Harnessing the Power of Stem Cells in Vaccine Against *Mycobacterium tuberculosis*

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Abstract

Mycobacterium tuberculosis (*Mtb*) remains one of the most successful human pathogens. Approximately two million people die of TB annually and eight to ten million new cases of active TB are documented each year. The only available vaccine against TB is Bacillus Calmette-Guerin (BCG). Despite that the efficacy of BCG varies considerably in protecting adults from pulmonary TB, BCG offers protection in children. Previous studies have shown that BCG is missing the most dominant T cell antigens of *Mtb* (e.g. ESAT-6, CFP-10) and that there is no direct link between BCG-specific T cells and protection against TB. Furthermore, multiple clinical trials using T cell-targeted vaccine approaches have failed to provide protection against *Mtb* infection. Therefore, the potential mechanism of protection offered by BCG vaccination is still unknown.

The importance of innate immune mechanisms is reflected by its remarkable diversity at almost every level of the evolutionary tree of life. Macrophages are a particularly ancient cellular compartment of innate immunity and the dominant cell type in which *Mtb* infects and replicates. We have recently shown that the fates of pulmonary macrophages are critical in both innate and adaptive immunity to *Mtb* infection. Thus, the success of *Mtb* in establishing chronic infection is dictated by pulmonary macrophages. Given the fact that bone marrow-derived monocytes are the main source of pulmonary macrophages during inflammatory settings and a recent study demonstrated that *Mtb* accesses the bone marrow and infect stromal stem cells, we speculate that *Mtb* hijacks the control center of hematopoiesis as a mechanism of long-term survival. Indeed, hematopoietic stem cells (HSCs) reside in a unique niche of the BM and are essential in host defense by rapidly replenishing both innate and adaptive immune cells following

infection. Although we know a great deal about the pulmonary immune responses to *Mtb*, our understanding of immunity to *Mtb* in the bone marrow is extremely limited. In the current study, we aim to define the cellular signatures involved in HSC activation/function during infection with avirulent (BCG) or virulent strains of *Mtb* (H37Rv) with the ultimate goal of developing a roadmap of protective versus detrimental immunity to this devastating pathogen.

Résumé

Mycobacterium tuberculosis (*Mtb*) demeure l'un des agents pathogènes les plus menaçant pour la santé humaine. Chaque année, il est estimé que deux millions de personnes meurent de la tuberculose et il est rapporté huit à dix millions de nouveaux cas de tuberculose active. Actuellement, le bacille de Calmette-Guérin (BCG) est le seul vaccin disponible contre la tuberculose. Alors que son efficacité dans la prévention de la tuberculose pulmonaire chez l'adulte reste très variable, la vaccination par le BCG confère une protection chez les enfants. De précédents travaux suggèrent que cette variabilité peut s'expliquer par le fait que le BCG manque les principaux antigènes des lymphocytes T de *Mtb* (i.e. ESAT-6, CFP-10). De plus, d'autres études ont montré qu'il n'y avait aucun lien direct entre les lymphocytes T spécifiques induits par le BCG et la protection contre la tuberculose. Enfin, plusieurs essais cliniques de vaccins ciblant les lymphocytes T se sont révélés inefficaces dans la protection contre l'infection par *Mtb*. Par conséquent, les mécanismes potentiels de protection conférés par la vaccination par le BCG restent encore inconnus.

Présente à presque tous les niveaux de l'arbre de l'évolution, l'immunité innée constitue un mécanisme de défense critique contre les maladies infectieuses. *Mtb* est capable d'infecter et de se répliquer dans les macrophages, un des plus anciens types cellulaires impliqué dans l'immunité innée. Nous avons récemment montré que dans les infections par *Mtb*, le sort des macrophages pulmonaires représentait une étape critique pour l'établissement des réponses immunitaires innée et adaptative et des infections chroniques par *Mtb*. Au cours des maladies inflammatoires, les macrophages pulmonaires ont pour principale origine les monocytes issus de la moelle osseuse (MO). Une récente

étude a démontré que *Mtb* pouvait accéder à la MO et infecter les cellules souches stromales. En se basant sur ces résultats, nous avons émis l'hypothèse que *Mtb* était capable de détourner l'hématopoïèse comme un mécanisme de la survie à long terme. Les cellules souches hématopoïétiques (CSH) localisées dans la MO sont essentielles pour les défenses de l'hôte en reconstituant rapidement les cellules immunitaires innées et adaptatives lors d'une infection. Contrairement à la réponse immunitaire pulmonaire qui est largement étudiée, notre compréhension de l'immunité contre *Mtb* dans la MO est extrêmement limitée. Dans cette étude, nous cherchons à définir les signatures cellulaires impliquées dans l'activation et la fonction des CSH lors d'infections par des souches avirulentes (BCG) ou virulentes de *Mtb* (H37Rv), avec pour but ultime la description des mécanismes immunitaires protecteurs ou préjudiciables en réponse à ce pathogène dévastateur.

In Loving Memory of

Mary Louise Dunn Obomsawin

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List of Abbreviations

AIDS −acquired immune deficiency syndrome

APCs −antigen presenting cells

BCG −Bacillus Calmette-Guérin

BM −bone marrow

DCs −dendritic cells

HIV −human immunodeficiency virus

HSCs −hematopoietic stem cells

IL-1β −interlueken-1β

IL-12 −interlueken-12

IRFs −interferon regulatory factors

KO −knock out

LKS+ −Lineage^{-} c-Kit^{+} Sca-1^{+}

LN −lymph nodes

LM −*Listeria monocytogenes*

LPS −lipopolysaccharides

MAVS −mitochondrial antiviral signaling protein

MDP −muramyl dipeptide

MHC I −major histocompatibility complex class I

MHC II −major histocompatibility complex class II

Mtb −*Mycobacterium tuberculosis*

MVA −modified vaccine virus Ankara

NLRs −NOD-like receptors

PAMPs –pathogen-associated molecular patterns

PGE2 –prostaglandin E2

PRRs −pattern recognition receptors

RLHRs −RIG-like helicase receptors

TB −tuberculosis

TLRs −toll-like receptors

TNF-α −tumor necrosis factor-α

TST −tuberculin skin test

Chapter I: Introduction

1.1 Epidemiology of Tuberculosis

For *Mycobacterium tuberculosis* (*Mtb*) to persist in individuals with apparently normal immune system implies that it has developed strategies to avoid, evade, and even subvert innate and adaptive immunity. As a result, *Mtb* remains one of the most successful human pathogens. Despite recent advances in diagnosis and treatment. Tuberculosis (TB) continues to pose a substantial threat to human health and ranks among the most prevalent and deadly infectious diseases. Approximately two million people die of TB annually and eight to ten million new cases of active TB occur each year due to the large reservoir of asymptomatic persons chronically infected with *Mtb* [1]. The approximate size of this latently infected reservoir is estimated to be one third of the world's population. From this latent pool, 5-10% are at risk of reactivation when the host immune response is no longer able to control the bacteria, inducing lung pathology, which increases the transmission risk and mortality. Immunocompromised individuals, such as TB patients co-infected with HIV, increases the risk of active disease from 5-10% in a lifetime to ~8% per year [2]. TB disproportionately affects the poor and thus the majority of global disease burden is concentrated in developing countries with the highest incidence rates of active TB in Sub-Saharan Africa and Southeast Asia. The Canadian artic Inuit community has had an increase of documented TB cases, roughly 1000 times the overall Canadian incidence rates [3]. However, cases in high-income countries are rare and more commonly found among recent and landed immigrants [4]. Considering the emergence and increase of multi-drug resistance tuberculosis [5], the development of an effective vaccine is undoubtedly critical to prevent TB. The only available vaccine to

protect individuals against TB is the *Mycobacterium bovis* derived Bacillus Calmette-Guérin (BCG) vaccine. However, the efficacy of this vaccine varies considerably and protection is not life-long [6, 7]. This highlights the urgent need for generating a high-efficacy vaccine against TB by employing novel and innovative approaches.

1.2 Immunopathogenesis of Tuberculosis

The primary route of infection occurs through the respiratory tract such as inhaling droplets containing *Mtb* expelled from coughing infected individuals [8]. TB begins and primarily remains as a pulmonary disease. Symptoms include chronic coughing, loss of appetite, weight loss, sputum production, fevers, and night sweats [9]. It is estimated that the number of *Mtb* bacilli required to cause disease ranges from 1 to 400 bacilli [10]. Once being inhaled, *Mtb* enters the distal parts of the lower respiratory tract, where they encounter and infect alveolar macrophages. *Mtb* has evolved into a parasite of the alveolar macrophages, where it not only survives but replicates inside the macrophages, which is typically a hostile environment. Due to its ability to inhibit the maturation of the phagolyosomal fusion, preventing the acidification of the phagosomal compartment, *Mtb* persists in the macrophage and actively replicate within the phagosome [11]. Macrophages normally excel at destroying the biological particles they engulf by phagocytosis instead Mtb kills them. Enabling the bacteria to disseminate to bystander cells and further promote the infection. Due to the virulence of the bacteria, *Mtb* is able to kill macrophages by exploiting their cell death program and induce necrosis rather than apoptotic cell death [12]. It is pivotal to maintain the plasma membrane integrity during cell death in order contain the pathogen within an apoptotic

vesicle that can be cleared by being engulfed via efferocytosis by other phagocytic cells. However, virulent *Mtb* induce necrosis and destroy the plasma membrane integrity by inhibiting the production of prostaglandin E_2 (PGE2), an eicosanoid which is essential to promote the plasma membrane repair mechanisms, resulting further dissemination [13]. Failure at eliminating or containing *Mtb* initiates a cascade of events that lead to a full-scale response of the innate immune system, involving production of cytokines and chemokines that induce the recruitment of other inflammatory cells, such as neutrophils, dendritic cells (DCs), and monocytes to the site of infection [14].

If the innate immune responses is unable to control the growth of *Mtb*, bacteria disseminate initially to the thoracic lymph nodes (LN) initiating the adaptive immune responses [15]. The central dogma of initiating an adaptive immune response towards tuberculosis is established with migratory DCs, the key immune cells responsible for the translocation of bacteria from the site of infection to the draining lymph nodes, priming a T lymphocyte mediated immune response. Interestingly, a recent study suggested a different manner on the translocation of *Mtb* from the primary site of infection to the lymph nodes. The inflammatory monocytes expressing CCR2 (a chemokine receptor that facilitates the egress of inflammatory monocytes from the BM) were responsible for the transport of *Mtb* from the lung into the draining LN where they may either transfer antigen to DCs or differentiate into antigen-presenting cells to prime *Mtb*-specific CD4 and CD8 T cells [16].

Regardless of the translocating mechanism, *Mtb* arrives in the draining LNs and conventional DCs prime naïve T cells. This leads to expansion of *Mtb* antigen-specific T cells and their migration into the lungs. There they will aid the innate immune cells to

further contain the mycobacterium by forming a granuloma. TB granulomas are primarily composed of infected macrophages, surrounded by uninfected DCs, macrophages, foamy macrophages, epitheloid cells, multinucleated giant cells, T and B lymphocytes, along with fibroblasts that encase this central mass with a fibrotic wall [17]. At the site of infection mainly $CD4^+$ T cells produce IFN-γ that activates antimicrobial capacity of *Mtb*-infected macrophages (e.g. ROS and NOS) and cytolytic $CD8^+$ T cells which are responsible for directly killing *Mtb*-infected cells via perforin/granzyme pathways [18]. Thus granulomas impede the spread of infection to extrapulmonary sites and ultimately contain the pathogen. However, the host immune system is unable to completely eradicate all bacteria and some enter a long-lived stationary phase known as latency. This is due to hypoxic and nutrient starving environment of granulomas that alters the *Mtb* metabolic state, becoming dormant, and thus surviving within a granuloma in a non-replicating state [19, 20]. This state of infection is considered latent as long as the immune system is able to sustain the granuloma's structure and obstruct the bacteria's reactivation.

