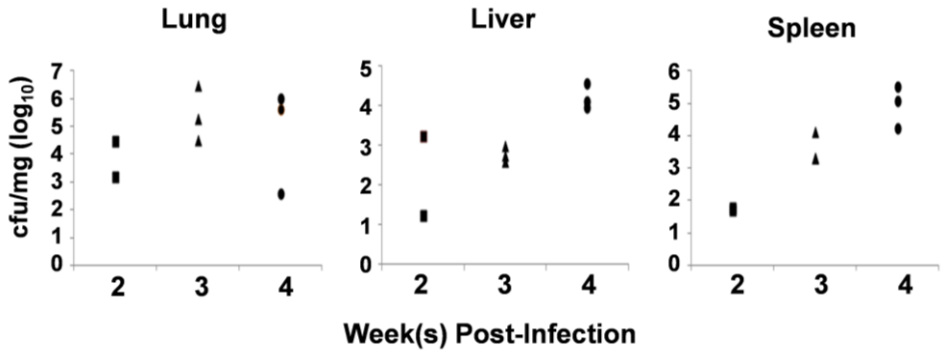
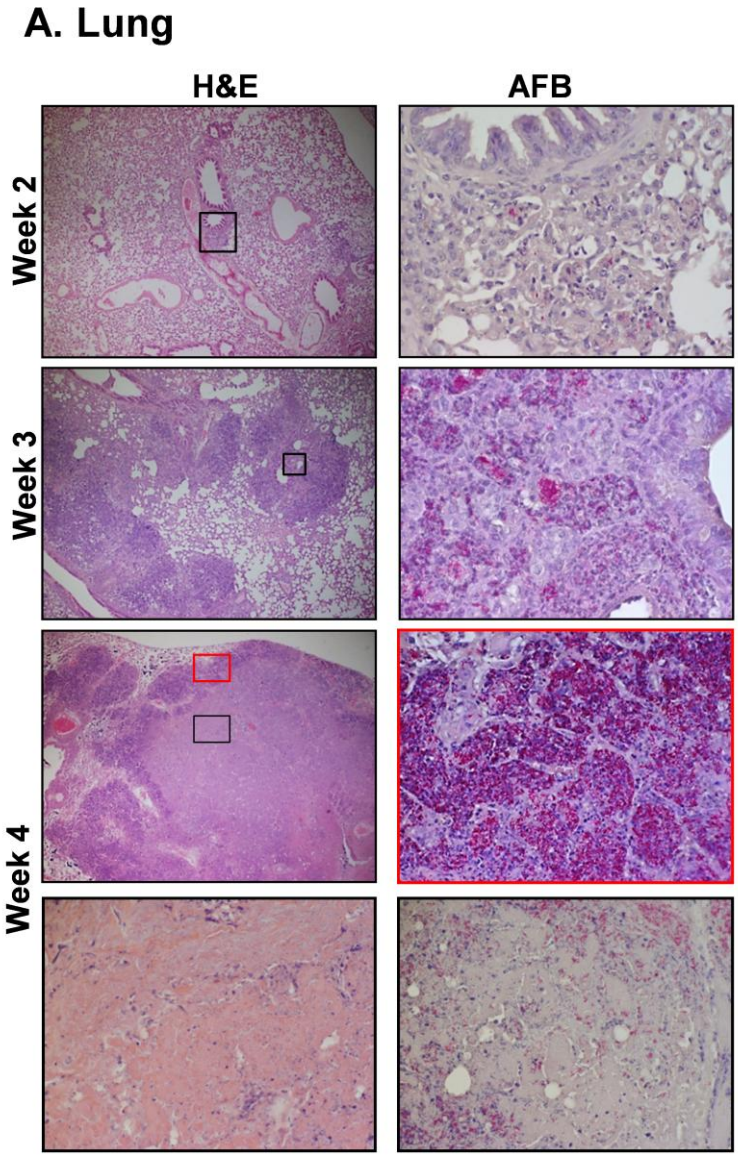


Figure 2.4: Progression of *M.tb* infection in the humanized BLT mouse. Animals (n=8) were infected i.n. with 10^6 CFU *tdTomato* H37Rv *M.tb* and bacterial load determined with serial dilutions on 7H11 agar plates. Colony-forming units (cfu) are shown per milligram (mg) of tissue from individual animals (n=2-3 per time point) at the 2, 3, and 4 weeks p.i.

Tissue Pathology in *M.tb*-infected BLT mice

To evaluate the development of TB in humanized mice, animals were sacrificed at specified time points and tissues obtained for cfu enumeration and pathology analysis, as previously described. Figure 2.5A shows H&E and AFB images at various time points from BLT mice infected with a high dose of *tdTomato* H37Rv *M.tb*. At 2 weeks p.i., multiple foci of mild interstitial inflammation are observed interspersed with areas of normal lung tissue. Few bacteria are observed and contained within the inflammatory areas. Bacteria are also observed in the bronchus (Appendix A) suggestive of endo-bronchial spread; although inflammatory cell obstruction is not seen at this time point. By week 3 p.i., severe interstitial inflammation is seen along with thickened alveolar walls and inflammation around bronchovascular bundles. Bacterial clustering is evident and primarily contained within these areas of inflammation. Additionally, bronchial obstruction is also observed as previously described for the low-dose group (Figure 1.8). At week 4 p.i., granuloma formation is clearly identified with cellular cuffing and central caseous necrosis. Bacteria are contained in clusters around the

periphery of the granuloma within alveolar pockets. Bacterial debris is also seen scattered throughout the necrotic center of the granuloma, along with pyknotic nuclei and cellular fragmentation (Figure 2.5A). Figure 2.5B shows liver pathology from BLT humanized mice infected with 10^6 *tdTomato* H37Rv *M.tb* at various times following infection. At week 3 p.i., multiple areas of organized inflammatory foci are observed and primarily located around the portal triade (Figure 2.5B). By week 4 p.i., inflammatory areas are located around the portal triade and throughout the tissue. At both time points, bacteria are relatively contained within the foci of inflammation. No significant obstructions are seen in the liver.



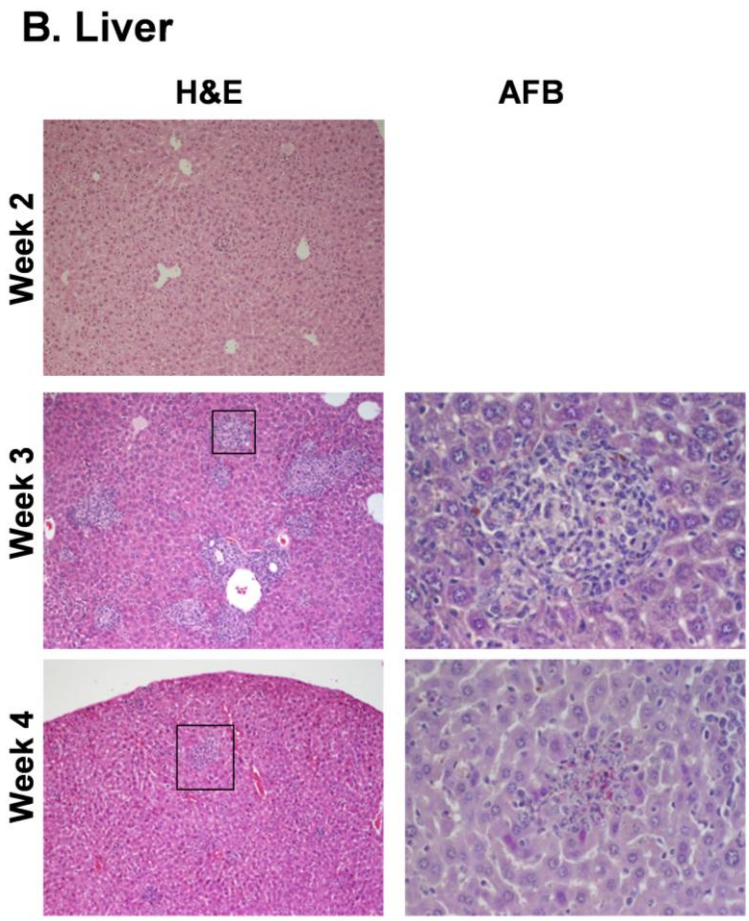


Figure 2.5: ***M.tb*-infected humanized mice have lung and liver pathology consistent with development of TB.** Animals were infected i.n. with 10^6 CFU *tdTomato* H37RV *M.tb* following establishment of human immune cell populations. Shown are images captured by brightfield microscopy following staining of infected tissues using hematoxylin and eosin (H&E) and Ziehl-Neelson to detect acid fast bacilli (AFB). Shown are images of tissue damage and inflammation localized to *Mtb* bacilli in formalin-fixed tissue sections of BLT mice infected i.n. with 10^6 cfu *tdTomato Mtb* H37Rv. Images are representative of mice sacrificed at 2, 3, and 4 wk pi described in Fig. 3. Shown in (A) lung tissue visualized by H&E staining (left panels, 4X). Localization and burden of bacilli are shown in right panels (40X) from the region indicated in the H&E image (black or red boxes), as visualized by acid fast and hematoxylin staining. (B) liver tissue at 2, 3, and 4 weeks post-infection (H&E 10X, AFB 40X of indicated region).

M.tb infection in control NSG mice without human leukocytes differs from that observed in humanized mice

To evaluate the contribution of the human immune system of the mouse in our studies, we also infected a small group (n=4) of non-humanized NSG mice (Figure 2.6) and used one non-infected NSG mouse as a control. These animals were infected with 10^5 CFU *tdTomato M.tb* H37Rv instead of 10^6 due to concerns for a greatly accelerated course of infection due to the lack of an adaptive immune system. Surprisingly, these animals did not all rapidly succumb to disease as expected, given the significant immune deficiencies of these animals. Of the 4 infected animals, 1 expired at <1 week and the remaining animals were euthanized at 2 (n=1) and 6 (n=2) weeks. The animal that died at <1 week died as a consequence of anesthesia complications, and was not thought to have succumbed to infection. To assess disease, tissues were harvested at 2 and 6 wk p.i. At week 2 p.i., scattered areas of inflammation are observed in the lung with slight interstitial and alveolar wall thickening (Figure 2.6A, top). Bacterial burden is diffuse with occasional interstitial macrophages containing relatively few bacilli. By week 6 p.i., massive interstitial thickening due to inflammation was observed along with intra-alveolar infection (Figure 2.6A, bottom). Bacterial clustering was seen and contained to areas of inflammation, primarily within alveolar sacs. At this time point, bacterial infection was further seen in the bronchial wall (data not shown). Within the liver at 2 weeks p.i., normal tissue architecture was observed, along with a few inflammatory foci and relatively few bacilli (Figure 2.6B). Throughout the liver at week 6 p.i., primarily neutrophilic areas of inflammation were seen containing bacteria (Figure 6B). The extensive granulomatous response observed in the humanized NSG mice was not

observed in these mice lacking reconstitution with human cells. The studies confirm that the results observed with a low-dose infection are not due to differences in dose.

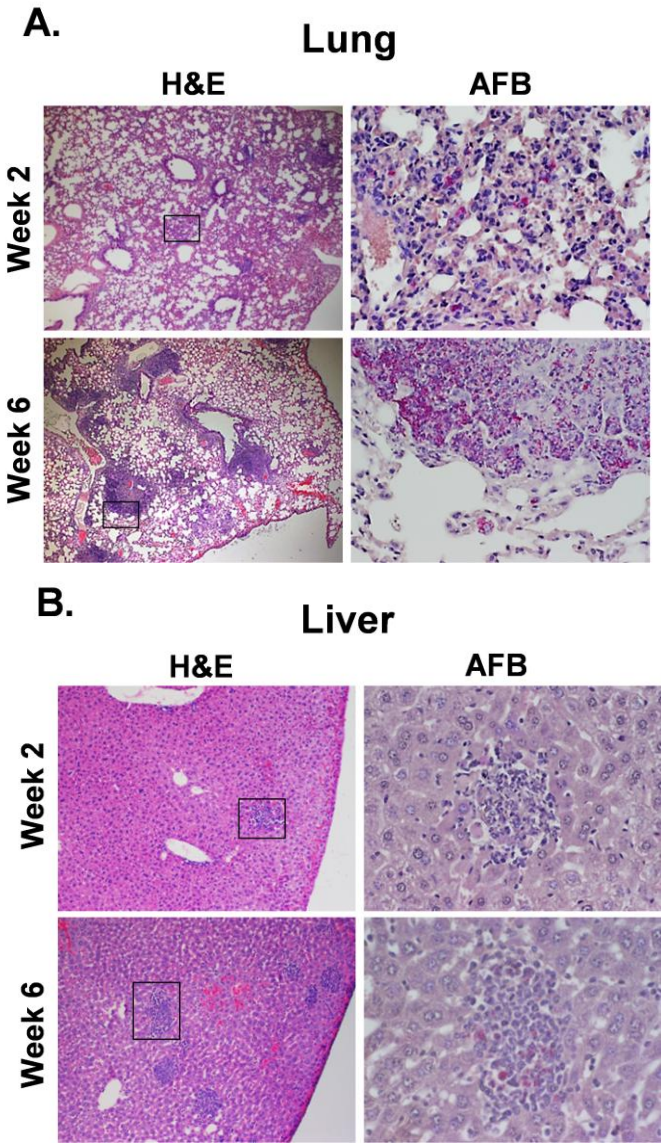


Figure 2.6: ***M.tb* infection in non-reconstituted NSG mice differs from that observed in humanized mice.** NSG mice that were not engrafted with human tissue or stem cells were infected i.n. with *Mtb*. Shown are images captured by brightfield microscopy following staining of infected tissues using H&E and AFB. Shown in (A) lung and (B) liver tissue and localization of AFB at 2 and 6 weeks post-infection (H&E 4X, AFB 40X). Results are representative of mice sacrificed at 2 (n=2) and 6 (n=3) weeks from a group of 5 animals.

Human T cells are recruited to and organize at sites of inflammation following *M.tb* infection.

The lymphocytic mantle, composed primarily of T lymphocytes, is a key feature associated with *M.tb* containment in the granuloma. Here, immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded tissue to determine if this classical feature, associated with both protective host response and immune-mediated pathology, occurs in our model. Consistent with human tissue and other animal models, T cells are characteristically organized to the outer area of sites of inflammation. At 3 weeks p.i., human $CD3^+$ T cells are detected in the periphery of lung granulomas and foci of infection in both lung and liver (Figure 2.8A). Similarly, $CD3^+$ T cells are also found in the center of larger lung granulomas, though in fewer numbers. These results suggest that the human T cells in the BLT mouse are able to respond to cytokines and chemokines expressed at sites of *M.tb* infection.

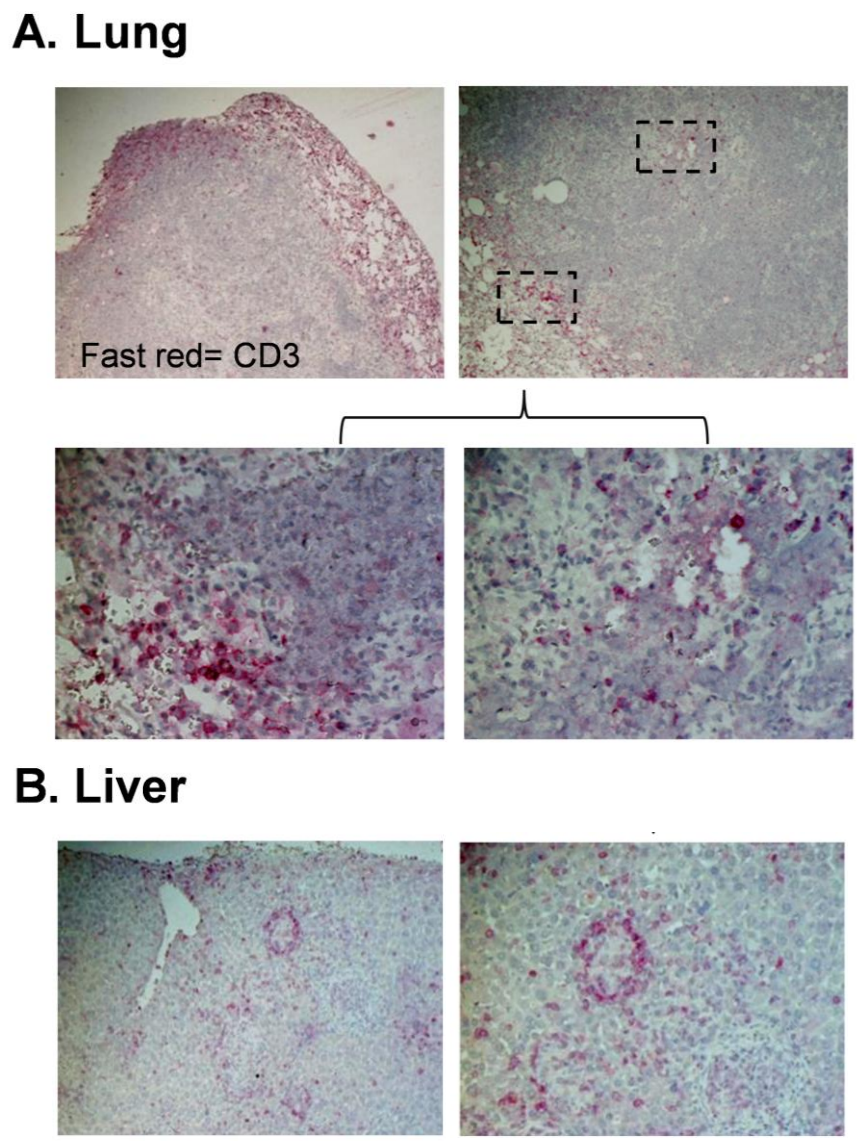


Figure 2.8: Human T cells are recruited to and organized in the periphery of lung granulomas and sites of inflammation following *M.tb* infection. Animals were infected i.n. with 250 CFU *tdTomato* H37RV *M.tb* following establishment of human immune cell populations. Formalin fixed paraffin embedded tissue sections were cut, dewaxed and stained with antibody to human CD3. Marker expression was visualized with Fast Red substrate and images captured by brightfield microscopy. (A) shown is localization of T cells relative to a granuloma periphery and center (top left; 4X, top right; 10X). Enlarged images of T cell staining in the indicated areas are shown in the bottom panels (40X). (B) Human T cells in portal tracts and sites of inflammation in the liver (top panel, 10X) and an enlarged area showing orchestration of T cells around an inflammatory foci (bottom panel, 20X). Shown are representative images (n=3) of animals sacrificed at 7 weeks pi.

DISCUSSION

These studies show, for the first time, that the humanized BLT mouse is able to become productively infected with *M.tb* and display TB disease similar to human infections. BLT mice were generated with a high level of human leukocyte reconstitution, including $CD4^+$ and $CD8^+$ T cells that can respond to activation with expression of effector molecules able to target *M.tb*. This represents a significant advance in our ability to study specific aspects of the human cell-mediated immune response to *M.tb*. These results provide support for the humanized BLT mouse as a promising small animal model to study *M.tb* infection. Moreover, we anticipate the BLT mouse model will be essential to understanding the mechanisms of immune dysregulation in *M.tb* infection during HIV co-infection.

To date, the BLT mouse has contributed to the understanding of various other infectious diseases including Dengue virus [439, 440], Epstein-Barr virus [435], *Plasmodium falciparum* [441], and *Salmonella* [442]. Perhaps, the most significant contribution is occurring with HIV research. The humanized BLT model has allowed for studies using natural routes of transmission, latency, and effectiveness of antiretroviral therapeutics [437, 443, 466].

In BLT mice, DCs have been shown to constitutively express markers for T cell interaction; these markers are up-regulated after stimuli and activate T cells [435]. The functional capacity of $CD14^+$ monocyte/macrophage populations in NSG mice reconstituted with $CD34^+$ fetal stem cells have been shown to be phenotypically immature, lacked expression of activation and antigen presentation markers [467]. Our studies in the BLT mouse model demonstrated $CD14^+$ cells from peripheral blood of

NSG BLT mice constitutively express HLA-DR, CD80, CD86, and CD40 (similar to DC populations in other studies [435]), and $CD14^+$ monocyte/macrophage populations are phenotypically mature and able to respond to stimuli. The functional maturity of macrophages is essential for the host's immune response for *M.tb* uptake and host response. A recent study supporting the phagocytic function of macrophages in humanized mouse models demonstrated phagocytic uptake and IFN-γ-mediated intracellular killing of *Salmonella* Typhi [468].

As previously discussed, cell-mediated immunity is essential for a protective response against *M.tb* infection. In the BLT mouse, we observed that IFN-γ is expressed, both constitutively and following stimuli, by $CD4^+$ and $CD8^+$ T cell in peripheral blood and tissues. $CD8^+$ T cells in the BLT mouse also constitutively express perforin; these results are similar to those observed by Schultz *et al*. [465] in the $CD34^+$ stem cell-reconstituted NSG model. Further, we were able to demonstrate that $CD4^+$, $CD8^+$, and NK cell populations express the antimicrobial granulysin protein, as well as perforin, upon activation.

Granulysin, a cytolytic granule protein secreted by cytolytic T cells and NK cells, kills mycobacteria via a perforin-mediated mechanism [469, 470]. Studies to identify the role of granulysin are primarily performed *in vitro* [470, 471] as a gene homologue for granulysin does not exist in the mouse [472]. In lung tissue, granulysin is selectively reduced at sites of TB infection indicating a potentially important role in the host's response to *M.tb* [473, 474]. Granulysin is also suppressed in T and NK cells during HIV co-infection [475-477]. The ability to detect for granulysin in the BLT mouse model will allow for further understanding of its role during *M.tb* infection. Moreover, granulysin

will be evaluated for use as a potential biomarker within BLT mice to predict the stage of infection.

A critical observation in these studies was the hallmark of human TB infection, "caseating" granulomas. *M.tb*-infected BLT mouse (<3 weeks p.i.) displayed lung pathology similar to other mouse models; consisting of interstitial thickening and relatively small foci of inflammation. By 4 weeks p.i., granulomas were observed with cellular accumulation, primarily T cells, along the periphery and caseous necrosis in the center. Similar to human TB disease, AFB were primarily found in the periphery. BLT mice also displayed bacterial dissemination with organized foci of infection and progressive bacterial load in the spleen and liver. Typically, granuloma formation has only been seen the guinea pig [415], rabbit [417], and NHP [478]. Traditional mouse model of TB, such as the BALB/c and C57BL/6, do not show the spectrum of granulomatous lesions associated with human TB disease. However, necrotizing granulomas have been observed in immunosuppressed mouse models with targeted deletions of important immune mechanisms, the IFN-γ deficient (GKO) and the Kramnik (C3HeB/FeJ) mouse models [133, 479-481].

The non-reconstituted NSG mouse did not reproduce the TB disease as observed in BLT mouse. NSG mice lacking human immune cell reconstitution developed an inflammatory disease that was characterized by small but organized foci of infection in the lung and dissemination to the spleen and liver. Surprisingly, most animals survived to 6 weeks and based on tissue pathology and bacterial load would likely have survived much longer than the planned time points. This is unexpected, as these animals are severely immune compromised. By 6 weeks p.i., however, the NSG mice failed to

develop the large, organized, granulomas that were observed in the BLT mice. Though this demonstrates the contribution of the human cells for development of important features of TB pathology in the model, it is still very interesting that these severely immune-compromised animals survived several weeks post infection. GKO mice succumb to the infection within 5-8 weeks after a low dose challenge of 50-200 cfu [482, 483]; while C3HeB/FeJ mice succumb to the disease within 4-12 weeks depending on dose [479-481]. Like these models, the NSG mice have retained considerably high level of functional murine neutrophils which appear to be playing an important role in bacterial infection [133, 481-484].

Of further importance and similar to human TB disease, human T cells were found at the granuloma periphery in BLT mice infected with *M.tb*. Although considered to be characteristic of granulomas and part of the *M.tb* containment mechanism, the accumulation of lymphocytes around the granuloma may permit for the entry of HIV-infected $CD4^+$ T cells [366]. Recruitment of T cells to sites of infection will allow for the study of cell-mediated immunity by HIV infection in and around granuloma lesions. The recruitment of T cells to these sites of infection will enables several avenues of investigation important for vaccine design, including studies of human memory T cell populations and effector molecule profiles associated with specific pathology/.

Overall, these studies provide support for BLT mouse as a model to understand *M.tb* infection. The expense of generating BLT mice may be greater than the traditional mouse, guinea pig, and rabbit models; although, compared to the expense of the NHP, BLT mice are considerably cheaper. The availability of immunological reagents is another benefit in utilizing this model which will greatly aid in deciphering *M.tb* disease

mechanisms and host immune response. Moreover, we have the ability to develop a small animal model for HIV/*M.tb* co-infection and begin to understand the additive effects of disease mechanism.

CHAPTER 5

HIV/M.tb Co-infection in the Humanized Mouse

HIV/*M.tb* co-infection is a significant health problem with massive morbidity and mortality. The ability of these pathogens to act in a synergistic manner results in an accelerated rate of HIV disease and an increased risk of new TB infection or reactivation [5, 379, 485]. The effective treatment of co-infected individuals has been hampered by various obstacles including high pill burden, higher toxicity and adverse effects, development of immune reconstitution inflammatory syndrome, and drug interactions [365, 486, 487]. As a result new drug compounds are rapidly needed to treat and reduce the global health threat of a continuously increasing co-infection rate. Treatment development can only begin with an understanding of the mechanisms that promote disease. The humanized mouse model has been developed to assist in identifying these mechanisms which will greatly aid in developing new treatments against HIV and *M.tb* in co-infected populations.