1.3 Innate Immune Response to *Mycobacterium Tuberculosis*

1.3.1 Pattern Recognition Receptors

Innate immune cells utilize a wide array of pattern recognition receptors (PRRs) to provide early detection, containment, and clearance of microbial pathogens. Three major types of PRRs recognize pathogens: Toll-Like Receptors (TLRs), NOD-Like Receptors (NLRs), and RIG-Like Helicase Receptors (RLHRs). TLRs are expressed both on the cell surface and on endosomes, and upon pathogen recognition activate NF-κB and

AP-1 transcription factors, leading to the production of inflammatory cytokines (TNF-α, IL-1, IL-6). In contrast, both the NLRs [21] and RIGRs [22] are intracellular. While the majority of RIGRs and NLRs are cytoplasmic, some have been localized to mitochondria. A critical adaptor for RIGR signaling is the Mitochondrial Antiviral Signaling protein (MAVS), which is localized in the mitochondria outer membrane and mediates activation of both NF-kB and interferon regulatory factors (IRFs) following infection [23]. While the essential role of type I IFNs in immunity to viral infection is well established, their protective activity against bacterial infection is more ambiguous. In fact, it has been suggested that type I IFN production during influenza infection sensitizes the host to bacterial superinfection. In line with this emerging concept, the type I IFN system increases host susceptibility to *Listeria, Streptococcus,* and *Mtb* [24].

During *Mtb* infection, alveolar macrophages are the first immune cells to recognize and respond to the bacteria. It is highly improbable for alveolar macrophages to eliminate the bacteria at the onset of the infection. Interestingly, there are anecdotal evidence indicating that few individuals are able to eliminate the bacteria from the outset while constantly exposed to active tuberculosis persons, suggesting they have a high innate immune response sufficient to control and clear *Mtb* infection [2, 25]. However, for the vast majority of individuals infected with *Mtb*, the adaptive immune response is ultimately required for containment of *Mtb*.

1.3.1.1 *Mtb & TLRs*

TLRs are crucial in innate resistance and initiating an adaptive immune response to infectious agents. TLRs are mainly expressed on the immune cell surface membrane

and bound on endocytic vesicle membranes, including macrophages and DCs. Different sets of TLRs are associated with specific responses to diverse microbial antigens. They trigger signaling pathways such as myeloid differentiation factor 88 (MyD88), which is required for downstream signaling for all TLRs except TLR3 that lead to the transcription of genes involved in the activation of innate host defense, mainly producing proinflammatory cytokines such as TNFα, IL-1β, IL-12 and nitric oxide [26, 27]. The TLRs known to be involved in TB are TLR2, TLR4, TLR9, and possibly TLR8 [26].

TLR2: TLR2 is important in the initiation of innate host defense against *Mtb* [28, 29]. TLR2 can form heterodimers with both TLR1 and TLR6. This is advantageous to the host because these heterodimers are capable to recognize more various mycobacterial cell wall structures [26, 29]. TLR2 recognizes *Mtb* and initiate cellular activation that leads to induction of key anti-mycobacterial mediators, including TNFα [27, 30], IL-12 [26] and nitric oxide [25]. The production of IL-1β is dependent on TLR2 and TLR6 [31]. *In vivo* models showed, TLR2 knock-out (KO) mice show defective granuloma formation following *Mtb* infection and have a greater susceptibility to infection compared to the WT and are unable to control chronic infection with *Mtb* [32, 33].

TLR4: Although TLR4 recognizes LPS, which is a component of gram-negative bacteria and mycobacteria do not express LPS, TLR4 appears to be involved in *Mtb* infection by potentially regulating the magnitude of inflammatory responses. In murine aerosol model of TB, TLR4 deficient mice were susceptible to high dose of infection (5000 CFU) [34],

but not low dose (100 CFU) [25]. Thus the physiological contribution of TLR4 in TB needs further investigation [27].

TLR9: TLR9 is located in endosome and responsible for recognizing unmethylated CpG motifs in bacterial DNA. *In vivo* studies demonstrated that *Mtb* induces IL-12 release by DCs in a TLR9-dependent manner [35]. It was also shown when mice were infected with a high infectious dose of *Mtb*, animals lacking TLR9, succumb earlier to infection than wild-type animals [36].

TLR8: Along with TLR9, TLR8 is also an endocytic vesicle PRR. TLR8 is known to recognize single-stranded RNA from pathogens such as RNA viruses. However, it may play a role in the innate response to *Mtb* infection. It was exhibited that there is an up regulation of TLR8 protein expression in macrophages after infection with BCG [37]. An *in vivo* study involving TLR8 KO mice challenged with *Mtb* exhibited an increase of neutrophils and macrophages numbers in the lung, but not lymphocytes. Additionally, TLR8 KO mice succumbed more rapidly to an *Mtb* infection than wild-type mice. Potentially this may also be linked to TLR8 mediated type I IFN production, as *Mtb* preferentially enhances type I IFN production that promotes bacterial growth. Therefore, TLR8 might play a regulatory role in immunity to TB [26] via type I IFN pathway.

1.3.1.2 *Mtb & NLRs*

NLRs are crucial cytosolic innate immune proteins that recognize intracellular bacterial peptidoglycan fragments and pathogen-associated molecular patterns (PAMPs)

and initiate an immune response by activating signaling pathways such NF-κB leading to a production of proinflammatory mediators [38]. The first two members of NLRs discovered were, NOD1 (nucleotide-binding oligomerization domain-containing protein 1) and NOD2 [39]. NOD1 is ubiquitously expressed in various cell types, whereas NOD2 is limited more in immune cells and different types of epithelial cells [40]. There are few studies investigating the functions of NOD1 responding to an *Mtb* infection.

NOD2: NOD2 responds to muramyl dipeptide (MDP), a peptidoglycan motif common to all bacteria, by activating the NF-κB pathway [41]. Mycobacteria produce a modified version of MDP, known as *N*-glycolyl MDP [42] which is more potent at mediating a host response by NOD2 compared to the regular form of MDP, *N*-actyl MDP [43]. Studies have demonstrated, NOD2 KO alveolar macrophages produced less proinflammatory cytokines IL-12 and TNFα and NOD2 KO mice are more susceptible to Mtb infection [44]. Similar to murine models, NOD2 was important in the production of IL-1β, 1L-6, and TNF-α in human alveolar macrophages infected with mycobacteria [45]. There was indication that NOD2 might play a role in bridging the innate and adaptive immunity as the MDP treatment in DCs leads to induction of autophagy and influencing antigen presentation in DCs [46].

1.3.2 Innate Cytokines

PAMPs and PRRs interaction initiate the first wave of cytokine production. Cytokines play a crucial role by activating cell defense mechanisms, in means of containing pathogens, initiating adaptive immunity, and resolving the inflammation. The

following section will highlight five major cytokines, produced by macrophages, involved in host defense against an *Mtb* infection.

Tumor Necrosis Factor-α (TNF-α)

TNF-α is a proinflammatory cytokine essential in formation of granulomas [47]. Mice deficient in TNF-α receptor or given anti-TNF-α treatment had an increase susceptibility to *Mtb* infection [48]. The importance of TNF-alpha in humans has been demonstrated when either latent TB patients with Crohn's or rheumatoid arthritis were treated with anti-TNF-α therapy that significantly increased their risk of developing active TB [49]. Thus, TNF-α is crucial for maintaining granulomas to protect individuals from active TB.

Interlueken-1β (IL-1β)

Initially IL-1β is produced in an inactive form called pro-IL-1β. It is upon cleavage by caspase-1, a product of the inflammasome, where it then becomes an active proinflammatory cytokine and participates in controlling *Mtb* infection [50]. Several studies have shown that IL-1β KO mice are unable to control the pulmonary *Mtb* infection leading to an increase of mortality [51]. Mechanistically IL-1β contributes to host defense against *Mtb* by promoting phagolysomal maturation and suppression of type I IFN in macrophages [52, 53].

Interlueken-12 (IL-12)

IL-12 is a critical cytokine in host defense against *Mtb* infection. Humans deficient in IL-12 signaling pathway are extremely susceptible to mycobacterial infection [54]. Similarly, IL-12 deficient mice are also susceptible to *Mtb* infection and IL-12 treatment significantly reduced lung bacterial burden [55]. It was later demonstrated that IL-12 is responsible for DCs maturation, triggering their migration to the draining lymph nodes to prime a T cell mediated immune response [56]. IL-12 also induces IFN-γ production [57] and drives a Th1 phenotype in naïve T cells [58]. Together, IL-12 is an important cytokine bridging the innate immune response to the adaptive immunity following *Mtb* infection [59].

Type I Interferon: Interferon alpha and beta (IFN-α, IFN-β)

IFN-α and IFN-β are important cytokines in providing anti-viral protection, but it is detrimental in immunity to mycobacterium infection. A study showed that *Mtb* can initiate type I interferon signaling which results in the suppression of IL-1β production [60]. Consequently, high levels of type I IFN can also lower the production of other key cytokines such as TNF-α and IL-12 increasing WT mice susceptibility to an *Mtb* infection [61]. Conversely, inhibiting the production of type I IFNs via PGE2 enhanced the host protection against *Mtb* [53] and we have also previously shown that PGE2 plays a protective role in immunity to TB [13, 62]. Overall, further studies are needed because the direct mechanisms involved between type I IFNs and host susceptibility during an mycobacterium infection are not well understood.

Type II Interferon: Interferon gamma (IFN-γ)

Though IFN-γ is not considered an innate cytokine nor secreted by macrophages, it is a crucial cytokine mainly produced by $CD4^+$ T cells to protect the host against *Mtb*. As stated before, the appearance of the signature cytokine IL-12 from phagocytes triggers the production of IFN-γ [57]. The production of IFN-γ promotes naïve $CD4^+$ T cells to differentiate towards Th1 T cell response, which is central in cellular immunity against intracellular pathogens [63]. IFN-γ synergizes with TNFα to activate macrophages to kill *Mtb* [64]. IFN-γ KO Mice succumb to death within weeks after challenge with *Mtb*. Along with activating macrophages, it has been shown that IFN-γ from CD4+ T cells may enhance functions of antigen specific $CD8^+$ T cells during an *Mtb* infection [65].

1.4 T cell priming

As *Mtb* is an intracellular pathogen T cell immunity is critical for controlling the infection. DCs are the main immune cells that are responsible for bridging the innate to adaptive immunity [66]. During *Mtb* infection, DCs phagocytize *Mtb* and present it's antigens to prime a T cell mediated immune response. There are two specific pathways for antigen presentation, exogenous and endogenous pathways, which are responsible for priming a distinct subset of T cells [67]. The exogenous pathway involves phagocytosis of exogenous antigens, snipping them into small peptides, loading the peptides on to major histocompatibility complex class II molecules (MHC II) and presenting them on the cell surface to prime $CD4^+$ T cells [68]. Unlike the exogenous pathway, the cytosolic pathway involves obtaining endogenous antigens preexisting in the cell's cytoplasm, transporting the antigen into an endosomal vesicle, snipping the antigen into smaller

fragments, loading the peptide on major histocompatibility complex class I (MHC I) molecules to prime $CD8^+$ T cells [69].

Following phagocytosis, *Mtb* persist in phagosomes of macrophages and DCs. Therefore, APCs preferentially utilize the exogenous pathway to load *Mtb* antigens on MHC II molecules to prime $CD4^+$ T cells. However, several lines of evidence have shown a role for $CD8^+$ T cells in controlling *Mtb* infection. It suggests that *Mtb* antigens can also be presented on MHC I molecules to prime $CD8^+$ T cells through a process called cross-presentation. Cross-presentation refers to the processing and presentation of extracellular antigens on MHC I molecules, whereas MHC I are normally associated with presenting cytosolic antigens to $CD8^+$ T cells [70]. Apoptosis, commonly known as programmed cell death, is associated with cross-presentation of mycobacterial antigens. Our lab has recently demonstrated the involvement of *Mtb* infected cells that die by apoptosis are engulfed by DCs via efferocytosis; a process involves in phagocytosis of apoptotic vesicles. The mechanism of efferocytosis requires 'eat me' signal, which is a unique set of surface markers (e.g. Annexin 1) on dying cells for facilitating their uptake by phagocytic cells, including DC for cross-presenting antigens to $CD8^+$ T cells [71]. . Ultimately, cross-presentation is essential in enhancing T cell mediated immunity to TB [70].