Currently, the NHP is the only available animal model for co-infection studies. Macaques are used based on their ability to develop TB disease and AIDS similar to humans [423]. During co-infection macaques develop increased viral load, reduced $CD4^+$ lymphocytes, accelerated disease progression to AIDS, disseminated *M.tb* infection, loss of antigen-specific T cells, granulomatous lesions, latent TB infection, and reactivation [488-491]. However studies with NHP are performed with SIV since primates are resistant to HIV infection [423]. This poses a significant limitation considering HIV and

SIV differ considerably including genetic heterogeneity, host receptor used for entry, and disease progression [492, 493].

Our studies show the humanized BLT mouse is able to develop HIV/*M.tb* co-infection characterized by increased *M.tb* disease severity and dysregulated cell-mediated immune responses. We show, following successful reconstitution with human leukocytes, humanized mice develop productive viral infection with a significant decrease in $CD4^+$ T lymphocytes, characteristic of human HIV disease. Pathology analysis demonstrates an accelerated rate of granuloma lesion development with necrotic centers in co-infected mice while *M.tb*-infected mice primarily display moderate to severe inflammation. Further, T lymphocytes are localized to sites of infection in both infection groups with an increasing number of CTL lymphocytes seen in co-infected mice. Expression of cytokine and chemokine levels correlated with disease pathology. These studies provide convincing evidence for further exploration of the humanized BLT mouse as a small animal model to understand the altered immune responses to *M.tb* infection during HIV co-infection.

RESULTS

Development of HIV/*M.tb* co-infection in the BLT mouse

An appropriate animal model to study HIV/*M.tb* co-infection has yet to be identified as HIV does not infect currently available animal models. To date no humanized mouse models, including the BLT humanized mouse, have not been used to study HIV/*M.tb* co-infection. We began our studies by engrafting NSG mice with human fetal tissues as previously described [435, 494]. Peripheral blood was analyzed prior to

infection (≥12 weeks post-engraftment) and reconstitution was successful with high human leukocyte levels detected (average 54% $CD45^+$, n=11) as shown in Appendix B. To assess the development of HIV/*M.tb* co-infection in the BLT humanized mouse, humanized mice (n=7) were intravenously (i.v.) infected with 2500 $TCID_{50}$ HIV (JR-CSF). At week 6 and 8 post-infection (p.i.), HIV viral load was detected in peripheral blood and tissues (spleen, liver, and lung) by HIV-1 gag RNA expression and HIV viral replication (capsid p24 antigen) as seen in Figure 3.1 A and B, respectively. HIV infection was further confirmed with immunohistochemistry (IHC) staining for HIV p24 antigen in the tissues (Figure 3.4B, Figure 3.5B,D).

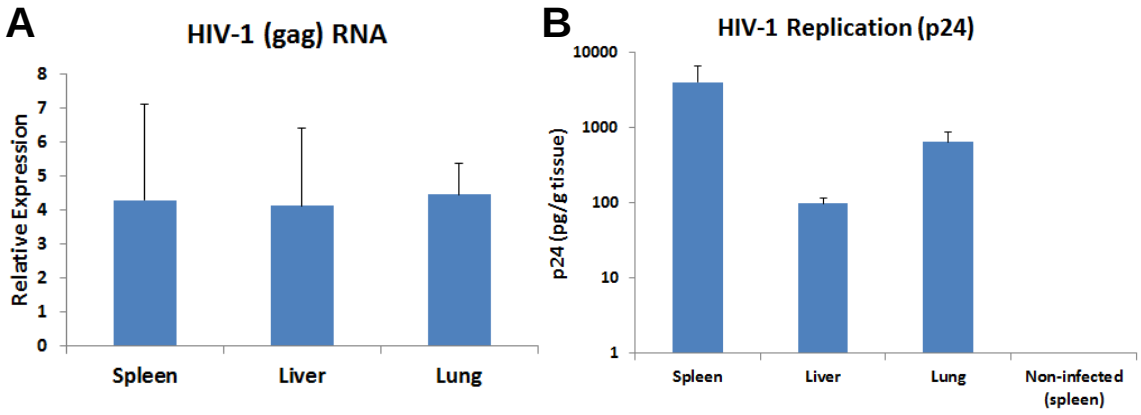


Figure 3.1: HIV-1 infection in BLT mice. Animals were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) following establishment of human immune cell population. At 8 weeks p.i., viral load was determined in tissues and peripheral blood. Shown in (A) is tissue RNA assayed for *gag* gene expression using real-time PCR. (B) shows tissue supernatants assayed to determine HIVp24 expression using ELISA, data shown in log_{10} scale.

Three weeks p.i., HIV-infected and non-HIV-infected (n=5) humanized mice were intranasally (i.n.) infected with 200 CFU *M.tb* H37Rv (n=5). The three week time point was used based on previous studies that evaluated viral load in plasma of HIV-infected BLT mice [436-438] and an independent study performed by our lab (data not

shown). Three weeks after *M.tb* infection in HIV-infected mice, bacterial load was barely detectable and limited to the lung of co-infected animals; while dissemination was beginning to be observed in a one of two *M.tb*-infected BLT mice analyzed (Figure 3.2A). By 5 weeks co-infection, bacteria had disseminated to various organs and were higher in co-infected mice (Figure 3.2B).

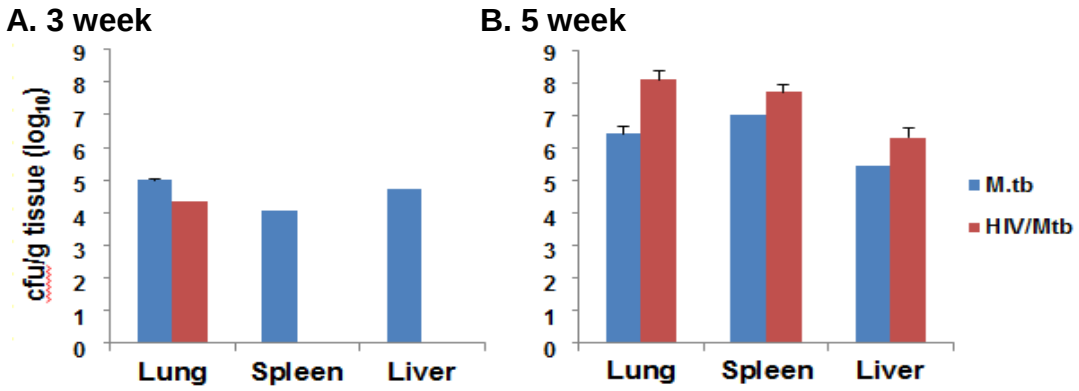


Figure 3.2: Bacterial load in *M.tb* and HIV/*M.tb* infected BLT mice. Animals were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) and 3 weeks later infected i.n. with 10^2 cfu H37Rv *M.tb*. At (A) 3 week and (B) 5 week p.i., cfu was determined using serial dilutions on 7H11 agar plates. Data is expressed as cfu per gram (g) of tissue from the average of *M.tb*-infected (lung n=2; liver, spleen n=1) and HIV/*M.tb*-infected (n=4) animals.

Depletion of $CD4^+$ T cells in HIV/*M.tb*-infected BLT mice.

HIV infection is characterized by a depletion in $CD4^+$ T cells [248]. At 3 and 5 weeks p.i., peripheral blood of HIV/*M.tb* co-infected mice was analyzed to determine changes in $CD4^+$ T cell percentages. At 3 weeks, no changes were observed (Figure 3.3, top). However, at 5 weeks p.i. a drop was observed in both groups (Figure 3.3B, top). Though significance was reached in HIV/*M.tb* co-infected mice, since animals are at an acute stage of infection, we are currently unable to conclude whether this is the effect of

HIV infection alone. It is thought that the reduction observed in *M.tb*-infected BLT mice represents cellular trafficking to the tissues after infection especially since $CD4^+$ T cells are essential for control of *M.tb* infection [98, 181]. The total number of circulating leukocytes was evaluated and found to remain the same, possibly suggesting a specific decline specific for $CD4^+$ leukocytes and potential increase in $CD8^+$ lymphocytes (data not shown).

Studies have also demonstrated an increase in $CD8^+$ T cells that correlated with $CD4^+$ T cell decline following HIV infection [436, 437]. At 3 weeks p.i., a drop in $CD8^+$ T cells was observed (Figure 3.3A, bottom) which may be also explained by cellular trafficking to sites of infection within the tissues and possible suggestive of $CD8^+$ T cell involvement early in infection at infection sites. However, $CD8^+$ T cell percentages did not change at 5 weeks (Figure 3.3B, bottom).

We further wanted to determine if these cells were proliferating as an indicator of CMI activation. Prior to sacrifice, animals were injected interperitoneally (i.p) with Brefeldin A (BFA) and monensin for 5 hours to allow for cytokine accumulation in the Golgi complex or ER [495]. Proliferation was detected using antibodies against human Ki-67 [496]. In HIV/*M.tb* co-infected mice at 5 weeks p.i., proliferation increased in $CD4^+$ and $CD8^+$ T cells compared to *M.tb*-infected mice; an increase from 3 and 5 weeks was also observed in $CD8^+$ T cells from co-infected mice (Figure 3.3C) though not significantly. These results suggest activation of the CMI response due to HIV/*M.tb* co-infection.

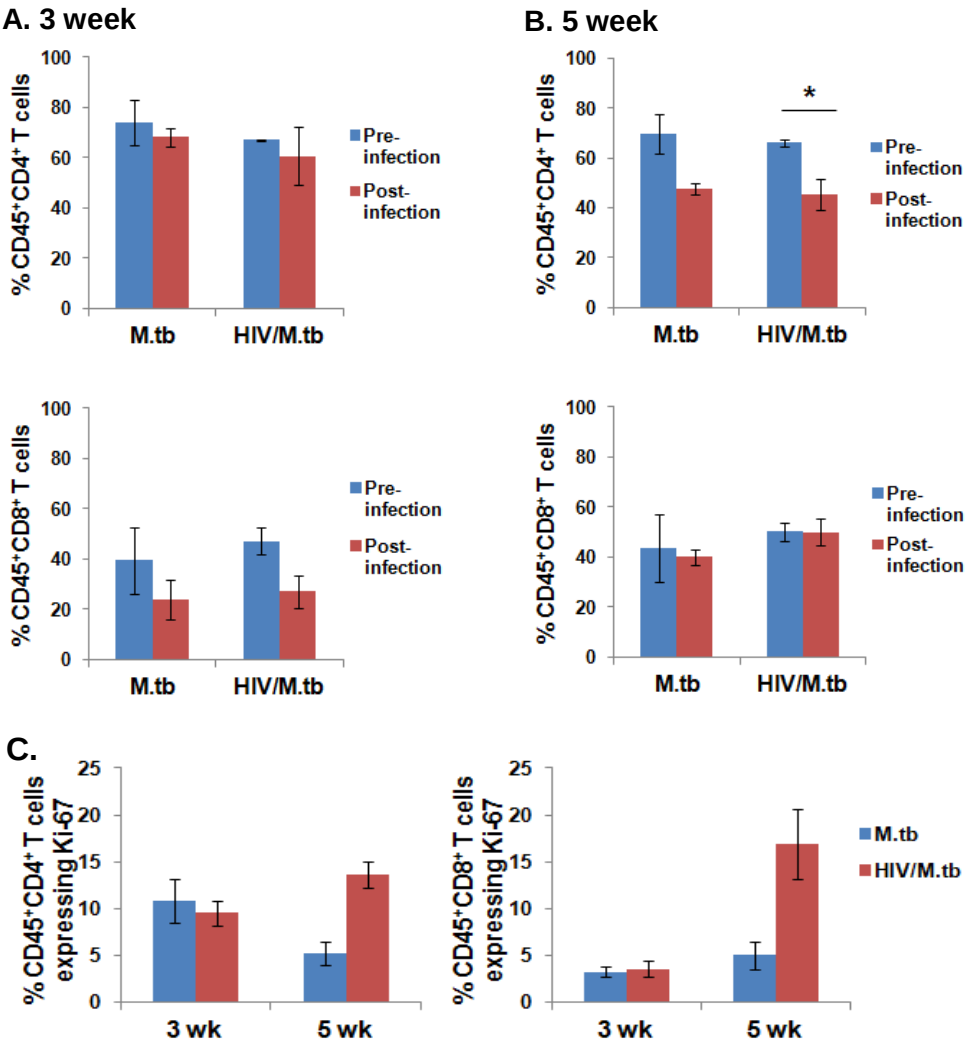


Figure 3.3: T cell levels and proliferative response in *M.tb* and HIV/*M.tb* infected BLT mice. Animals were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) and 3 weeks later infected i.n. with 10^2 cfu H37Rv *M.tb*. Prior to sacrifice, animals were injected i.p. with 250µg BFA and 500 µg monesin for 5 hours. At specified time points post-infection, peripheral blood was obtained and flow cytometry performed to determine surface expression of cellular phenotype markers (CD45, CD3, CD4, and CD8) and proliferation by nuclear antigen Ki-67. Show are percentage (%) of $CD4^+$ and $CD8^+$ T cell levels pre- and post-infection at (A) 3 weeks using n=2 HIV/*M.tb* co-infected and n=2 *M.tb*-infected and (B) 5 weeks using n=4 HIV/*M.tb* co-infected and n=2 *M.tb* infected mice. Proliferation is shown in (C) by Ki-67 expression in $CD4^+$ and $CD8^+$ T cells. Data is shown as the % T cells based on the $CD45^+$ population. To determine statistical differences, a two-tailed Student t-test was used; *$p<0.05$

HIV disrupts immune responses to *M.tb* infection in BLT mice

It has been proposed that HIV infection dysregulates the immune response to *M.tb* by down-regulating the $CD4^+$ Th1 responses [280]. Alternatively, CTL responses have been shown to control HIV infection early in infection and possibly during chronic stages, as previously discussed. To determine whether and how HIV affects immune responses during *M.tb* infection in BLT humanized mice, flow cytometry was performed on peripheral blood at specified time points to detect expression of the Th1 cytokine, IFN-γ, and the CTL molecule, granulysin. Prior to sacrifice, animals were injected i.p with BFA and monensin to allow for cytokine accumulation. Immune responses were detected as early as 3 weeks p.i. with co-infected mice expressing increased IFN-γ-$CD4^+$ T cells and no differences in granulysin expression (Figure 3.4 A,B). At 5 weeks p.i., increased IFN-γ expression in two HIV/*M.tb* co-infected BLT mouse is seen while expression is depressed in the other two BLT mice (Figure 3.4A). Co-infected BLT mice expressed greater levels of granulysin than mice only infected with *M.tb* (Figure 3.4B). An individual HIV/*M.tb* co-infected mouse (M2) did not appear to elicit an immune response; which might be explained by low viral (83 pg/g HIV p24 in the lung) and lower bacterial load ($8X10^3$ cfu in the lung) when compared to the other co-infected mice.

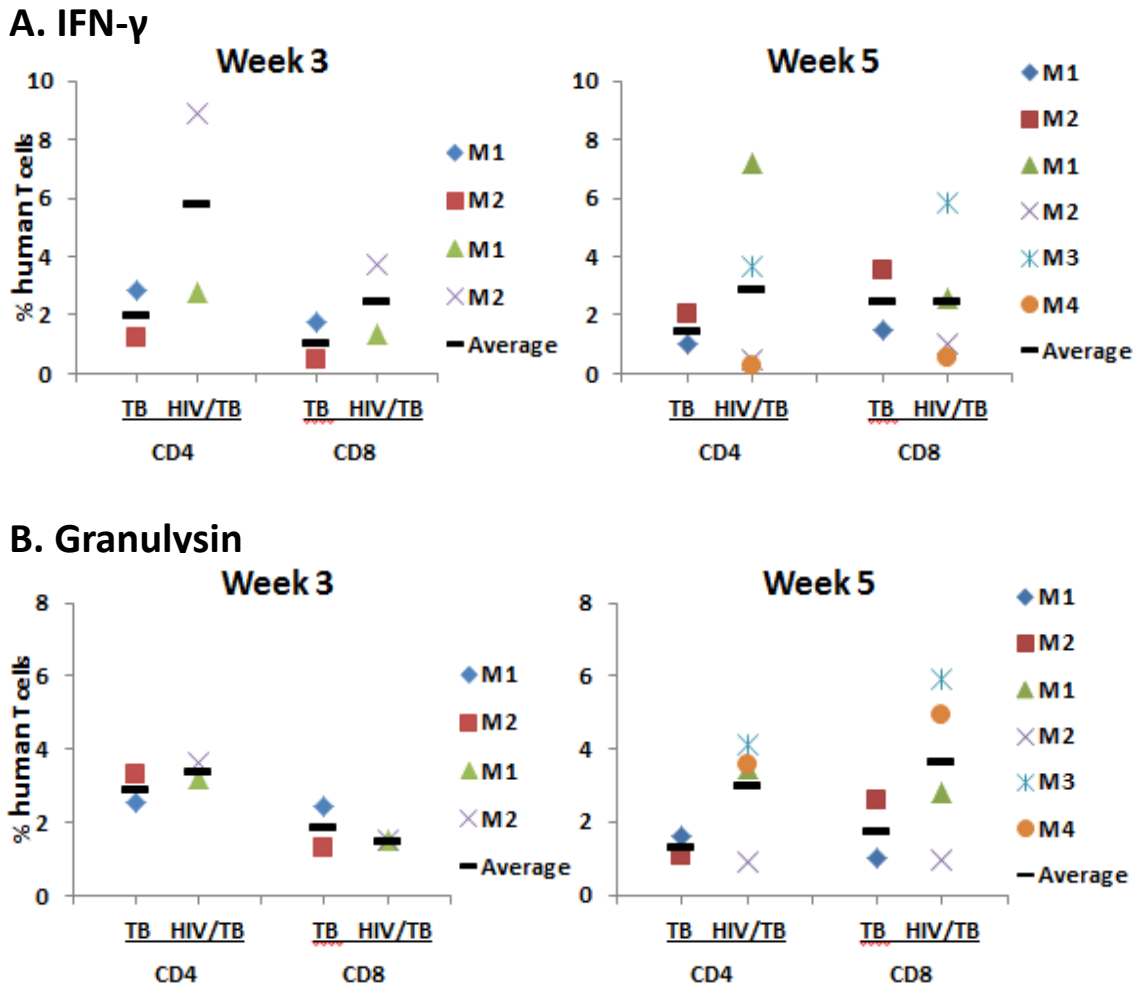


Figure 3.4: HIV affects immune response to *M.tb* infection in BLT mice. BLT mice were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) and 3 weeks later infected i.n. with 10^2 cfu H37Rv *M.tb*. Prior to infection, mice were injected i.p. with 250µg BFA and 500 µg monesin for 5 hours. At specified time points, peripheral blood was obtained and flow cytometry performed to evaluate IFN-γ and granulysin cytokine levels. Data is shown for individual animals as a percentage (%) of $CD4^+$ and $CD8^+$ T cells based on the $CD45^+$ population. Black line represents the average of each group.

Effects of HIV infection on the cytokine profile of *M.tb*-infected BLT mice.

To determine additional effects of HIV infection on *M.tb* in humanized mice, including whether co-infected mice developed the Th1 to Th2 cytokine bias, a human

Bioplex ELISA was performed. Following BFA and monensin injection, organs were obtained and homogenized as previously described, and supernatants collected. Results of cytokine analysis from lungs are shown and separated based on their CD4 subtype or function: Th1 (IFN-γ, IL-12, IL-2), Th2 (IL-4), Th17 (IL-17), pro-inflammatory (IL-1β, IL-6, IL-8, TNF-α, G-CSF), and chemokines (MCP-1, RANTES, MIP-1α, MIP-1β, and IP-10). Overall, significant differences between *M.tb*-infected and HIV/*M.tb* co-infected animals were not observed and the Th1 to Th2 cytokine bias development was not seen (Figure 3.5). Th1 cytokines IFN-γ, IL-12, and IL-2, are primarily increased in the individual co-infected BLT mouse which also has increased circulating IFN-γ and granulysin (Figure 3.8A). Th2 cytokine IL-4 is detected in both infection groups albeit with slight increase in the co-infected mouse with the organized granuloma (Figure 3.5A). The Th2 cytokine, IL-10, was not detected in either infection group (data not shown). IL-17, produced by γδ T cells in *M.tb* infection but also produced by Th17 to recruit neutrophils to sites of infection [143], is increased in the majority of HIV/*M.tb* co-infected mice (Figure 3.8B). An individual co-infected mouse is lacking IL-17, although is expressing various pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and G-CSF (Figure 3.8C), also known to be involved in neutrophil recruitment and TNF-α, essential for *M.tb* control [108]. In general, the majority of co-infected BLT mice expressed G-CSF, TNF-α, and IP-10, a chemokine that is expressed by infected cells to promote a pro-inflammatory response [497]. The chemokines detected are primarily being expressed by an individual co-infected mouse (M2, Figure 3.5D); this is the same mouse which is lacking circulating IFN-γ and granulysin (Figure 3.4). This response may be demonstrating an early stage of infection, involving recruitment of immune cells to the infection site.

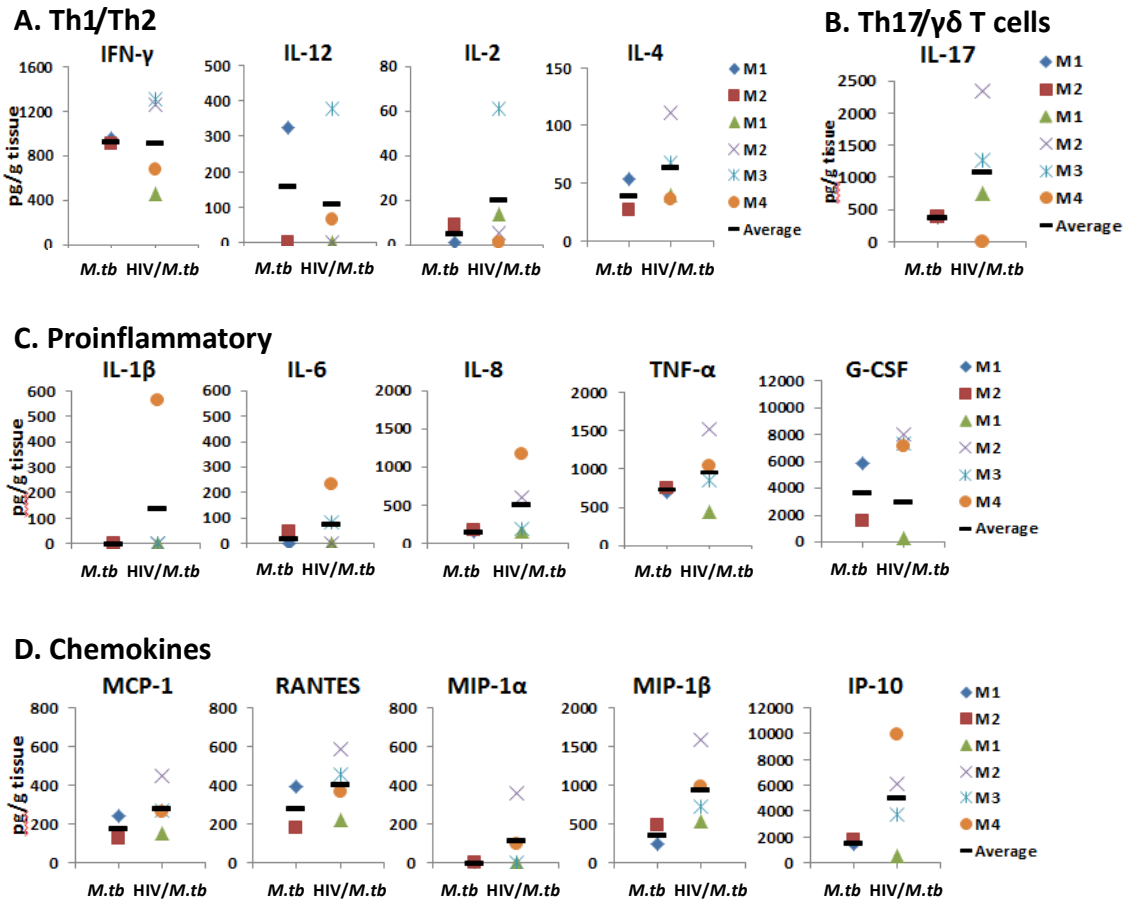


Figure 3.5: Effects of HIV infection on the cytokine profile of *M.tb*-infected BLT mice. Animals were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) and 3 weeks later infected i.n. with 10^2 cfu H37Rv *M.tb* for 5 weeks. Prior to sacrifice, animals were injected intraperitoneal (i.p) with 500 µg of monesin and 250 µg (BFA) for 5 hours. Lung tissue was collected and homogenized as previously described. Supernatants were collected and assayed for total cytokine levels using the 27-plex human cytokine Bioplex kit. Data are shown as average from *M.tb* (n=2) and HIV/*M.tb* (n=4) BLT mice. Shown in (A) is lung tissue pathology visualized by H&E staining (left panels, 4X) and AFB staining (right panels, 20X) from the indicated regions in the H&E image. Shown in (B) localization of HIVp24 staining in lung tissue (right, 10X; left, 20X).

Comparative tissue pathology of HIV/*M.tb*-infected and *M.tb*-infected BLT mice.