1.5 Adaptive Immune Response to Mycobacterium tuberculosis

1.5.1 T lymphocytes

T cells are central for protection against the development of tuberculosis. $CD4^+$ T cells proliferate and migrate to the site of infection and produce IFN-γ and TNFα to

initiate macrophages host defense against the intracellular bacteria. It is well documented that $CD4^+$ T cells are crucial in immunity to TB, as reduction of $CD4^+$ T cells in HIV co-infection markedly increase TB reactivation [72]. Activated CD8+ T cells also expand and migrate to the pulmonary infection sites providing protection. Similarly MHC-I KO (lacking CD8 T cells), MHC-II KO (lacking CD4 T cells), or TCR-alpha/beta KO (lacking both CD4 and CD8 T cells) mice are profoundly susceptible to *Mtb* infection [73, 74].

1.5.2 B-lymphocytes

B cells are generally ignored in the study of immunity against *Mtb* due to its antibody-mediated immunity, as humoral immunity is an important immune response for extracellular pathogens [75]. The role of B cells have been comprised of antigen presentation [76], cytokine production to modulate T function [77], and antibody production [78]. Yet, B cells are a prominent component of tuberculosis granulomas, within in mice and humans [79]. It has been suggested that B cell antibodies may have direct contact with the bacterium when the infected cell becomes necrotic and *Mtb* disseminates to infect other regions [75]. Overall, the role of B cells remains unclear in immunity to TB.

1.6 Extrapulmonary Infection of Mycobacterium tuberculosis

Though 70% of TB cases are predominantly pulmonary disease [80], mycobacterium can be also isolated from peripheral lymphoid organs in diverse populations ranging from asymptomatic individuals to those suffering from AIDS [81]. In

a TB mouse model, mycobacterium dissemination to the regional LN initiates the anti-*Mtb* T cell response [15]. In addition, *Mtb*-specific T cells have been found in the BM during pulmonary TB [81, 82]. However, the mechanism by which *Mtb* spreads to the BM and its ultimate effect on host-mediated control of TB is not clear. The BM is the seat of hematopoiesis where HSCs constantly undergo asymmetric division giving rise to the full repertoire of immune cells such as myeloid and lymphoid cell types. It also is an active site of mature leukocyte circulation suggesting the ability of this primary lymphoid organ to serve as a site of host defense. For example, previous studies have shown that memory T cells accumulate in the BM, and upon secondary immunization, migratory DCs can reactivate these cells to produce IFN-γ [83]. However, the relevance of this response to protective immunity has not been fully investigated. The translocation of *Mtb* to the BM may have significant consequences on the pathogenesis and chronicity of TB, considering this privileged site lacks a physical barrier to exclude returning immune cells and is also home to hematopoietic stem cells (HSCs), the earliest precursor of immune cells.

1.7 Bone Marrow Hematopoietic Stem Cells

1.7.1 Introduction

HSCs are responsible for sustaining and producing mature cells of the blood and immune system. It is estimated that HSCs produce over 500 billion blood cells each day [84]. Despite the enormous production of mature blood cells HSCs make up only around 0.01-0.001% of total nucleated cell in the BM [85]. In stem cells, multipotency, self-renewal capacity, and quiescence are three central properties featured in HSCs [84]. At a

homeostatic condition, the majority of HSCs are quiescent, some dividing only five times in a mouse lifetime [86], while others divide more frequently constantly proliferating and differentiating maintaining the threshold of required blood cells while pertaining in their BM niche.

1.7.2 Bone Marrow Niche

HSCs mainly reside in a specialized niche of the BM [87]. Despite that the bone is continuously undergoing remodeling to grow, heal damage, and regulate calcium and phosphate metabolism [88], the BM provides a microenvironment that is able to maintain and regulate hematopoiesis. Along with hematopoietic cells, the BM is constituted of other cells such as mesenchymal stem cells, osteoprogenitors, osteoblasts, osteocytes and chondrocytes, which all contribute to bone homeostasis [84]. Mesenchymal cells, such as osteoblasts and osteoclasts, are responsible for mediating mobilization and homing of HSCs within the BM [88]. The association of HSCs mobilization utilizing the peripheral blood and finally homing to the BM led investigations of the HSCs distribution within the primary lymphoid organ. The distribution of HSCs is not uniform within the BM. Their positioning is proportioned near the sinusoidal endothelial region, which is an area near a blood vessel, and others residing to a more peripheral region like the endosteal lining of the trabecular bone [89]. By microscopy the majority of HSCs (80%) are localized peripheral to the endosteal region and the rest (20%) are in close proximity to the perivascular niches in proximity to the central vein in BM [90, 91]. The various stages of HSCs activity (e.g. quiescent or proliferating) determines their location within the BM. Quiescent HSCs reside more in a hypoxic environment close to the endosteal region.

While active HSCs reside near perivascular niches enabling them to receive stress signals to mobilize, proliferate and differentiate [92].

1.7.3 HSCs and Infection

As HSCs are progenitors of immune cells, which are responsible for protecting the host from pathogens, recent studies indicate that HSCs also play a crucial role in response to several acute and chronic infections. For example, it has been observed in models of acute *Escherichia coli* infection [93] as well as chronic *Mycobacterium avium* infection [94] there was a significant expansion in HSCs including the Lineage^{-}, c-Kit^{+}, Sca-1^{+} (LKS+) population, markers expressed on a distinct subset of HSCs responsible for differentiating into immune cells. This proliferation enhances differentiation of lineage cells necessary to preserve the host integrity by rapidly replacing progeny lost during infection [95]. However, persistent stress signals can over stimulate HSCs, resulting in BM exhaustion and even complete depletion of HSCs with devastating systemic effects on the immune compartment [96-98]. Although the exact mechanisms of HSC proliferation/expansion after infection are not well understood, there have been recent studies that proposed several mechanisms to explain this phenomenon [99]. This includes:

Direct Infection

HSCs lack the molecular machinery required for phagocytosis, many pathogens including *Salmonella*, *Listeria*, *Yersinia*, and *M. avium* fail to directly infect HSCs. However, the possibility that a virulent strain of *Mtb* can infect HSCs has no been

experimentally tested. Notably, recent data indicates that *Mtb* infects BM mesenchymal stem cells, which are phagocytic [81, 100].

Pattern Recognition Receptor Signaling

HSCs express both cell surface Toll-like receptors (e.g. TLR-4 that recognizes bacterial LPS, and the heterodimer of TLR1 and TLR2, that recognizes lipopeptides [101, 102]) and cytosolic NOD-like receptors (NOD1 and NOD2 which can sense bacterial peptidoglycan) [103]. Exposure to either TLRs or NOD PRR ligands lead HSCs to enter the cell cycle and to induce the generation of its progenies, including monocytes and macrophages – a process called myelopoiesis., The differentiated immune cells later egressed from the BM to mobilize to the site of infection [101, 103, 104]. Magias et al, 2011 elegantly demonstrated the influence of TLR activation on the differentiation of HSCs. By transplanting HSCs LKS+ cells from BM of mice into TLR2 KO mice, TLR4 KO mice, and MyD88 KO mice, they showed that the stimulation of PRRs leads to HSCs proliferated and differentiated towards a myeloid lineage cell type [105]. Thus the activation of PRRs is the major deriver for HSC activation during infection.

Inflammatory Cytokine Stimulation

Quantitative changes in HSC activity are regulated by cytokines relevant to TB. For example, both type I IFNs (IFN-α and β) and type II IFN (IFN-γ) along with TNF-α promote quiescent HSCs to proliferate and differentiate to replenish downstream myeloid immune effector cells during bacterial infections [94, 96, 98, 104, 106]. However, as type I and type II IFN have opposing effects in the clearance of *Mtb* by macrophages [53,

107], IFN stimulation on HSCs during a mycobacterium infection is not well understood. It was demonstrated with *in vitro* and *in vivo* models, following a *M. avium* infection the increased activation of HSCs was IFN-γ dependent, highlighting the pivotal role of IFNs in guiding HSC responses to mycobacterial infection [94]. Despite the influential role of type II IFNs, chronic stimulation from either type I or type II IFN may be detrimental and cause impairment of HSC function, leading to stem cell exhaustion [94, 98]. Further investigation is needed to determine the role of IFN-dependent pathways in regulation of HSC responses and its consequences following infection with virulent strains of *Mtb*.

1.8 Bacillus Calmette-Guérin Vaccine

1.8.1 Introduction

Vaccines are important tools to protect the host from microbial infections. Since 1921, Bacillus Calmette-Guérin (BCG) vaccine is the only approved vaccine against TB. It has been administered to billions of individuals since its implementation, making it the most widely used vaccine in the world [108]. Today, 104 million children are vaccinated, by intradermal injection in the deltoid region, each year [2]. Nearly a century of use, BCG still remains controversial with known variations in vaccine efficacy [109]. The capacity to protect adults is still debated since clinical trails have provided estimates of protection against TB ranging from 0% to 80% [110]. However, in children it is effective of preventing military and disseminated TB in children [111]. Thus, BCG appears to be more of a preventive treatment to limit the progression of infection to disease rather than preventing the onset of infection. Despite an incomplete understanding of the basic mechanisms of immunity conferred by BCG against the development of TB, researchers

have continuously sought to improve it by adding various agents (i.e. cytokines, chemokines, lipid mediators, nucleotides, and antigenic components from *Mtb*) to the vaccine; all with variable levels of success [108]. A recent setback in enhancing the BCG vaccine was the MVA85 vaccine. This vaccine used an attenuated modified vaccinia virus Ankara (MVA), which lacked all virulence factors, including the ability to replicate within human cells but expressed Mtb antigen-85A. This vaccine was used as a booster shot after the initial BCG vaccine. The booster shot was quite successful inducing protection in early animal models and phase I human clinical trails. However in phase II human studies, it showed no additional protection against infection or development of disease [112]. Currently there are sixteen TB vaccine candidates that are in clinical trails and a few entering phase II studies. The vaccine candidates include five vaccines based on whole cell mycobacteria and the rest based on various sub-unit of *Mtb* antigens formulated with adjuvants or presented in recombinant viral vectors [113].

1.8.2 History of route of BCG Delivery

BCG was conceived and developed between 1905 and 1921 at the Pasteur Institute, first in Lille and later in Paris, France [114] by Albert Calmette, a French physician, and his colleague, Camille Guérin, a veterinarian. They understood the principle of vaccination, set by Edward Jenner's discovery of using cowpox, a lesser pathogenic relative of smallpox, to engender immunity to the more threatening bacterium [115]. Therefore to produce a vaccine against *Mtb*, Calmette and Guérin isolated *M. bovis* from infected cow's udder and grew it on potato slices. Calmette added a detergent, specifically bile, to the potato culture medium, to prevent the bacteria from clumping. As

a result of the bile, unique colony types arose, which were less virulent [116]. And over the course of 230 serial cultures, which took more than thirteen years, Calmette and Guérin concluded that they obtained an attenuated strain of *M. bovis* due to its inability to produce tubercles and its inability to restore its virulence in animals models by various methods of delivery; intravenous, subcutaneous, intraperitonial inoculation or oral ingestion [114, 117]. Furthermore, re-inoculation experiments using a virulent strain administered to animals after vaccination indicated protection lasting at least 18 months [114].

In France during the early 1920's, tuberculosis was a serious disease in children. The mortality rate of infants was high and increased to 25% if the child was in contact with a family member suffering with tuberculosis. And it was in 1921, where Calmette and Guérin took it upon themselves to vaccinate the first human, a newborn, whose risk of tuberculosis was considered high due to house relatives having tuberculosis. After vaccination the child grew up healthy. Calmette continued vaccinating endangered children and observed that BCG-vaccinated children were more resistant compared to other infants' that were not vaccinated. Therefore, they decided to extend the benefits of the vaccine to non-endangered children [117].