Evaluation of pathology in HIV/*M.tb* co-infected and *M.tb*-infected humanized BLT mice was performed at specified time points. Similar to previous studies, the right lung lobe and a lobe of the liver was used to evaluate pathology and bacterial load following H&E staining and AFB staining. Additionally HIV p24 capsid protein was detected using IHC to determine viral replication at sites of infection in the lung and liver. Figure 3.4 shows H&E, AFB, and p24 staining at week 3 from BLT mice infected with either 200 CFU of *M.tb* only or 2500 $TCID_{50}$ HIV-1 and 200 CFU *M.tb*. The lung of *M.tb*-infected BLT humanized mice displayed scattered areas of slight interstitial thickening and inflammation primarily along bronchovascular bundles; additionally plasma cells can be seen throughout the cellular infiltrate (Figure 3.6A). B cell development has been identified in other studies involving HIV-infected humanized BLT mice [436, 438]; however, because of the limited role of humoral immunity in TB, our studies do not measure antibody levels. At this same time point, an early stage granuloma formation is also observed, including the presence of multi-nucleated giant cells (MGC, Figure 3.6A). Bronchial obstruction is also seen in *M.tb*-infected mice (data not shown), similar to previous studies (Figure 2.7B). Compared to *M.tb*-infected BLT mice, *M.tb*-infected BLT mice with HIV displayed increased interstitial thickening, along with inflammation around bronchovascular bundles (Figure 3.6B). HIV, as observed by p24 expression, is scattered throughout the inflammatory foci. At this stage of infection, bacteria are not detected in either group by Ziehl-Neelson stain.

Liver pathology is displayed in Figure 3.6C and D from BLT mice at 3 weeks p.i., respectively. *M.tb*-infected mice are observed to have healthy liver with slight cellular infiltration around the portal veins (Figure 3.6C), while larger areas of inflammatory foci are observed in HIV/*M.tb* co-infected mice (Figure 3.6D). Neither bacteria nor HIV, however, are detected in *M.tb*-infected or HIV/*M.tb* co-infected mice.

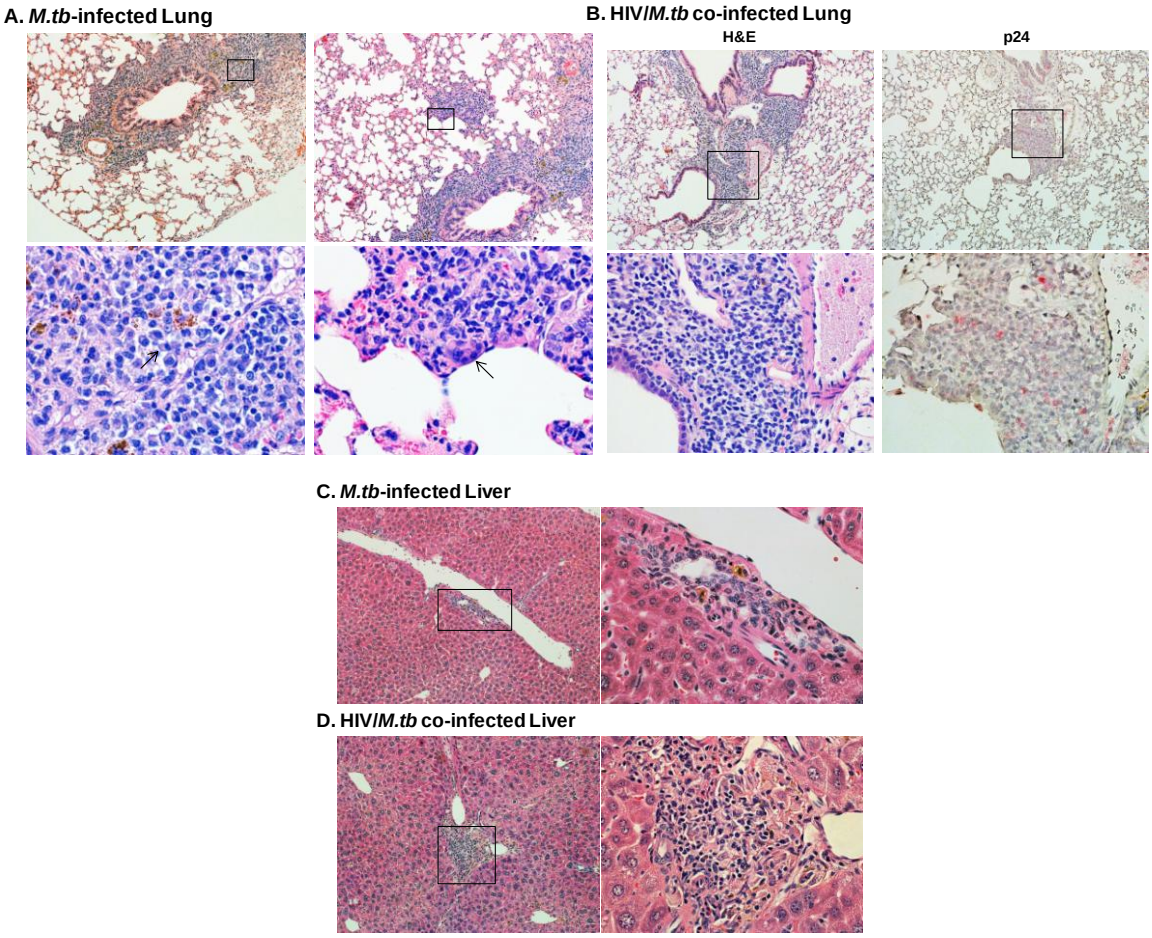
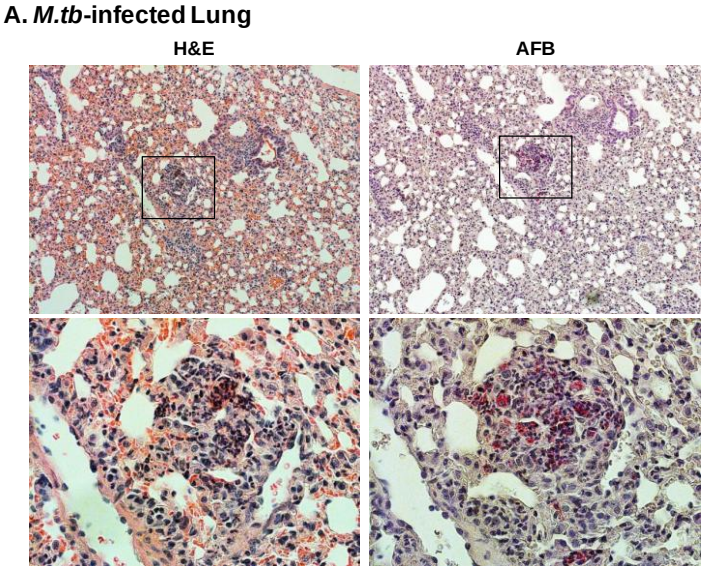


Figure 3.6: Tissue pathology in BLT mice with *M.tb*-infected or HIV/*M.tb* co-infection at 3 weeks p.i. Animals were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) and 3 weeks later infected i.n. with 10^2 cfu H37Rv *M.tb* after successful reconstitution of human leukocytes. Shown are images of tissue inflammation in formalin-fixed sections captured by microscopy following staining using hematoxylin and eosin (H&E), the Ziehl-Neelson method to detect for acid-fast bacilli (AFB), and HIV p24 capsid protein detected using IHC. Images are representative of mice sacrificed at 3 wks p.i. Shown in (A) is H&E staining of lung tissue in *M.tb*-infected mice showing inflammation around bronchial, vessel bundles with plasma cells indicated by the arrow in the left panel and early granuloma formation with a multinucleated giant cell indicated by the arrow in the right panel (top panel, 10X; bottom panel, 60X of the region indicated). (B) H&E staining of lung tissue in HIV/*M.tb* co-infected mice showing inflammation around bronchial, vessel bundles in the left panel and localization of HIV p24 expression by IHC detecting with Fast-red staining in the right panel (top panels, 10X; bottom panels, 40X of the region indicated). Shown in (C) is liver tissue visualized by H&E in *M.tb*-infected mice and (D) liver of HIV/*M.tb* co-infected BLT mice (left, 10X; right, 40X of the indicated region).

At 5 weeks p.i., *M.tb*-infected BLT humanized mice have extensive interstitial inflammation and thickened alveolar walls throughout the lung as seen in Figure 3.7A. In addition, small areas of cellular accumulation are observed with bacteria clustered within these inflammatory foci. Figure 3.7B shows the formation of a solitary organized granuloma (Ghon's focus) and relatively healthy lung structure throughout the tissue of a HIV/*M.tb* co-infected mouse. The inner region is necrotic with pyknotic nuclei while the outer edge is surrounded by activated macrophages. A massive amount of bacteria are seen throughout the granuloma, whereas p24 is localized to the periphery. Interestingly, this is the same BLT mouse, M3, which elicited a strong Th1 response characterized by IL-2, IL-12, and IFN-γ (Figure 3.5A). This may demonstrate the attempt of an immune response to control infection. It is also of importance to note that another HIV/*M.tb* co-infected BLT mouse, M4, displayed severe pathology included caseous necrosis (Appendix C). This mouse corresponds to the BLT mouse that expressed elevated pro-inflammatory cytokines IL-1β, IL-6, and IL-8 (Figure 3.5C). The lung pathology of this mouse demonstrates an array of characteristics, including alveolar pockets compact with mononuclear cells and AFB surrounding a caseous necrotic area with pyknotic nuclei, cellular fragmentation, and bacterial debris. Within this necrotic region, activated macrophages are observed surrounded by necrotic neutrophils. An accumulation of cells along with necrotic debris and AFB are also seen spreading into the bronchus, a characteristic typical of an *M.tb*-infected individual that would be expelling bacteria upon coughing [455]. HIV p24 was not isolated around or within the necrotic lesion although is localized at inflammatory foci throughout the tissue and around bronchovascular bundles. Compared to the granuloma formation from previous studies with *M.tb*-infected

BLT mice (Figure 2.5A; Figure 2.7B), the lesion displayed by this HIV*M.tb* co-infected appears disorganized with more inflammatory cells. Overall, different spectrums of HIV/*M.tb* co-infection are observed, M3 shows an efficient immune response able to contain and possibly control infection (Figure 3.7) and M4 shows a dysregulated inflammatory response causing extensive tissue damage, caseous necrosis, and uncontrolled infection (Appendix D).

Shown in Figure 3.7C and D is liver pathology from infected BLT mice at 5 weeks p.i. *M.tb*-infected mice have histologically normal healthy liver with isolated areas of organized foci of inflammation and relatively low levels of bacteria contained within the foci (Figure 3.7C). HIV/*M.tb* co-infected mice displayed organized areas of inflammation around portal veins and scattered throughout the liver tissue with bacteria isolated within the inflammatory foci (Figure 3.7D). HIV p24 can be seen localized to the periphery of the inflammatory foci, although viral replication is primarily seen in around portal veins and dispersed throughout the liver.