BCG was first administered by oral ingestion, even though most of Calmette's animal models were administered by subcutaneous inoculation [117]. Calmette reasoned the natural vector for bovine tuberculosis infection to occur was by oral ingestion [114]. In addition, the advantages of oral vaccination compared to other delivery methods of vaccination was due to its simplicity of administering the vaccine thus the method could

be entrusted to medical personal other than physicians including midwives and visiting nurses to perform, compared to needle injections [108, 114].

Up until 1927, BCG was confined within the country of France due to the Pasteur Institute maintaining a monopoly over the vaccine. Therefore, the production and standardization of the vaccine was maintained; inoculation by oral ingestion. However, after 1927 it was distributed internationally and consensus of administering standardization broke down [114]. Arvid Wallgren, chief medical officer at the children's hospital in Göteborg, Sweden, publically argued that oral delivery of BCG was not the way of vaccinating children because it was arduous to know the amount BCG was absorbed by the intestinal tract and the variation of BCG absorbed was likely different from child to child. Therefore to be certain the infant was vaccinated with the same dose of BCG, Wallgren encouraged vaccination by injection, specifically intradermal inoculation, and by this method a child could show tuberculin sensitiveness indicating evidence that the vaccination had taken place [118]. Despite the critics and having acknowledged inconsistent results in terms of tuberculin skin test (TST) sensitivity, signifying immunity, by BCG oral ingestion, Calmette disregarded the TST as reliable indicator of BCG's immunizing potency [114]. Thereafter, intradermal administration was widely developed in Scandinavian countries after 1927, whereas the oral route remained dominant in France until the end of World War II [114]. In 1947, the Danish Red Cross, along with the Norwegian and Swedish Red Cross started the first mass BCG vaccination campaign across war-torn Europe due to the raising rates of tuberculosis. Since the post-war mass vaccination program in Europe, BCG administered method was standardized to the intradermal route [114].

The implementation of BCG vaccination in Canada was pioneered by the renowned Canadian Physician Dr. Armand Frappier. Frappier received his medical degree from Université de Montréal and moved to Paris to study tuberculosis and BCG at the Pasteur Institute. He became convinced that BCG was both effective and safe against tuberculosis. Frappier returned to Quebec (Canada) with a flask containing a strain of BCG and led the first BCG vaccination campaigns in the 1940s and 1950s to the rural Canadian communities, which suffered higher rates of death from tuberculosis [119]. From his efforts, Frappier was one of the first to confirm the efficacy and safety of BCG within North America.

1.8.3 Novel BCG Approach

Despite the lack-luster of T cell-targeted vaccines, BCG is still feasible because of its effectiveness in early childhood and thus offers critical proof-of-concept evidence that protective vaccination against TB is achievable. However, the mechanism of protection offered by BCG vaccination is most likely independent of T cell-mediated immunity, due to the most prominent T cell antigens of *Mtb* (e.g. ESAT-6, CFP-1o) missing in BCG [116] and there being no direct link between BCG-specific T cells and protection against TB [120], but rather relies on innate immune cells. In support of this hypothesis, it was recently shown that BCG could confer resistance to heterologous secondary infection by epigenetic reprogramming of monocytes and altering cell metabolism [121, 122]. These epigenetic changes may occur even prior in immune cell lineage differentiation ultimately in progenitors of all immune cells, HSCs, depending on the delivery of the vaccine and exposure to HSCs. This idea of having a high potential in generating a more

enhanced innate immunity (the first immune response) by vaccines has been largely ignored and may offer a new innovative approach for protecting individuals against TB.

1.9 Central Hypothesis

The central hypothesis of the current study is that ***the protection mechanism of BCG vaccination is mediated via innate immunity.*** To test this hypothesis we design three objectives:

1) Characterize the effect of a virulent strain of *M. tuberculosis* on the HSCs' LKS+ compartment.
2) Determine the effect of the an avirulent strain of *M. bovis* (BCG) on the HSCs' LKS+ compartment
3) Evaluate the protection provided by BCG, utilizing macrophages derived from stimulated LKS+ stem cells against *M. tuberculosis*.

Chapter II: Materials & Methods

2.1 Mice

Six to ten weeks old C57BL/6 mice were from Jackson Laboratories. All experiments were conducted in accordance with the guidelines of the animal research ethics board of McGill University.

2.2 Bacteria strain and infection

BCG-tice and virulent M. tuberculosis strain H37Rv were grown at 37°C under constant shaking in 7H9 medium (BD Biosciences) containing 0.2% glycerol (Fischer), 0.05% Tween 80 (Sigma-Aldrich) and 10% albumin-dextrose-catalase (BD Biosciences). CFU were determined by plating whole BM samples suspended in 1 mL of media and performing 100uL aliquots of serial 10-fold dilutions in PBS-Tween 80 (0.05% Tween 80) on Middlebrook 7H10 medium (BD Biosciences) containing 0.5% glycerol (Fischer), 10% oleic acid-albumin-dextrose-catalase (Sigma) and PANTA (BD Biosciences). Colonies were counted after 21 days. For pulmonary infection, ~100 bacteria of *M. tuberculosis* strain H37Rv were delivered via the aerosol route using a nose-only exposure unit (Intox Products) and adequacy of infection was ascertained by enumeration of bacteria from the lungs of animals (n=3/group) 24 hours after infection. For intravenous infection, 10^6 H37Rv or BCG-tice bacteria were resuspended in 200ul of PBS and delivered via the retro-orbital vein. For subcutaneous infection, 10^6 BCG-tice bacteria were resuspended in 100ul of PBS and delivered via base of the tail. For infection with *L. monocytogenes* (LM), frozen stocks were thawed and diluted in PBS, and mice were inoculated with 10^4 LM resuspended in 200ul PBS and delivered via the

retro-orbital vein. LM was grown in brain heart infusion (BHI) media (DIFCO Laboratories) to OD_{600nm} = 0.4-0.8. At mid-log phase, bacteria were harvested, frozen in 25% glycerol, and stored at -80°C. CFUs were determined by plating whole BM samples suspended in 1 mL of PBS-Titron X-100 (0.05%) (Sigma) and performing 100ul aliquots of serial 10-fold dilutions on BHI-agar (BD Biosciences) plates. Colonies were counted after growth at 37°C for 24-36 hours.

2.3 Generation of BMDM

Murine bone marrow derived macrophages (BMDM) were prepared from aseptically dissected and flushed tibias and femurs. BM cells were seeded in BMDM medium (RPMI supplemented with 10% FBS, 2 mM L-glutamine, 1% MEM, 1% NEAA, 1 mM sodium pyruvate, 2% HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin – all from Gibco, Invitrogen) containing 30% L929 supernatant in tissue culture flasks and were allowed to differentiate into macrophages for 7 days. At day 3, fresh medium containing L929 supernatant was added. Macrophages were then used from day 7 onwards. Purity was usually higher than 99%, as evaluated by flow cytometry.

2.4 *In vitro* macrophage infection

Macrophages ($1x10^6$) were seeded into 6-well culture dishes and allowed to adhere for 24 hours. Macrophages were then challenged with an MOI of 1 of growing H37Rv between the OD of 0.5 – 0.9 AU. After 4h infection with H37Rv, macrophages were washed 3 times with culture medium and were cultured in 1mL of complete RPMI at 37°C. At varies timepoints (4hr, D3, D5) after infection, cells were then harvested and transferred

into sterile eppendorf tubes, where they were centrifuged for 10 minutes at 4000 rpm. The supernatant was aspirated. The pellet was resuspended by adding 500 uL of water. The macrophages were incubated at room temperature for 5 minutes and then 500 uL of PBS-tween 80 (0.05%) was added. The eppendorf tubes were vigorously vortexed. The CFU were determined by plating serial dilutions in PBS-Tween 80 (0.05% Tween 80) on Middlebrook 7H10 medium (BD Biosciences) containing 0.5% glycerol (Fischer), 10% oleic acid-albumin-dextrose-catalase (Sigma) and PANTA (BD Biosciences). Colonies were counted after 21 days.

2.5 Generation of chimeric mice

Five of ten CD45.1+ C57BL/6 mice were intravenously vaccinated with 10^6 BCG-tice while the other five were intravenously injected with 200 uL of PBS. After a two-week period, BM from femurs and tibiae were flushed with RPMI medium. BM cells of the same group, were collected, spun at 1,700 rpm for 7 minutes and resuspended in RPMI medium containing 10% FBS. As recipient mice, CD45.2+ C57BL/6 were irradiated with 9 Gy. These mice were kept under antibiotic treatment (128mg polymixin B and 1.1g neomycin trisulfate [Sigma-Aldrich] per liter of drinking water) for 10 days after irradiation. Sixteen hours after irradiation, $4x10^6$ BM cells were intravenously transferred from either BCG-iv or PBS CD45.1+ mice to the recipient mice. Chimeric mice were used after 12 weeks, at which time the hematopoietic compartment was reconstituted as determined by flow cytometry.

2.6 Flow cytometry

Single-cell suspension ($2x10^6$) was obtained from BM, spleens, lymph nodes, and lungs [before staining, lungs were processed and incubated at 37°C for 1 hour in a mixture of collagenase - (300 U/organ) (Sigma)]. With each panel, LKS+, T-lymphocytes and Monocyte/Macrophages, cells were stained with fixable viability dye eFluor 506 (eBioscience) for 30 min at 4°C and washed twice with 0.5% BSA/PBS. Then each panel was also incubated with anti-CD16/32 in 0.5% BSA/PBS at 4°C to block non-specific Ab interaction with Fc receptors. After this they were stained for the following markers specific for their panel. For evaluation of LKS+ population (only for LKS+ single cell suspension was $3x10^6$), cells were stained with a Biotin antibodies for Lineage negative cells; anti-TER-119/Erythroid Cells, anti-CD5, anti-CD8a, anti-CD4, anti-CD45R/B220, anti-Ly-6g/Ly-6C, and anti-CD11b (eBioscience) for 30 min at 4°C. The cells were washed twice and stained with Streptavidin APC-Cy7, anti-CD117 (c-Kit) APC, anti-Ly-6A/E (Sca-1) PE-Cy7, anti-CD135 (Flk2/Flt3) PE, anti-CD34 FITC, and anti-CD34 eFluor450 (eBioscience) for 30 min at 4°C. For evaluating LKS+ population with the chimeric mice, anti-CD48 and anti-CD150 were replaced with anti-CD45.1 (PerCp-Cy5.5) and anti-CD45.2 (eFluor450). After two washes, with 0.5% BSA/PBS, cells were fixed with 1% paraformaldehyde. For evaluation of T-lymphocyte population, cells were stained for anti-CD45.1, anti-CD45.2, anti-CD3, anti-CD8, anti-CD4, anti-CD19 and anti-CD44 markers (eBioscience) for 30 min at 4°C. The cells were washed twice and fixed with 1% paraformaldehyde. For evaluation of Monocyte/Macrophage population, cells were stained for anti-CD45.1, anti-CD45.2, anti-CD3, anti-CD19, anti-CD11b, anti-CD11c, anti-F4/80 and anti-Siglec-F markers (eBioscience) for 30 min at 4°C. The cells

were washed twice and fixed with 1% paraformaldehyde. Flow cytometry was performed using BD LSR II (BD Biosciences) with FACSDiva Software Version 6.1.2 (BD Biosciences) and analysis was performed using FlowJo Software Version 10.0.6 (Tree Star).

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Software, San Diego, California, USA). Data was compared using a two-tailed Student's *t* test or two-way ANOVA with Sidak's multiple comparison test and are expressed as mean ± SEM. Differences were considered significant if $p<0.05$.

CHAPTER III: Results

3.1 Immune evasion: Virulent *M. tuberculosis* Persists in the Bone Marrow

Its estimated that one third of the world's population is infected with *Mtb*. This implies that *Mtb* is a successful pathogen due to its ability to avoid, evade, and even subvert a normal human immune system. Despite the immune system's attempt of containing the site of infection by various and numerous immune cells resulting in the formation of granulomas. It has been suggested that the pathogen is able to survive within a granuloma, modulating its metabolic state to become dormant and persisting in the harsh hypoxic environment of a granuloma [123, 124]. Only recently it has been shown that after one-month post aerosol infection, *Mtb* was detected in murine BM [81, 82]. As BM is home to the HSCs LKS+ compartment, which are the progenitors of both the innate and adaptive immune system, they play a central role in response to infections. Thus, the gating strategy to identify BM LKS+ stem cells is elaborated on **Figure 1**.

We initially investigated the presence of *Mtb* in the BM after a low aerosol infection dose (50-100 CFU H37Rv) **(Figure 2A)**. We found that between 14 and 30 days post-infection, *Mtb* transalocates into the BM and the number of bacteria increases over time (**Figure 2B**). The LKS+ stem cells significantly expand their population resulting in differentiation of immune lineage cells necessary to replace progeny lost during infection [99]. However, the frequency of LKS+ cells slightly increased at early timepoints post-infection (**Figure 2C and 2E**), while it was significantly increased at later time point (day 97) after infection (**Figure 2D**). This significant difference on day 97 in LKS+ population coincides with the increased number of *Mtb* in the BM.

To delineate if this increase of LKS+ population was primarily due to presence of *Mtb* in the BM, we intravenously injected H37Rv (10^6 CFU) **(Figure 3A)**. We found that at day 7 post-infection there were 10^4 bacteria in the BM (**Figure 3B**), which was associated with significant increased in the LKS+ frequency (**Figure 3C, 3D, and 3E**).

To delineate the link between the persistence of mycobacteria in the BM and its effect on the LKS+ population, we next used *Listeria monocytogenes* (LM), another intracellular pathogen causing acute infection and capable of accessing the BM **(Figure 4A)**. We showed that after intravenous injection of LM (10^4 CFU), the bacteria was only detectable in the BM at day 3 post-infection and was completely cleared by day 7 after infection (**Figure 4B**). Interestingly, the expansion of the LKS+ population also occurred at day 3 post-infection, which then returned to baseline after 7 days of infection (**Figure 4C, 4D, and 4E**). Taken together, these results suggest that both virulent intracellular bacteria are able to access the BM and expand the LKS+ population. Though unlike LM, *Mtb* is able to persist in the BM and has sustained effects on the LKS+ population.

3.2 Immune Protection: BCG access to BM is critical for protective immunity

As *Mtb* access into the BM is critical in understanding chronic infection, we investigate whether BCG is able to access the BM by subcutaneous injection and have an expansion effect on the LKS+ population. Mice were subcutaneously vaccinated with BCG (10^6 CFU) and one colony appeared from one mouse out of five on day 1 and day 7 post immunization in the BM (**Figure 5A**). Thus it is appears that BCG cannot gain access to the BM after subcutaneous vaccination. As expected, there was no difference in frequency of HSCs LKS+ population at any timepoints after vaccination **(Figure 5B)**.

To investigate the consequences of BCG access to BM and immune response, next we intravenously vaccinated mice with the same dose of BCG (10^6 CFU). We determined that at day one post BCG-vaccination there was 10^3 CFU in the BM that peaked at day 30 and gradually decreased after 210 days post BCG-vaccination (**Figure 5A**). The expansion of the LKS+ population also correlates with the number of BCG in the BM (**Figure 5B**). Collectively, these data indicate that following subcutaneous vaccination, BCG highly unlikely access the BM and modulate HSCs.

3.2.1 Protective Effect Dependent to Bone Marrow

During an *Mtb* pulmonary infection, macrophages are recruited to the site of infection to contain and prevent the dissemination of mycobacteria. Macrophages recognize and phagocytize the bacteria and eliminate *Mtb* through various mechanisms [125]. Therefore, the antimicrobial function of macrophages is essential in immunity to *Mtb*. Thus in order to understand the potential contributions of BCG in the BM, we initially generated Bone Marrow Derived Macrophages (BMDM) at different timepoints after subcutaneous or intravenous BCG-vaccinated mice and then infect them with H37Rv (MOI=1) *in vitro*. Remarkably, BMDM form intravenous BCG-vaccinate mice had significant lower bacterial load compared to subcutaneous and control (PBS) group on day 5 post-H37Rv infection (**Figure 6A**). The protection of BMDM from intravenous BCG vaccinated mice was sustainable even after two or five months post vaccination (**Figure 6B-C**).

To support that the macrophage protective effect stemmed from the LKS+ compartment due to the presence of BCG within the BM we next generated chimeric

mice. One group of CD45.1+ mice receiving BCG intravenous vaccination and the other administered PBS. After two-weeks vaccination, the entire BM (4 x 10^6 BM cells) were then transferred to irradiated CD45.2+ mice retaining two separate groups, one receiving BM from BCG-vaccinated CD45.1+ and the other group receiving BM form PBS-Control CD45.1+ mice. After 12 weeks engraftment, mice were aerosolized with H37Rv (<50 CFU). Mice were then sacrificed at 4, 8, and 20 weeks after infection and both immunological as well as protection were determined (**Figure 7A**).

Two weeks post-BCG vaccination of the CD45.1+ mice, the frequency of LKS+ population was significantly increased compared to PBS-control group (**Figure 7B**). Despite the possibility of BCG being transferred along with the BM cells to the recipient mice, no BCG was detected in the BM or Spleen 7 or 12 weeks post engraftment **(Figure 7C and 7D)**. Importantly, mice were effectively engrafted (**Figure 7E and 7F**).

One month after H37Rv aerosol challenge, we assessed the LKS+ population and observed that the absolute number were not significantly different between groups **(Figure 8A)**. The T lymphocyte frequency (**Figure 8B**) and absolute numbers within the lungs were the same as well (**Figure 8C**). Along with the BM and lung CD45.1+ F4/80+ CD11b+ frequency (**Figure 8D and 8F**) and absolute numbers were equal (**Figure 8E and 8G**). However, the lung bacterial burden was significantly lower in the chimeric mice receiving the BCG-vaccinated BM compared to PBS-control group (**Figure 9A**). Two months after *Mtb* infection, the lung bacterial burden was still significantly lower in the chimeric mice receiving the BCG-vaccinated BM (**Figure 9A**), and even with the BM (**Figure 9B**), or spleen (**Figure 9C**). Five months after *Mtb* infection, the lungs bacterial

burden still remained significantly low in the chimeric mice receiving the BCG-vaccinated BM (**Figure 9A**).

The LKS+ frequency were the same relative size (**Figure 10A**) and along with the absolute number (**Figure 10B**) and absolute number of CD45.1+ F4/80+ CD11b+ within the BM and lungs (**Figure 10B and 10C**). These results collectively suggest that the access of BCG to BM significantly enhances HSC activity to generate immune progenitors that are wired to provide effective protection against *Mtb*. Interestingly, this protective effect was sustainable even after eight months of BCG vaccination.

Chapter IV: Discussion

Humans are the only host for *Mycobacterium tuberculosis*. Coexisting with *Homo sapiens* since our migration out of Africa, *Mtb* continues to coevolve with modern humans, establishing a clear indication of a long-term intimate interaction between *Mtb* and humans [126]. Consequently, *Mtb* is an extremely successful human pathogen. *Mtb* is responsible for claiming over a billion lives in the last two hundred years, more than small pox, HIV, or any other infectious disease in history [127]. However, TB became under better control following the discovery of antibiotics and BCG vaccine (attenuated form of *Mycobacterium bovis*). However, despite theses scientific advantages, one third of population is infected with *Mtb* and approximately two million people annually die of TB [128]. Drug resistance is a major concern in considering the future trajectory of TB worldwide, and is even more heterogeneous in its distribution. Of particular concern in recent years has been the rise of drug-resistant TB in China (in which a quarter of all TB cases are resistant to either isoniazid or rifampin) and India (which has witnessed the emergence of "totally drug resistant" strains) [129, 130]. Moreover, after more than half century of BCG vaccination, we still do not know how exactly BCG provides protection in only children, but not adult, and to what extend this protection is mediated via T cells. An emerging hypothesis is that that BCG protection is mainly mediated through innate immune pathways [121, 122]. Thus a better understanding of innate immunity to *Mtb* may provide a novel approach in developing vaccine to effectively prevent this devastating disease.

Previous studies [81, 82] have demonstrated that one of the major sites for extrapulmonary *Mtb* infection is the BM. In the current study, we have also shown the

kinetic of *Mtb* translocation from the lungs to BM and furthermore demonstrated that the presence and growth of bacteria directly have an effect on the progenitors of the immune cells, HSCs LKS+ population. Translocation of *Mtb* to the BM might be an evasive mechanism to avoid detection and effectively control or modulate HSCs for their own survival. It is speculated that the bacteria infect other cell lineages other than immune cells harbored in the BM, such as the possibility of *Mtb* infecting stem cells [81]. It is not known, whether *Mtb* preferentially or rather remain indifferent towards infecting quiescent cells than actively dividing cells. However the prospect of stem cells being a safe haven for *Mtb* may be an opportune self-preserving mechanism due to stem cells' ability to self renew and stay dormant for lengthy periods of time. It is not clear whether mycobacterium can infect the progenitors of immune cells, HSCs, but we were able to demonstrate that the LKS+ population increases during a BM infection. Then comparing a BM infection by another intracellular pathogen, *Listeria*, we noted that in sharp contrast, H37Rv BM infection was persistent and has long-term effects on the LKS+ population. The ramifications of constant stimulation resulting in an expansion of the LKS+ population may ultimately result in exhaustion of the stem cells leading to total collapse of the immune system [131]. Considering infection of BM by *Mtb* is an evasion mechanism, we next investigate the potential consequences of BCG vaccine on BM HSCs.

Since the route of BCG vaccination in humans is intradermal, we thus subcutaneously vaccinated mice with BCG. Surprisingly, BCG was not able to gain access to the BM via subcutaneous administration and having no effect on the LKS+ population. On the contrary, when the mice were given the same amount of BCG by

intravenous injection, detection of BCG was obvious at day one post vaccination and the LKS+ frequency increased after two weeks and returned to base line over a seven month period. Considering only the BMDMs from BCG-iv mice were able to provide protection against an in vitro infection of H37Rv, the access of BCG to BM appears to be an important mechanism for protection. Most importantly, we were also able to translate this *in vitro* observation to *in vivo* by generating chimeric mice. The aim of generating chimeric mice was to have a fully developed immune system derived only from the HSCs LKS+ population that either had access to BCG or not, prior to H37Rv challenge. We were forced to perform these experiments using whole BM transplant, as the engraftment of active sorted LKS+ population has not been successful [94]. It was also important that BCG was not detectable in the recipient mice after engraftment took place. A potential reason of this outcome may be due to the simple procedure of centrifuging of the whole BM cells from BCG-vaccinated mice that separate cells from bacteria before transferring to the irradiated mice. Therefore, it was highly unlikely that live BCG was transferred to the irradiated mice that would likely jeopardize the survival of immunocompromised irradiated mice. Following the 12-week successful engraftment, mice were challenged with *Mtb* and it was striking to observe that at different timepoints post-*Mtb* challenge, only the BCG chimeric mice were significantly protected to infection. Interestingly, this was independent of the number of LKS+, monocyte-macrophage, and T lymphocyte population, which is an indication of differences in quality of immune cells rather than quantity in providing protection. BCG has been shown to change the epigenetics of immune effector cells (monocyte/macrophage) [31]. However, in our study, we showed that access of BCG to the BM might alter the HSCs LKS+ population to produce

progenitors highly effective in significantly reducing the bacterial burden load. Therefore, when the BCG chimeric mice were infected with H37Rv, we foresee that the monocyte-macrophage innate immune response was likely more efficient in protecting the mice than the control mice. However, we still do not know how BCG reprograms HSCs for generating "educated macrophages", which prevent *Mtb* growth. At same time, we envision that virulent *Mtb* may also reprogram HSCs for generating "permissive macrophages", which promote bacterial growth. We are currently investigating the transcritptomic and epigenomic differences between BCG-infected, H37Rv-infected, and non-infected macrophages and LKS+ cells.