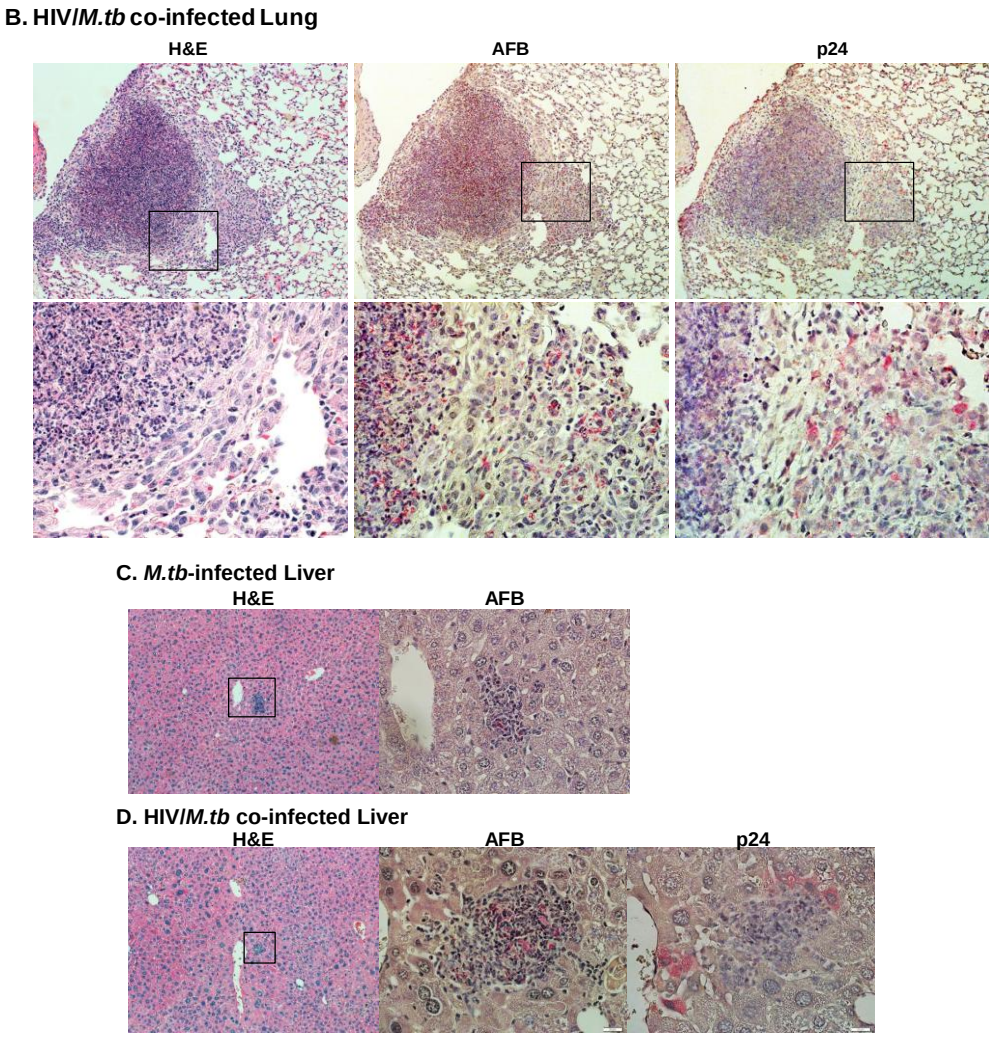


Figure 3.7: Tissue pathology in *M.tb*-infected or HIV/*M.tb* co-infected BLT mice at 5 weeks p.i. Animals were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) and 3 weeks later infected i.n. with 10^2 cfu H37Rv *M.tb* after successful reconstitution of human leukocytes. Shown are images of tissue inflammation in formalin-fixed sections captured by microscopy following staining using H&E, AFB, and HIVp24. Images are representative of mice sacrificed at 5 wks p.i. Shown in (A) is lung tissue pathology in *M.tb*-infected mouse visualized by H&E in the left panel and AFB staining in the right panel (top panel, 10X; bottom panel, 40X of indicated region). (B) lung tissue pathology from a HIV/*M.tb* co-infected mouse visualized by H&E in the left panels, AFB in the middle panels, and p24 in the right panels (top panel, 10X; bottom panel, 40X of the indicated region). Liver is shown in (C) *M.tb*-infected mouse visualized by H&E (10X) and AFB staining (40X). (D) liver tissue from a HIV/*M.tb* co-infected mouse visualized by H&E (10X), AFB (40X of the indicated region), and HIV p24 (40X of the indicated region).

Recruitment and localization of human leukocytes at site of infection during HIV/*M.tb* co-infection and *M.tb* infection of BLT mice

As previously discussed, T lymphocytes typically contain bacterial infection by localizing to the periphery of *M.tb* granulomas. It was previously demonstrated that human T cells organized at sites of infection and inflammation (Figure 2.8). To determine if HIV affects T lymphocyte localization, IHC was performed on formalin-fixed, paraffin-embedded tissues. At 3 weeks p.i., $CD3^+$ T lymphocytes are localized at inflammatory sites around bronchovascular bundles for both HIV/*M.tb* co-infected and *M.tb*-infected BLT mice (Figure 3.8A). At 5 weeks p.i., $CD3^+$ T cells are organized to sites of inflammatory foci in *M.tb*-infected mice; whereas in co-infected mice T cells were detected throughout the granuloma (Figure 3.8B). It was determined, by an independent pathologist that based on the orientation of the tissue section, the image presented for Figure 3.8B is the outer region of the granuloma structure; as expected, T lymphocytes are localized on the periphery of the lesion. In the HIV/*M.tb* co-infected mouse with severe necrosis, $CD3^+$ T lymphocytes are readily seen throughout the lung, primarily localized to sites of inflammation near bronchial, vessel bundles, and isolated inflammatory foci. T lymphocytes are also scattered around the caseous necrosis lesion (Appendix D). Further IHC staining determined that a majority of these cells are $CD8^+$ T cells.

Based on the elevated expression of the cytokines responsible for neutrophil activation and recruitment, specifically IL-17 in *M.tb*-infected BLT mice with HIV, myeloperoxidase (MPO) staining was performed to determine whether neutrophils are localized to infection sites. Increased MPO is detected in BLT co-infected HIV than mice

only infected with *M.tb* at 5 weeks p.i. (Figure 3.8C). This demonstrates neutrophils are playing a major role in the immune response during co-infection which appears to be induced by HIV disease.

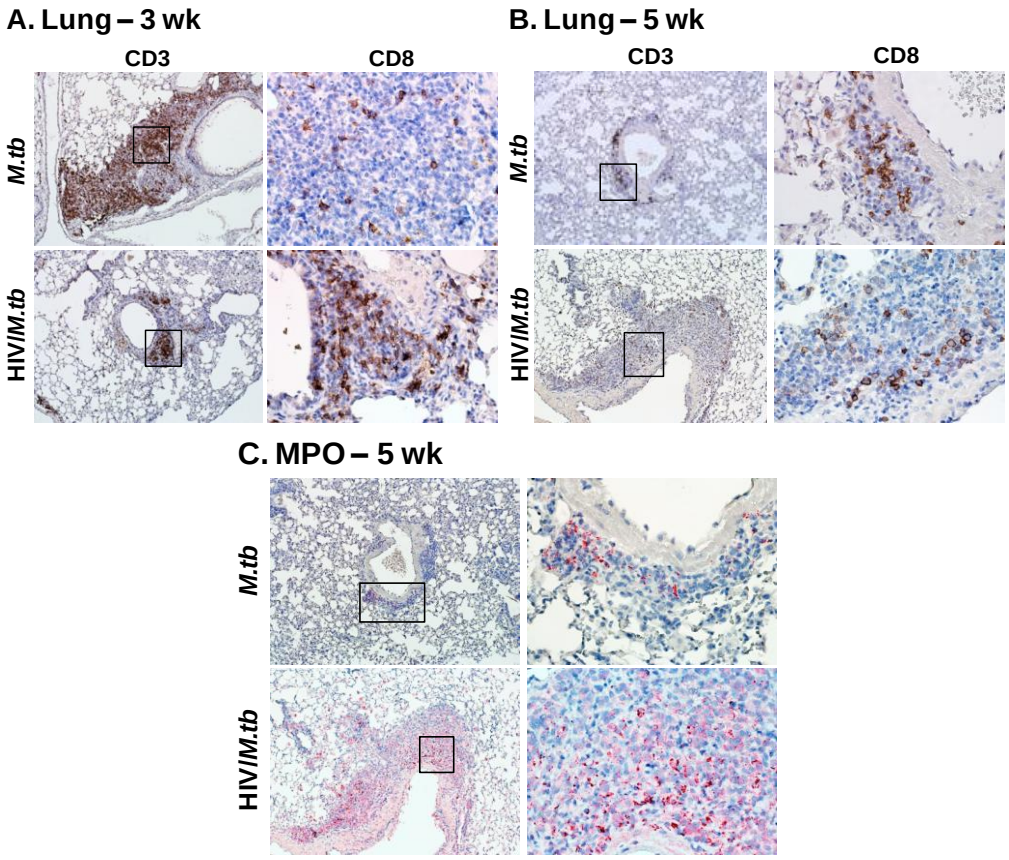


Figure 3.8: Recruitment and localization of human leukocytes at sites of infection during HIV/*M.tb* co-infection and *M.tb* infection of BLT mice. Animals were infected i.v. with 2500 $TCID_{50}$ HIV-1 (JR-CSF) and 3 weeks later infected i.n. with 10^2 cfu H37Rv *M.tb* following successful reconstitution of human leukocytes. Formalin-fixed, paraffin-embedded tissue sections were cut, dewaxed, and stained with human CD3 and CD8 antibody using (DAB) substrate or MPO using Fast Red substrate. Images are representative of mice sacrificed at (A) 3 and (B,C) 5 wks p.i. Shown is $CD3^+$ T cell localization in inflammatory areas of a representative *M.tb*-infected (top) and HIV/*M.tb* co-infected (bottom) mouse. Enlarged images of $CD8^+$ T cells staining of the indicated regions are shown in the right panels. (C) Neutrophil recruitment by MPO stain using Fast Red substrate of inflammatory areas in *M.tb*-infected and HIV/*M.tb* co-infected mice. (left panels, 10x; right panels, 40x)

DISCUSSION

An understanding of the mechanisms used by HIV to alter the host response during *M.tb* is critical to the development of treatments that will reduce and prevent the massive levels of morbidity and mortality associated with HIV/*M.tb* co-infection. Following successful reconstitution with human leukocytes, humanized BLT mice develop active and disseminated HIV disease concurrently with TB disease. The characteristic depletion of $CD4^+$ T cells during HIV infection is demonstrated in co-infected humanized mice in addition to altered effector molecule and cytokine/chemokine responses. Furthermore, an accelerated rate of TB disease progression is seen in co-infected mice with granuloma development and necrosis. These studies establish, for the first time, the ability of the humanized BLT mouse to develop productive HIV and *M.tb* co-infection. This model will greatly advance our understanding of HIV/*M.tb* co-infection disease mechanisms and contribute to our knowledge of the immune responses need to circumvent disease progression. HIV infection accelerates the rate of TB disease or reactivation of LTBI [367, 499], likewise, Mb infection leads to an increased progression of HIV disease [395, 423, 500]. Over time, co-infected BLT mice developed higher bacterial load and increased pathology (granuloma, necrotic lesions) compared to M.tb-infected humanized mice. Additionally, the co-infected mice with granuloma lesions expressed higher viral replication compared to co-infected mice with less pathology (data not shown). These results suggest that HIV and M.tb co-infection in the humanized mouse may represent disease progression similar to humans and even the NHP co-infection model, although further exploration of the BLT mouse as a model are needed to validate these observations.

Several studies using the humanized BLT mice have shown a reduction of $CD4^+$ T cells [436-438], similar to human HIV infection. A drop in $CD4^+$ T cells was seen in both infection groups, with a significant drop observed in *M.tb*-infected mice co-infected with HIV possibly demonstrating the effect of HIV on immune suppression. However, further studies are needed to validate these findings. Our studies did not permit for comparisons between co-infected and HIV-infected BLT mice, however an accelerated $CD4^+$ T cell decline has been observed in co-infected compared to HIV-infected humans [498] and NHPs [489]. In NHPs, the drop in $CD4^+$ T cells appears to be the result of bacterial immune activation. In co-infected BLT mouse, T cells are activated, as indicated by the expression of Ki-67; similar to the study by Brainard *et al*. [438] which demonstrated increased T cell proliferation in HIV-infected humanized mice. Moreover, our studies demonstrate HIV localizes to inflammatory foci and granulomas where AFB are present. HIV viral replication is increased in lung at sites of *M.tb* infection [393]. Taken together, this may suggest HIV localizes to sites of infection causing immune suppression. Future studies will determine whether HIV-induced $CD4^+$ T lymphocyte reduction is exacerbated by the recruitment and activation of target cells to sites of *M.tb* infection.

In addition to $CD4^+$ T lymphocyte effector molecules (IFN-γ), cytolytic $CD8^+$ T cell effector molecules – perforin and granulysin - are important for the control of TB [499]. The control of HIV infection is inhibited by the improper differentiation of $CD8^+$ T cells into effector cells [500, 501]. In other studies of HIV-infected humanized BLT mice, $CD8^+$ T cell levels increased along with increased granulysin expression [438]. In our studies, though lymphocyte levels were not elevated, $CD8^+$ T cells were able to produce more granulysin when compared to *M.tb*-infected BLT mice; perhaps

demonstrating that these $CD8^+$ T cells were highly activated. In subsequent studies, the development of antigen-specific T cells will be evaluated to identify immune recall produced to control infection and inhibit disease progression.

At the present time, we did not observe a change from a Th1 to Th2 cytokine profile. Th1 and pro-inflammatory molecules were increased in HIV/*M.tb* co-infected mice with moderately elevated Th2 cytokines in a few co-infected mice. Though the Th1 to Th2 bias paradigm has generally been accepted, other studies have also failed to observe a Th1 to Th2 bias in co-infected individuals [249, 502, 503]. Additional studies in the humanized BLT mouse with longer disease progression may define the cytokine response generated by co-infection. Nonetheless, the cytokine profile observed for individual mice appears to correlate with disease pathology.