Furthermore, to build upon this experiment and support our speculation we should do two things: 1) repeat the chimeric experiment having the same two groups intravenous BCG vaccination and PBS injection but after BCG vaccination wait for a longer period of time before transferring it to irradiated mice. This potentially might provide enough time for LKS population to return to their inactive statuses, which are, then will be suitable for successful engraftment for generating chimeric mice. This experiment will provide direct evidence that LKS+ population are responsible for providing protection to TB; and 2) To ultimately support BCG intravenous injection provides better protection than a subcutaneous injection, we need to vaccinate C57BL/6 wild-type mice either intravenous or subcutaneous and then challenge them with H37Rv. We expect that intravenous BCG-vaccinated mice will be more protective than subcutaneous BCG-vaccinated mice to pulmonary *Mtb* infection. These experiments will collectively support further our central hypothesis

It was necessary to investigate the historical literature and determine how the method of BCG vaccination came about to intradermal vaccination. For many years, BCG was orally administered to infants in France however in Scandinavian countries subcutaneous injections were reported to be more efficacious. The dichotomy of route of vaccination continued till after World War II. As Europe's infrastructure was obliterated and disease became rampant, the cases of tuberculosis also increased. It was by serendipity that the Scandinavian Red Cross started a BCG vaccination campaign across Europe and the intradermal injection was their preferred method. From that point forward, administrating BCG via intradermal was adopted as the accepted method to induce immunity against tuberculosis. However, our study clearly indicates that BCG has no access to BM following subcutaneous vaccination, which appears to be critical for generating protective immunity. Intravenous vaccination of BCG appears to be a pivotal contribution towards protection within the murine model, but it acknowledges the fact we still do not understand how protection in children via intradermal vaccination or with oral (no longer a practiced method) vaccination occurs, let alone why intradermal vaccination protection is limited to children and does not transpire protection within adults. The variables and differences between adult and children involving immunity are not clearly understood and must be further investigated in respect to this lack of protection between these two cohorts. A possible explanation the high variability of BCG protection among adults (0 to 80%) and protection within children, may be due to improper administration of BCG by intradermal injection and instead unintentionally injecting the vaccine deeper than the dermal level of the skin improving the chances of the bacteria to gain better access to

blood circulation and reach the BM that providing better protection. However, these are speculations.

Alternatively, a better understanding of BCG inducing HSCs reprograming may reveal a unique protective pathway that can potentially be exploited using PRRs such as TLRs and NODs. It has been shown that both these PPRs are important in activating and mobilizing HSCs during infection [101, 103, 104]. NOD2 signaling is pivotal in both innate and adaptive immunity to *Mtb* and the mycobacterial form of MDP (N-glycolyl-MDP) is potent in inducing NOD2 modification in monocytes providing protection to heterologous secondary infection [132]. Given these published observations and our preliminary results of BM progenitors exposure to BCG providing enhanced protection against virulent *Mtb in vitro* and *in vivo*, we will further explore whether BCG induces protective innate immunity through epigenetic reprogramming of stem cells (progenitors of Macrophages) via pattern recognition receptors.

Chapter V: Conclusion

Ever since German scientist Robert Koch first discovered *Mycobacterium tuberculosis (Mtb)* to be the infectious agent of tuberculosis in 1882, it has defied efforts by scientists to prevent or cure the disease. The presence of *Mtb* DNA in ancient Egyptian mummies (dated 1550-1080 BC) suggests a long-standing evolutionary relationship between this bacterium and humans. Despite the world-wide application of BCG vaccination and other anti–*Mtb* interventions, *Mtb* remains one of the deadliest human pathogens killing annually two million people. Eight to ten million new cases of active tuberculosis occur each year due in large part to asymptomatic people chronically infected with *Mtb*. The ability of *Mtb* to persist in individuals with apparently normal immune systems implies that *Mtb* has developed strategies to avoid, evade, and even subvert our host defense system.

Considering *Mtb* also desires to be a "*residence"* of the BM, which is home to stem cells that produce all immune cells, *Mtb* potentially hijack the *control centre* of the immune response. Although we know a great deal about the immune response to *Mtb* in the lung, our understanding of BM immunity to *Mtb* is extremely limited. The tropism of *Mtb* for the BM may also explain why it is an incredibly successful pathogen, as the vast majority of our efforts have been dedicated to controlling its growth in the lung while the root of the disease may be seeded in the BM. Our data suggest that access of BCG (an avirulent mycobacteria and major component of the current anti-TB vaccine) to the BM is critical in augmenting protective immunity to *Mtb* in mice. These data indicate that the BM may be a *privileged site* for generating protective (e.g. BCG vaccine) or detrimental (virulent *Mtb*) immunity to TB. In this study we have showed a novel approach to

understand a poorly-defined area of immunology that not only expands our knowledge regarding the pathogenesis of TB, but also provides significant insights into designing novel vaccine strategies against TB.

Figures and Figure Legends

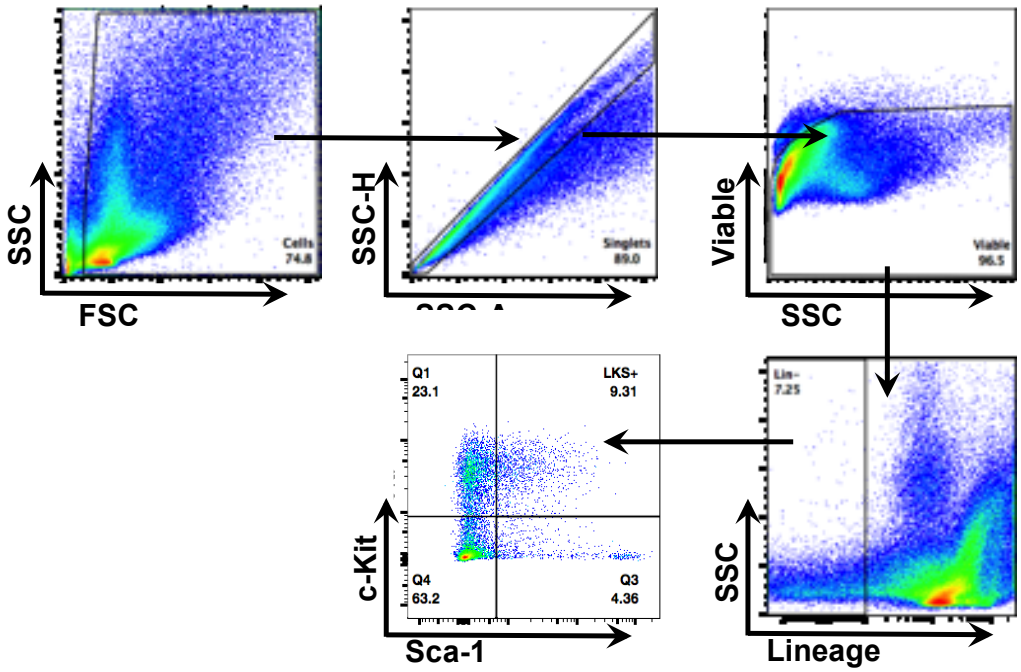

Figure 1. Gating Strategy to identify the Bone Marrow LKS+ Population. BM cells from femurs and tibia of C57BL/6 mice. Initial gates sequentially selected for size and granularity, singlet and viable BM cells. Following the gating of lineage negative cells, the LKS+ population was identified based on expression of c-Kit+ and Sca-1+ markers.

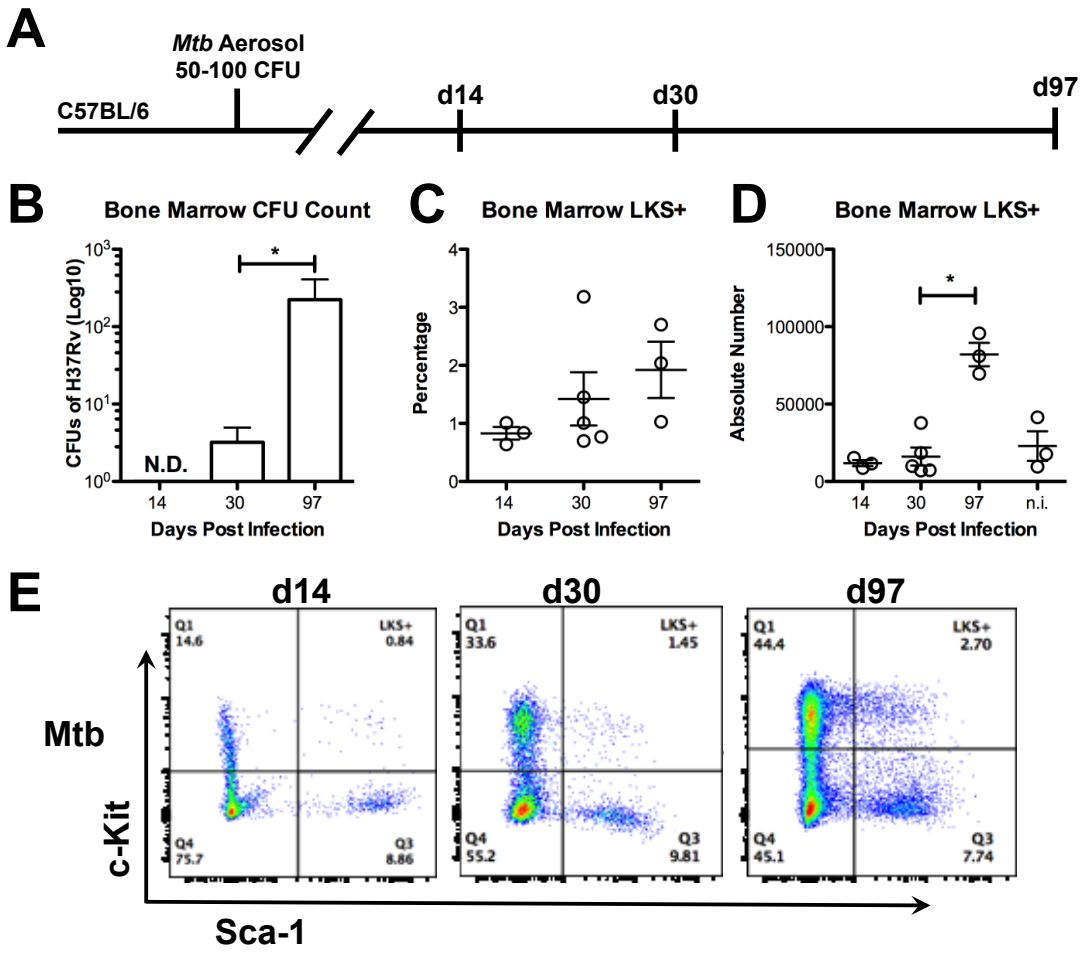

Figure 2. The Kinetics of Bone Marrow LKS+ Population after *Mycobacteria tuberculosis* Aerosol Infection. (A) Aerosol infection with 50-100 CFUs of virulent *Mtb* (H37Rv). Hematopoietic Stem Cell LKS+ population and H37Rv burden load were assessed on day 14, 30 and 97. **(B)** H37Rv CFUs in BM 97 days post infection. **(C)** Kinetics of LKS+ frequency. **(D)** Kinetics of LKS+ absolute number. **(E)** Hematopoietic Stem Cell LKS+ population from BM were identified by Flow Cytometry. At least three mice were individually analyzed and the data are given as mean value ± SE. *, P<0.05 (2way Anova).