In general, there were no to slight differences in Th1 cytokines. However, Th1 cytokines were produced to elevated levels in an individual co-infected BLT mouse, possibly promoting bacterial and viral control. IL-12 and IFN-γ are essential for *M.tb* control by promoting a Th1 phenotype and activating macrophages, respectively [98, 108, 504]. It is suspected that this co-infected BLT mouse is attempting to control *M.tb* by containing the bacilli within a well-organized granuloma observed in the lung tissue. The abundance of macrophages and lymphocytes on the periphery lead us to believe there is a continuous influx of monocytes and lymphocytes associated with granuloma maintenance. In patients with HIV, elevated IL-12 and IFN-γ expression is correlated with lower viral load [504]. IL-12 and subsequent IFN-γ impairment is associated with TB and HIV disease progression [500]. IL-2, which induces T-lymphocyte expansion [505], may reduce HIV replication by down-regulating the expression of CD4 and CCR5

receptors [351, 506]. Viral replication is not inhibited in this mouse since viral load was highest compared to the other co-infected mice. High viral load could be the result of additive effects of IFN-γ and IL-12 expression which are increasing the recruitment and activation of macrophages and T cells, providing an abundance of target cells for viral replication.

Chemokines are leukocyte chemoattractants, which recruit and activate various cells to infection sites [148]. Multiple chemokines (MCP-1, MIP-1α, MIP-1β, RANTES) were elevated in HIV/*M.tb* co-infected BLT mice compared to *M.tb*-infected mice; although an individual co-infected mouse in particular expressed levels higher than its cohorts. MCP-1, MIP-1α, MIP-1β, and RANTES production is an early response to *M.tb* infection, possibly acting to inhibit bacterial growth through the recruitment of inflammatory cells [167, 507, 508]. In addition, these chemokines suppress HIV infection by binding macrophage receptor CCR5 that is also required for viral entry [351, 509]. TNF-α was also increased in this mouse, which is important since TNF-α can induce MIP-1, MIP-1α, MIP-1β, and RANTES production [351, 510]. This co-infected mouse displayed mild tissue pathology (isolated foci of inflammation with mostly healthy lung structure; data not shown) and low viral load when compared to the other co-infected mice. The cytokine and chemokine profile expressed by this co-infected BLT mouse may demonstrate early stage of infection with initial inflammatory cell recruitment aimed at controlling infection as seen in human TB patients [508, 511]. However, the lack of HIV-infected BLT mice as controls limits our ability to make definitive conclusions on whether reduced viral load was a result of increased chemokine expression.

While it is well established that TNF-α is required for control of *M.tb* infection, it is also known to induce severe pathological effects such as tissue necrosis [512]. This is evident from the varying pathological stages observed in HIV/*M.tb* co-infected BLT mice. Elevated levels of TNF-α are observed in the co-infected mouse with a granuloma supporting the notion that this cytokine is involved controlling infection. On the other hand, elevated TNF-α is seen in the co-infected mouse with severe pathology and caseous necrosis development. Excessive TNF-α, along with MCP-1, can also activate HIV replication at TB disease sites [500, 513]. Expression of IL-1β and IL-6 also correlates with increased viral replication [395]. Interestingly, the co-infected BLT mouse with caseous necrosis secreted elevated pro-inflammatory cytokines IL-1β and IL-6. Future studies using HIV-infected BLT mice as controls will need to be performed to validate the role of *M.tb*-mediated cytokine production in viral replication since such conclusions cannot be made from the current preliminary study.

The recruitment of neutrophils and macrophages is responsible for initial granuloma formation [166, 168]. As previously discussed, IL-6 or IL-21 and TGF-β induce Th17 differentiation which secrete IL-17 and G-CSF; both of which recruit and activate neutrophils to sites of infection [143]. High IL-17 expression is also a correlate of protection during HIV infection [500, 514]. In HIV/*M.tb* co-infected BLT mice, IL-17 was elevated in all mice except the individual mouse displaying caseous necrosis. IHC staining for MPO validated neutrophil involvement at tissue sites of infection within these mice. This further demonstrates the attempt of the immune response within these co-infected BLT mice to control infection. Although bacterial load remains high so the function of neutrophils in our model remains to be determined. Considering γδ T cells are

an important source of IL-17 expression during *M.tb* infection [143], additional studies will need to be performed to determine the source of IL-17 in *M.tb*-infected BLT mice with HIV. Moreover, since IL-6 was not expressed by the co-infected mice that secreted high IL-17 levels, it is safe to assume that IL-21 is mediating this response, although future studies will be performed to identify the mechanism of IL-17 secretion in BLT mice during infection.

Though the co-infected BLT mouse with caseous necrosis did not express IL-17, it did express IL-6 and IL-8. Similar to TNF-α, IL-6 is a multifunctional cytokine produced early during *M.tb* infection [108, 515]. During the chronic stages of *M.tb* disease, IL-6 stimulates bacterial growth [108, 516] and inhibits TNF-α and IL-1β production affecting granuloma formation to promote bacterial dissemination [108, 517]. IL-6 along with TNF-α can also activate HIV replication [351, 518]. It is possible that the elevated levels of IL-6 levels seen in the co-infected BLT mouse with caseous necrosis represent the destructive effects of IL-6. Furthermore, extensive neutrophil accumulation was observed in the tissues of this co-infected mouse. This may be a result of elevated IL-8, a chemokine produced by macrophages and lymphocytes that recruits and activates neutrophils to sites of infection during *M.tb* infection [505]. Similar to other co-infected mice, regardless of neutrophil accumulation, bacterial load does not seem to be controlled.

IP-10 is a chemokine produced by monocytes, endothelial cells, epithelial cells, fibroblasts and keratinocytes, to promote a Th1 pro-inflammatory response against *M.tb* [497]. Various cytokines can drive the production of IP-10 including IFN-γ, IL-2, IFN-α, IL-17, TNF-α, and IL-1β [497]. Similar to IFN-γ, IP-10 is expressed in response to

antigen [519-522]. In HIV/*M.tb* co-infected patients, regardless of CD4 cell counts, IP-10 was detected similar to or better than IFN-γ [523-525]. The use of IP-10 as a detection marker for *M.tb* disease state could be of great use considering humanized BLT mice expressed high levels of this cytokine with even greater amounts in HIV/*M.tb* co-infected mice. As IP-10 is becoming a promising alternate marker to IFN-γ in detecting *M.tb* [497], future studies using the humanized BLT mice will evaluate IP-10 as another potential biomarker – similar to granulysin – to predict the stage of *M.tb* infection.

As stated by Hunter [457], observational studies of human TB pathology samples do not readily permit for reproducibility and statistical analysis. Nonetheless, great effort is taken at identifying consistencies within pathology samples. Our group has determined that reproducibility can be difficult to achieve among groups of humanized BLT mouse. As presented in the current study, variability was observed on the pathological and immunological characteristics of individual mice from mild inflammation to granuloma formation to caseous necrosis. It can be speculated that these mice displayed accelerated rate of infection (i.e. higher bacterial and viral load, reduced lymphocyte count and function, increased cellular infiltration, and accelerated rate of disease pathology) compared to *M.tb*-infected BLT mice and as is expected for co-infected patients.

The development of bronchial obstructions with AFB accumulation, previously discussed, has become a standard observation in our *M.tb*-infected BLT mice allowing for the spread of bacteria into the lung. Cholesterol crystal formation, previously observed, did not develop in this group of either *M.tb* or HIV/*M.tb* co-infected mice. However, the development of multi-nucleated giant cells (MGCs) in *M.tb*-infected mice is a feature previously not observed. MGCs result from the fusion of macrophages,

induced by *M.tb* infection, and lack the ability to take up bacteria [526]. However, they are able to present antigen, suggesting their role in eliminating bacteria by fusing with other infected macrophages [526]. *M.tb*-infected BLT mice with HIV did not have MCGs, possibly the result of immune alterations observed in these mice. Similar observations might be expected for immune suppressed individuals with an immune system which is unable to induce an adequate immune respond to infection [457]. Another interesting feature, found only within the necrotic lung of an HIV/*M.tb*-infected BLT mouse, was foamy macrophages. The lipids from foamy macrophages are thought to contribute to TB persistence by providing a nutrient source [457, 458]. Foamy macrophages are also involved in caseation through the release and accumulation of lipids in granulomas [457, 459], possibly explaining the phenomenon seen in this mouse.

The detection of plasma cells is another interesting observation. B cells and humoral immunity have been regarded as not contributing in protection against *M.tb* infection [527]. In studies with B-cell deficient mice, immune responses vary from reduced, to delayed, to having no effect [528-531]. The use of monoclonal antibodies has shown protective effects [532-535], though the use of traditional mouse models in these studies raises concern regarding their reliability. Currently, the role of B cells in *M.tb* infection remains unknown; as a result, the BLT mouse could provide as a model for future studies.

Overall, these studies support the humanized BLT mouse as a tool for HIV/*M.tb* co-infection research. The ability of co-infected BLT mice to develop progressive and disseminated infection, immune suppression, and disease pathology, demonstrates that it can provide valuable information on the pathogenesis of co-infection *in vivo*. The use of

HIV instead of SIV will also allow for a more accurate understanding of the disease mechanisms associated with co-infection in the human. Moreover, the humanized BLT mouse can serve as a model for treatment testing.

CHAPTER 6

Conclusions/Future Directions

The research presented in this dissertation represents a significant advance in the field of TB. The humanized BLT mouse should be an ideal model to study *M.tb* infection and HIV/*M.tb* co-infection because of the potential to reproduce TB disease pathology seen in NHPs and humans. This model will allow us to gain a detailed understanding of the mechanisms behind disease development. It should be made clear that much of what is known about the pathogenesis of HIV and *M.tb* co-infection is speculative, based on studies performed *in vitro*, *ex vivo*, or using human autopsy samples. The BLT mouse model of HIV/*M.tb* co-infection will enable us to understand the effects of HIV on the immune response that increases susceptibility to *M.tb*, and clear the way for new treatment designs and testing.

The humanized BLT mouse was chosen as the model to reproduce HIV/*M.tb* co-infection for its excellent reconstitution of human leukocytes and the potential to overcome the limitation of the human tropism of HIV. These results further indicate the development of pathology in the lung is consistent of human TB disease with characteristic granuloma formations (foamy macrophages, lipid deposits, cholesterol crystals, multinucleated giant cells, and bronchial obstruction). Furthermore, lymphocytes are localized to the periphery of granulomatous lesions along with the bacilli. BLT mice are also able to develop productive HIV infection with reduced $CD4^+$ T cell levels and an array of granulomatous lesions that express phenotypically distinct cytokine profiles. Ultimately, the ability of the model to develop these responses will enable us to study

immune response mechanisms during *M.tb* infection and altered by HIV co-infection that cannot be demonstrated in the human host or are unknown.

The reconstitution of circulating human cells will allow us to study the function of individual cell populations and cell-to-cell interactions *in vivo* that are induced as a protective response or altered by the pathogen to promote disease progression. Macrophages have been shown to produce reactive oxygen species (ROS) upon infection [97], a mechanism of controversy within the human. HIV inhibits microbicidal activity by an unknown mechanism [388], possibly inhibiting ROS or NO production. This humanized mouse model would enable us to identify correlates of immune protection, such as ROS or RNS, and how HIV prevents its response. HIV has also been shown to prevent macrophage phagocytosis of pathogens [389], though this has not been demonstrated during *M.tb* infection. In those studies, involving other OIs, the mechanism of inhibition is not known. Endocytosis of *M.tb* allows entry through various surface receptors including complement, macrophage mannose receptor, and type A scavenger receptors [108]. During HIV co-infection with *Candida albicans*, a down-regulation of complement receptor, CR3, was observed [383]. It is possible that a reduction in phagocytosis is mediated by the down-regulation of receptors required for *M.tb* entry into the macrophage, possibly targeting them for degradation similar to MHC molecules and CD4 receptors. Other macrophage-mediated responses that we could evaluate, which are altered by HIV, include changes in cytokine and chemokine levels. Moreover, we have the ability to evaluate other innate immune responses including NK and DC cell functions. The role of NK cells in *M.tb* infection has been largely underrepresented until recently. NK cells are able to secrete effector molecules such as IFN-γ and granulysin

[154, 477]. *In vitro*, they have been shown to directly lyse *M.tb*-infected macrophages, induce $CD8^+$ T cell-mediated lysis of *M.tb*-infected macrophages, and eliminate T-regulatory cells induced by *M.tb* [536]. Previous studies in our lab have demonstrated that granulysin induced by NK cells is reduced during HIV infection [477]. Thus further studies to determine how HIV impairs NK cell activity during *M.tb* infection would be valuable.

Various effector molecules (IFN-γ, granulysin) are expressed constitutively and upon activation in BLT mice. As the role of IFN-γ has been extensively studied with regard to *M.tb* infection and HIV/*M.tb* co-infection, our studies will focus on defining the functional role of granulysin during bacterial infection and co-infection. The detection of granulysin was interesting and further validated the model as a human surrogate since mice do not express granulysin. As a correlation has been shown between granulysin expression and disease status [537], our studies would evaluate the potential of granulysin to be used as a diagnostic marker of disease. Additional studies could involve the use of granulysin neutralization in humanized mice. Granulysin has also been shown to act in an antiviral manner, inhibiting replication and inducing apoptosis [538]; however the same was not observed with HIV replication [539]. Nonetheless, it would be of interest to evaluate the potential of granulysin as an antiviral *in vivo*.

Perhaps the most important observation during *M.tb* and HIV/*M.tb* co-infection in the humanized BLT mouse is the formation of granulomas; the hallmark of TB infection which is not seen in most traditional mouse models. During *M.tb* infection, granulomas appear well-organized and characteristic of granuloma lesions in human disease with lymphocytic cuffing, peripheral localization of bacteria, and caseous necrosis within the

center; although granulomas in animals co-infected with HIV appear more disorganized, with bacteria and lymphocytes scattered throughout the lesions. Though co-infected animals are producing cytokines and chemokines representative of a protective immune response, it is possible that we are observing an early infection response and a single animal is rapidly progressing to disease. Additional studies will be taken to longer time points, perhaps between 6 to 8 weeks considering *M.tb*-infected mice with low dose infection did not display granulomatous lesions until this time. Even so, the information obtained from the granulomatous lesions in co-infected mice has provided a multitude of avenues of pursuit going forward with this research.

The pulmonary lesions in our studies appear to be developing rapidly and become necrotic with caseation within weeks; in humans these types of granulomas typically take months perhaps years to develop possibly the result of treatment delaying progression. For a variety of reasons, primarily ethical consideration, we are not able to study disease progression and granuloma formation in humans so what is known is based on single moments in time irrespective of when infection was acquired and distorted by the attempt to treat. This model will allow us to understand TB disease as before antibiotics were available, when disease progressed without limit, to follow the process from initial infection to disease and understand the natural course of infection. In the liver, lesions appear primarily necrotic with numerous bacilli. Though this type of granuloma is not seen in humans or animal models today, this form of disseminated infection was seen in human TB disease during the pre-antibiotics era, though infrequently. Rich [540] called these types of lesions “acute caseating miliary tuberculosis”, consisting of a loose accumulation of mononuclear cells undergoing necrosis throughout the lesion rather than

within the center. As we continue with our studies, it will be interesting to observe the multitude of granuloma formations described pre- and post-antibiotics.

The array of cytokines expressed in HIV/*M.tb* co-infected tissue provides various opportunities for discovery. The most prominent finding was the elevated levels of IL-17 in animals which appear to correlate with a protective immune response. The role of IL-17, including whether Th17 cells or γδ T cells are the source, will be a continuing avenue of research in our group. IL-17 recruits neutrophils to infection sites and plays a protective role to control bacterial infection [143]. Contradicting studies regarding the role of neutrophils have also been identified, showing that neutrophils are not protective and rather they may contribute to disease pathology [154, 155, 541]. The controversy surrounding neutrophils dates back to Rich [540] suggesting that polymorphonuclear (PMN) cells contribute to the cavity formation (liquefaction) of granulomas resulting from the proteolytic enzymes released by PMN cells within necrotic regions [155, 540]. In agreement with this, we observed that the necrotic granulomas observed in our model contained high levels of neutrophils while the caseating granulomas lacked these cells. As a result, the function of these cells will be of great interest to study at different stages of infection. Of further interest is the effect of HIV on recruitment of neutrophils during *M.tb* infection and granuloma formation. The future development of IL-17 neutralization and neutrophil-depletion in humanized mice will provide further insight into these mechanisms.

As previously demonstrated [366], we were able to verify that HIV localized to sites of *M.tb* infection, more specifically granuloma lesions in BLT mice. These observations will allow us to identify specific populations targeted which leads to the

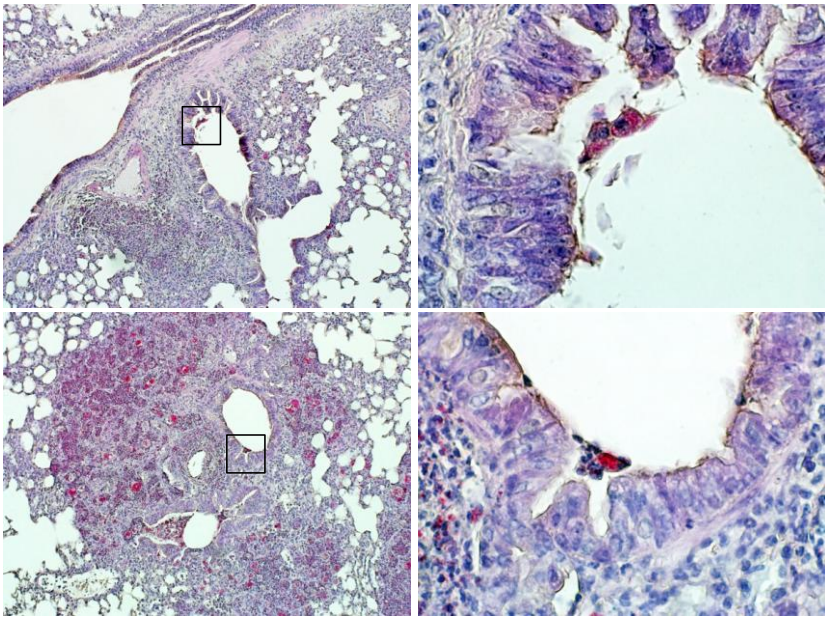
disruption of the granuloma, such as *M.tb*-specific $CD4^+$ T cells or even macrophages involved in maintaining the granuloma structure. Furthermore, the mechanism of depletion can be determined, whether it is through direct infection to reduce the CD4 receptor, by inducing apoptosis, or indirectly by inhibiting macrophage-derived cytokines such as IL-12 that promotes Th1 development.

These studies evaluated a disease course that began initially with HIV infection followed by *M.tb* co-infection. In the future, we anticipate assessing a disease course that begins with newly acquired *M.tb* infection or LTBI followed by HIV infection. Our approach to latency development within the humanized BLT mouse will follow what has been shown with in Cornell mouse model [413] with alterations as deemed necessary to infection dose, infection period, and treatment length.

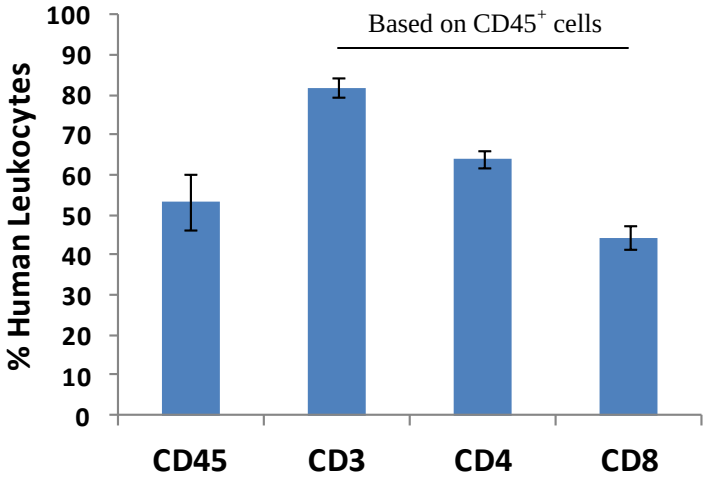
The BLT mouse model will be a tremendous asset to the field of *M.tb* and HIV/*M.tb* co-infection, expanding the knowledge regarding the mechanism of disease and immune evasion. The observations that we have already made will promote hypothesis-driven research to begin to understand the pathogenesis of HIV/*M.tb* co-infection. This new understanding of important immune mechanisms will lead to the development of new drug compounds, therapeutics, and vaccines to prevent or treat *M.tb* infection or reactivation in HIV-positive individuals. We also anticipate providing the field of therapeutic and vaccine development with a model for testing treatments prior to use in humans.

Appendices

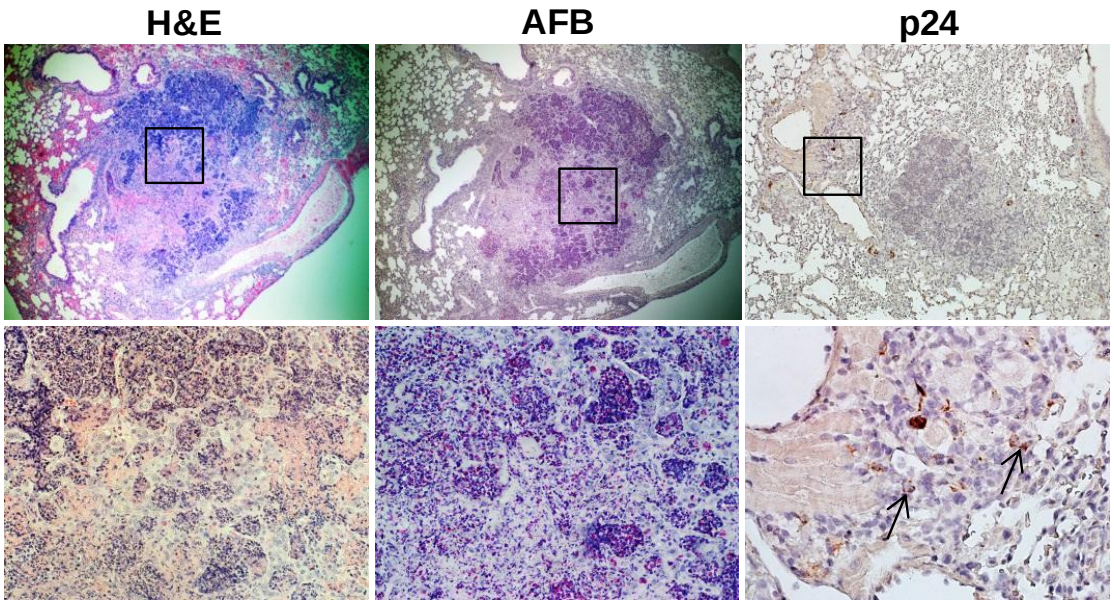
Appendix A. Endobronchial bacteria in *M.tb*-infected BLT mice at 3 weeks p.i.



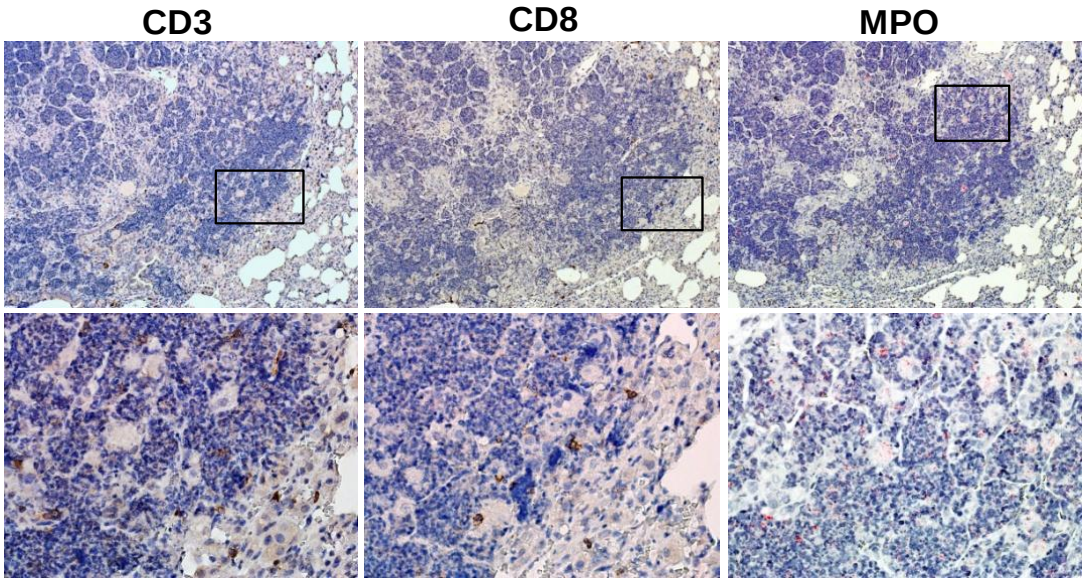
Appendix B. Human Leukocyte Levels in BLT Humanized Mice Prior to HIV/*M.tb* Co-infection



Appendix C. Caseous necrotic lesion in HIV/*M.tb* co-infected lung tissue visualized by H&E (left: top, 4X; bottom, 20X), AFB (middle: top, 4X; bottom, 20X), and HIV p24 using DAB stain shown with arrows (right: top, 10X; bottom, 40X)



Appendix D. Leukocyte localization in caseous necrotic lesion from HIV/*M.tb* co-infected lung tissue . $CD3^+$ T cells (left: top, 10X; bottom, 40X), $CD8^+$ T cells (middle: top, 10X; bottom, 40X), and MPO using DAB stain (right: top, 10X; bottom, 40X). $CD3^+$ and $CD8^+$ T cells stained with DAB; MPO stained with Fast Red



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