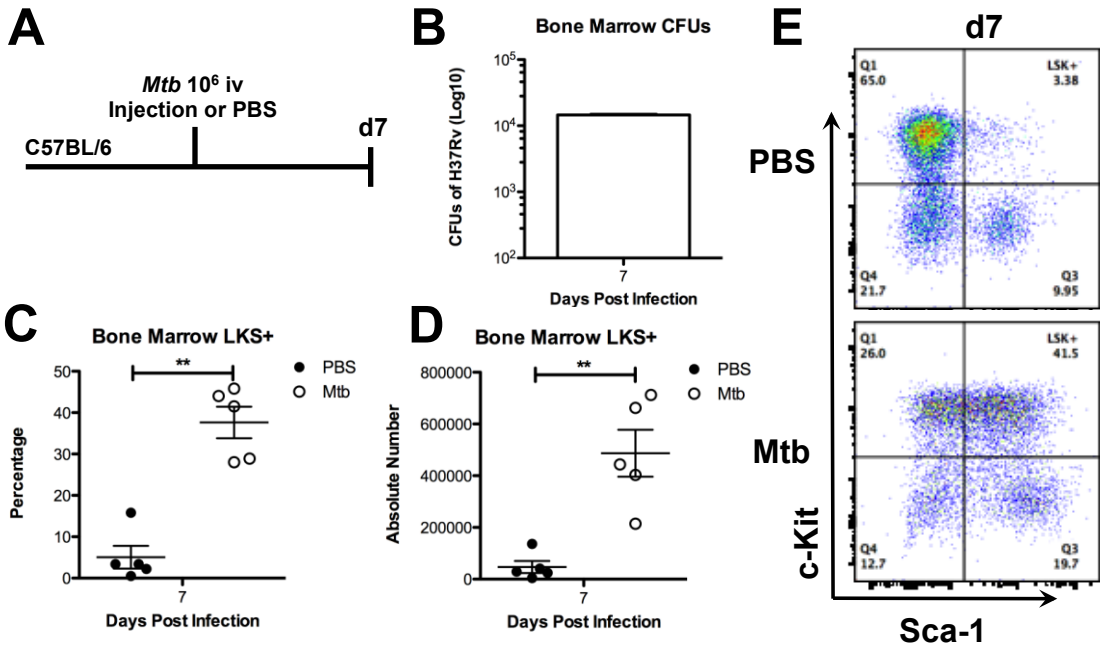

Figure 3. Enhanced LKS+ Population Associates with Increased Growth of *Mtb* in the Bone Marrow. (A) Mice were intravenously infected with 10^6 *Mtb* (H37Rv) or injected with PBS. Hematopoietic Stem Cell LKS+ population and burden load in BM were assessed on day 7. **(B)** H37Rv CFUs in BM 7 days post infection. **(C)** Comparative LKS+ frequency at 7 days. **(D)** LKS+ absolute number at 7 days. **(E)** Hematopoietic Stem Cell LKS+ population from B57BL/6 were identified by Flow Cytometry. Five mice were individually analyzed and the data are given as mean value ± SE. *, P<0.05; **, P<0.01; (t-test)

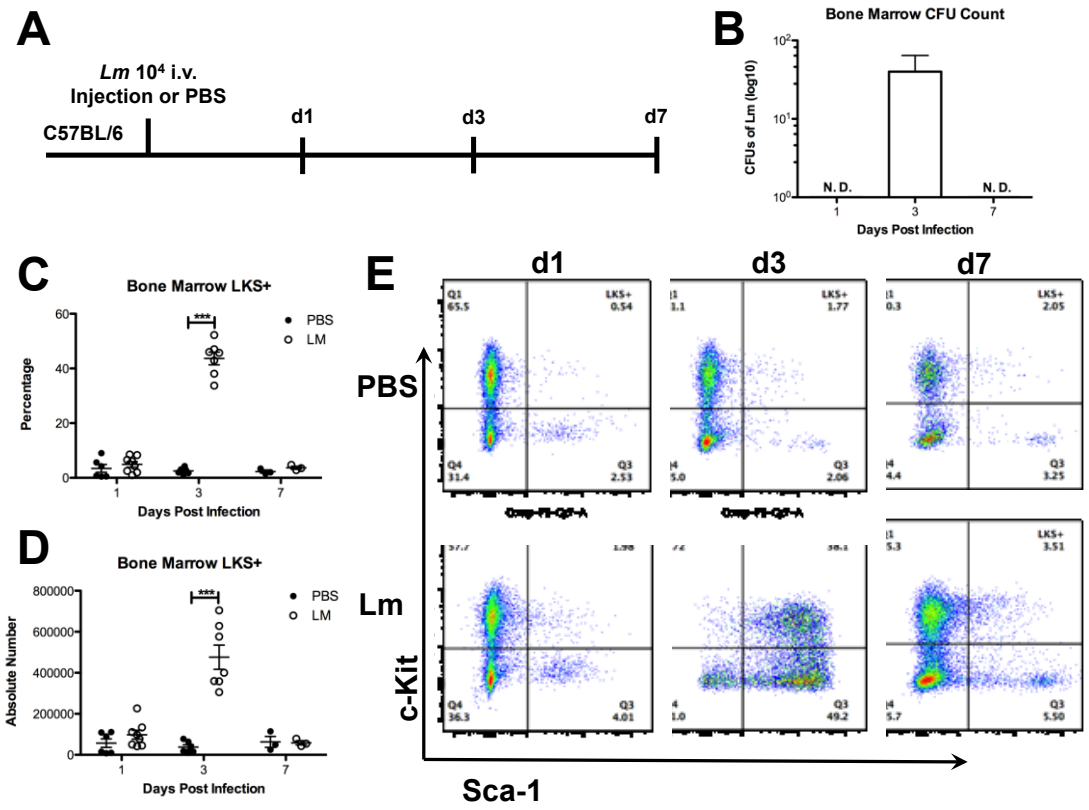

Figure 4. The Kinetic of Bone Marrow LKS+ Population following *Listeria monocytogenes* Infection (A) Mice were intravenously infected with 10^4 *Listeria monocytogenes* (LM) or injected with PBS. Hematopoietic Stem Cell LKS+ population and LM burden load in BM were assessed on day 1, 3 and 7. **(B)** LM CFUs in BM post infection. **(C)** Kinetics of LKS+ frequency. **(D)** Kinetics of LKS+ absolute number. **(D)** Hematopoietic Stem Cell LKS+ population from BM were identified by Flow Cytometry. At least three mice were individually analyzed and the data are given as mean value ± SE. Data grouped from two experiments. *, P<0.05; **, P<0.01; ***, P<0.001 (2way Anova).

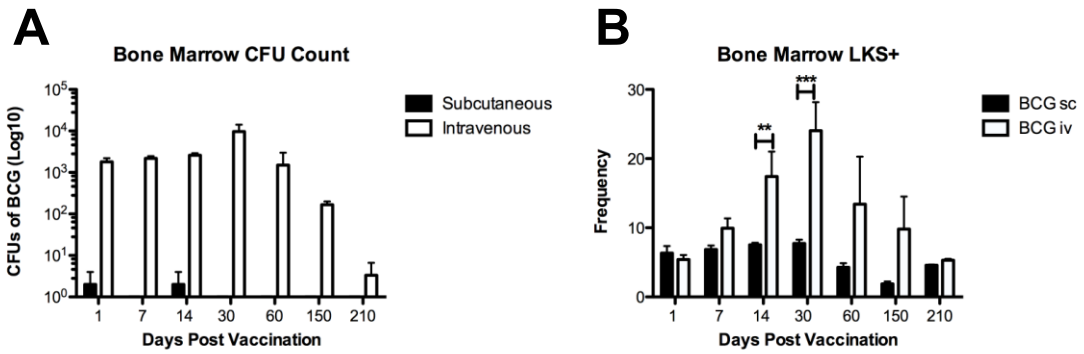

Figure 5. The Kinetic of Bone Marrow LKS+ Population following Intravenous versus Subcutaneous BCG Vaccination. C57BL/6 mice vaccinated with 10^6 BCG either via intravenous or subcutaneous injection. Assessed the CFU burden load and LKS+ frequency. **(A)** Detection of BCG after intravenous vaccination compared to subcutaneous route. **(B)** Kinetics of LKS+ frequency. At least three mice were individually analyzed and the data are given as mean value ± SE. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (2way Anova).

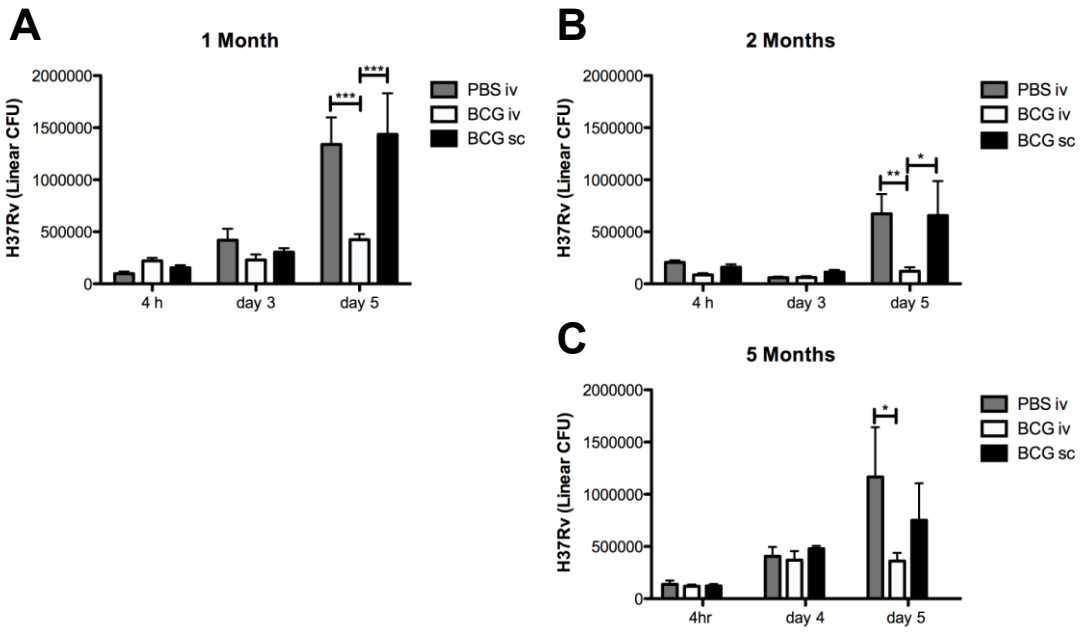

Figure 6. Enhanced Protective Capacity of Bone Marrow Derived Macrophages Vaccinated with BCG Intravenous versus Subcutaneous. Bone Marrow Derived Macrophages (BMDMs) from C57BL/6 mice vaccinated with 10^6 BCG either via intravenous or subcutaneous injection or PBS infected with H37Rv (MOI=1) for 4 hours and assessed the CFU burden load 4 hours, 3 and 5 days post infection. **(A)** BMDMs from mice 1-month post vaccination. **(B)** BMDMs from mice 2-months post vaccination. **(C)** BMDMs from mice 5-months post vaccination. Three sample replicates were individually analyzed and the data are given as mean value ± SE. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (2way Anova).

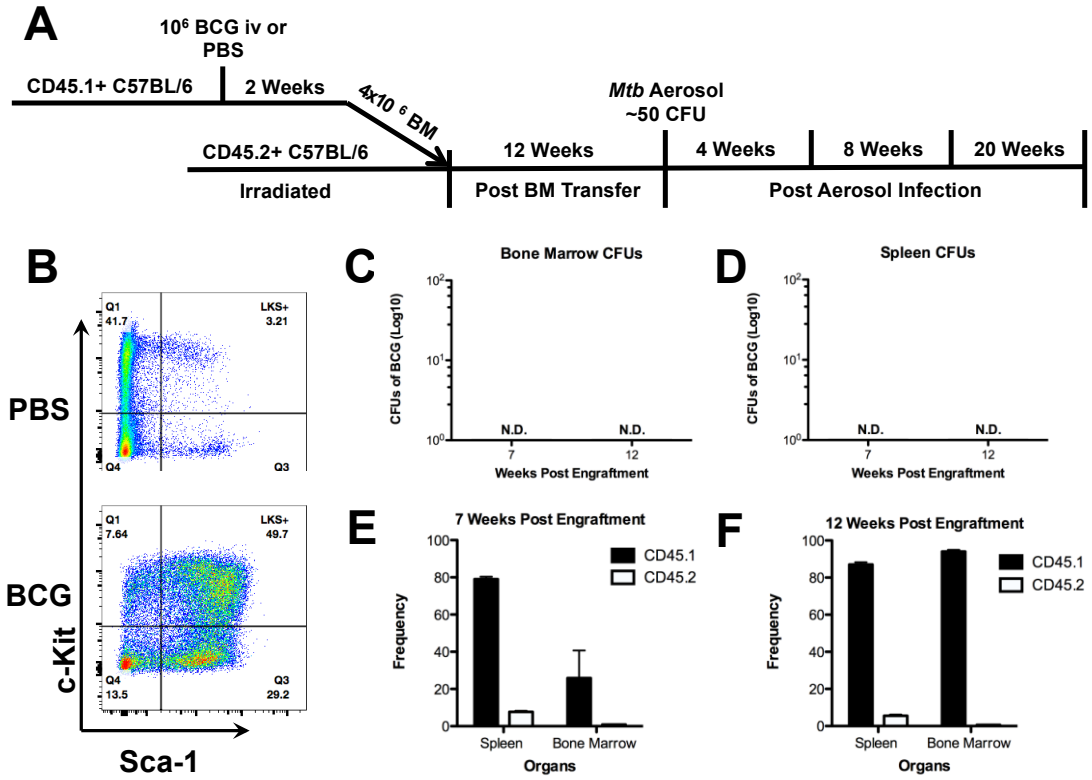

Figure 7. Generation of Chimeric Mice using Bone Marrow from BCG Intravenous Vaccinated Mice. (A) Chimeric mice model: CD45.1+ C57BL/6 were either vaccinated with 10^6 BCG or injected with PBS. After two weeks, 4 x 10^6 BM cells were transferred to irradiated CD45.2+ C57BL/6 mice. 12 weeks were allotted for proper engraftment. The mice were then challenged with H37Rv (~50 CFU) and CFUs, LKS+ and Immune effector cells were assessed 4, 8, and 20 weeks after aerosol infection. **(B)** Frequency of BM LKS+ population, from either PBS injected or BCG vaccinated mice, transferred into irradiated CD45.2+ mice **(C)** BCG CFUs detected in BM of CD45.2+ mice 7 and 12 weeks post whole BM transfer. **(D)** BCG CFUs detected in Spleen of CD45.2+ mice 7 and 12 weeks post whole BM transfer. **(E)** Donor versus Recipient 7 weeks post engraftment **(F)** Donor versus Recipient 12 weeks post engraftment.

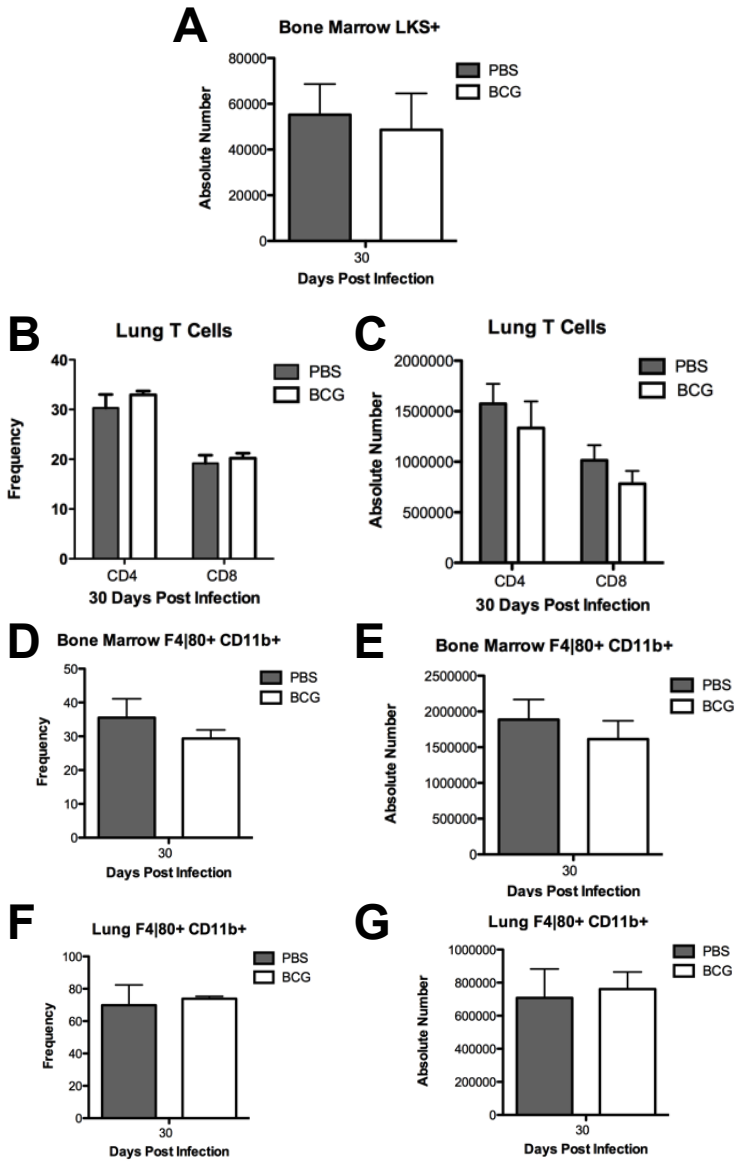

Figure 8. The Number of LKS+ and Immune Effector Cells are Equal in Chimeric Mice after Four Weeks H37Rv Infection. Absolute number of BM CD45.1+ LKS+ cells **(A),** frequency **(B)** and absolute number **(C)** of CD45.1+ T cells in the lungs. Frequency **(D)** and absolute number **(E)** of CD45.1+ F4/80+ CD11b+ cells in the BM. Frequency **(F)** and absolute number **(G)** of CD45.1+ F4/80+ CD11b+ cells in lungs. Five mice were individually analyzed and the data are given as mean value ± SE. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (2way Anova).

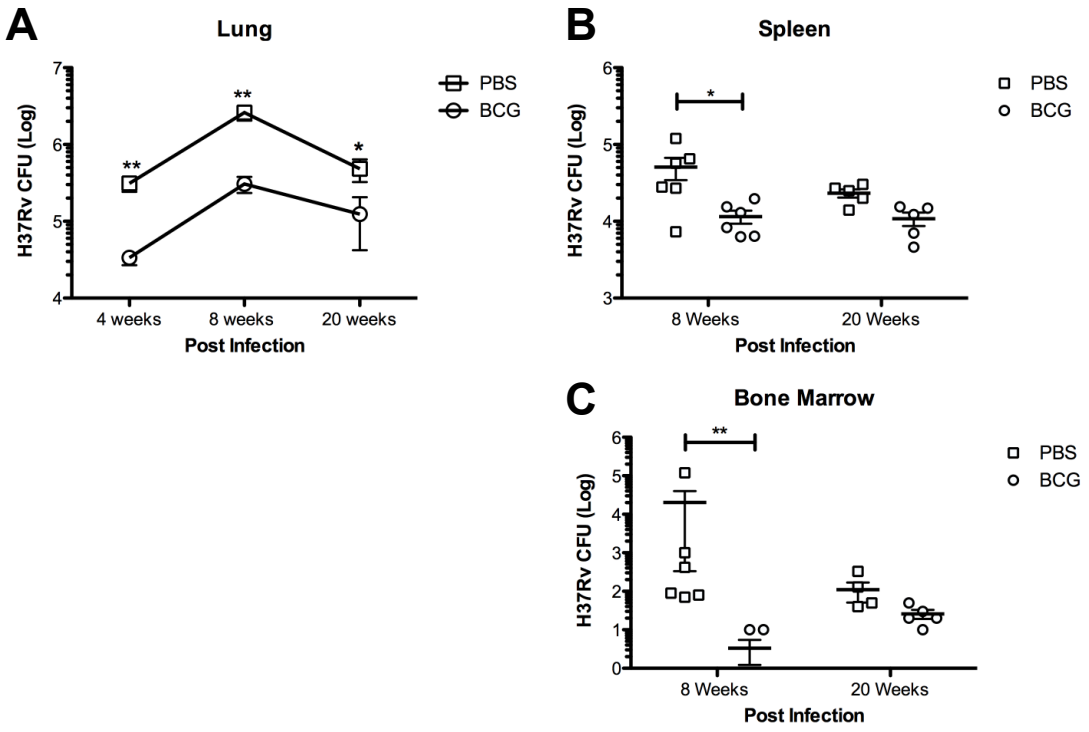

Figure 9. BCG Vaccinated Chimeric Mice were Significantly Protected Against *Mtb* Infection. (A) The pulmonary bacterial burden load. **(B)** The spleen bacterial burden load. **(C)** The Bone Marrow bacterial burden load. At least five mice were individually analyzed and the data are given as mean value ± SE. *, P<0.05; **, P<0.01; ***, P<0.001 (2way Anova).

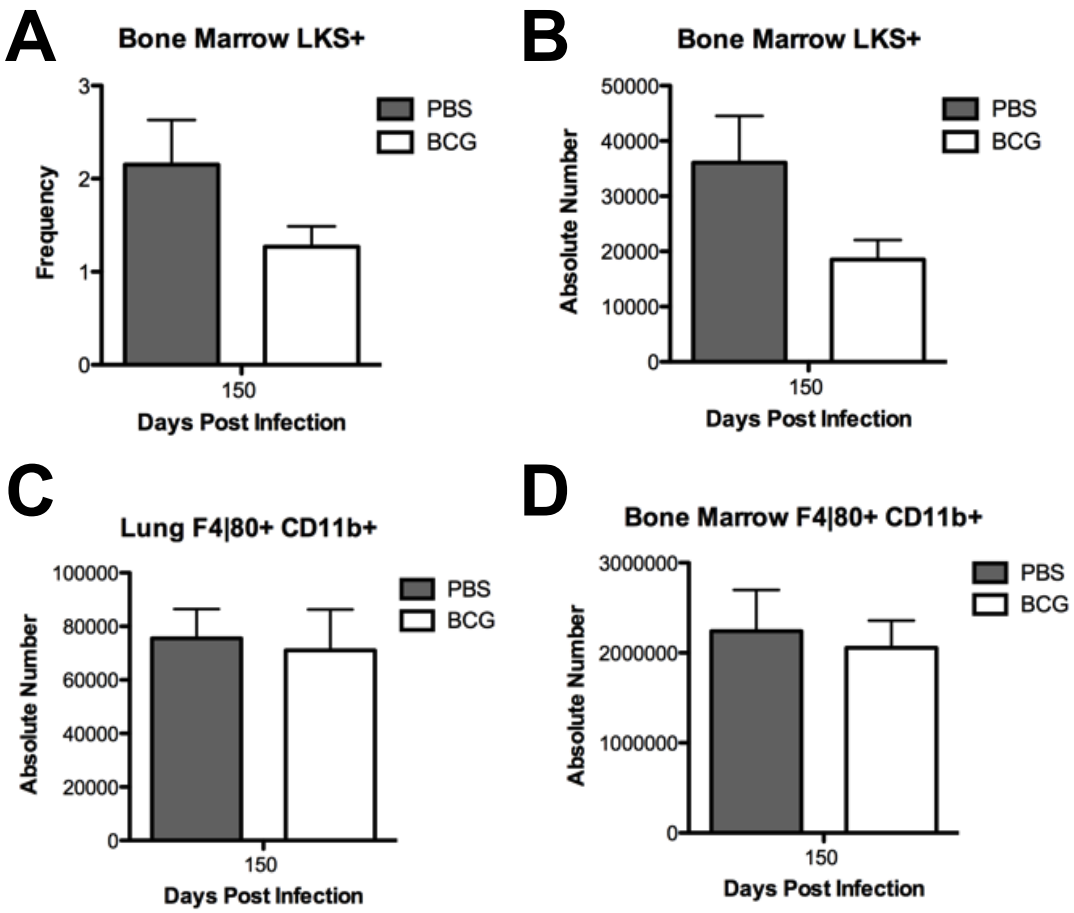

Figure 10. The number of LKS+ and Immune Effector cells are Equal between BCG vaccinated or PBS generated Chimeric Mice after 20 Weeks H37Rv infection. (A) Frequency of Bone Marrow CD45.1+ LKS+ cells (**B**) Absolute number of BM CD45.1+ LKS+ cells **(C)** Absolute number of CD45.1+ T cells in the Lungs. **(D)** Absolute Number of CD45.1+ F4/80+ CD11b+ cells in BM. At least five mice were individually analyzed and the data are given as mean value ± SE. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (2way Anova).

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