BCG-INDUCED TRAINED INNATE IMMUNITY IN ALVEOLAR MACROPHAGES AND THEIR ROLE IN EARLY PROTECTION AGAINST PULMONARY TUBERCULOSIS

By MARYAM VASEGHI-SHANJANI, B.Sc.

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TITLE: BCG-Induced Trained Innate Immunity in Alveolar Macrophages and Their Role in Early Protection Against Tuberculosis

AUTHOR: Maryam Vaseghi-Shanjani, B.Sc. (McMaster University)

SUPERVISOR: Professor Zhou Xing, M.D., Ph.D.

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Lay Abstract

Pulmonary tuberculosis (TB) is a disease of the lung and is now one of the leading causes of human mortality worldwide. For more than eight decades, parenterally administered Bacillus Calmette–Guérin (BCG) vaccine has been globally used as the only approved vaccine against TB. Recently, it has also been observed that BCG vaccination provides protection against other diseases unrelated to TB and reduces childhood mortality in many developing countries where it is routinely administered to children shortly after birth. The mechanisms underlying the off-target protective effects of BCG vaccine remains largely under-investigated. In this project, we investigated how the BCG vaccination enhances the immune system responses against TB and other unrelated infectious diseases. A better understanding of how the BCG vaccination modulates our immune system will provide us with the knowledge that will be useful in the development of more effective vaccination strategies against infectious diseases.

Abstract

Pulmonary tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M.tb*) is the leading cause of infectious disease-related death worldwide. The critical role of adaptive immunity in anti-TB host defense has been firmly established and thus, current efforts in developing novel vaccination strategies against TB are primarily focused on generating protective adaptive immunity at the infection site, the lungs. Innate immunity has not been a target for vaccination strategies against TB due to the belief that innate immune cells cannot exhibit memory-like characteristics which are known to be central to the long-lasting immunity created by vaccines. Also, the importance of innate immunity in anti-TB host defence has been historically overlooked. However, over 25% of individuals that are heavily exposed to *M.tb* clear infection without any detectable conventional T cell immune responses, suggesting a crucial role for innate immune cells in bacterial clearance. Interestingly, the early protection in these individuals is associated with their Bacillus Calmette-Guerin (BCG) vaccination status. Epidemiological studies have shown that BCG is capable of providing protection against numerous infectious diseases unrelated to TB in an innate-immune dependent manner. Such observations suggest that the innate immune system exhibits memory-like characteristics, capable of remembering the exposure to the vaccine and thereby responding in an augmented manner to future systemic infections. Nonetheless, it still remains unknown whether parenteral BCG immunization modulates the innate immune cells in the lung and airways, and if so, what role the trained innate immune

cells play in early protection against pulmonary TB. Using a subcutaneous BCG immunization and pulmonary TB challenge murine model, we show that early protection against *M.tb* is independent of adaptive responses in the BCG immunized host. Our data suggest that enhanced early phase protection is mediated by the BCG-trained memory alveolar macrophages that we have shown to be functionally, phenotypically, metabolically, and transcriptionally augmented following immunization. These novel findings suggest a significant anti-TB immune role for the innate immune memory established in the lung and airways following parenteral BCG immunization and have important implications for the development of novel vaccination strategies against pulmonary TB.

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List of Abbreviations

ACK – Ammonium-Chloride-Potassium RBC lysis buffer
Ad – adenoviral
AdHu5 – human adenovirus serotype 5
Ag – antigen
Ag85A – antigen 85A
AIDS – acquired immunodeficiency syndrome
AM – alveolar macrophage
ANOVA – analysis of variance
APC – antigen presenting cell
BAL – bronchioalveolar lavage
BCG – Bacille Calmette-Guérin Vaccine
CCR – CC type chemokine receptor
CD – Cluster of Differentiation protein
CFU – colony forming unit
DC – dendritic cell
dLN – draining lymph node
ECAR – Extracellular Acidification Rate
FBS – fetal bovine serum
FasL/Fas - FasL is a type-II transmembrane protein from TNF family that binds with its receptor FasL to induce apoptosis
HIV – human immunodeficiency virus
i.n. – intranasal
i.p. – intraperitoneal
i.t. – intratracheal
IFN – interferon
Ig – immunoglobulin
IL – interleukin
IM – interstitial macrophage
iNOS – inducible nitric oxide synthase
IP-10 – interferon gamma-induced protein 10
IRF – interferon regulatory factor
LAM – lipoarabinomannan
M.tb – Mycobacterium tuberculosis
MCP-1 – monocyte chemotactic protein 1
MDM – Monocyte derived macrophage
MDR – multi-drug resistant
MHC-II – major Histocompatibility Complex II
MIG – Monokine induced by gamma interferon
MIP – macrophage inflammatory protein
MLN – mediastinal lymph node
MOI – multiplicity of infection (# of bacteria to # of cells)

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
NK – natural killer cell
NO – Nitric Oxide
NOD – nucleotide-binding oligomerization domain
PBS – phosphate-buffered saline
PRR – Pattern recognition receptor
RNA – Ribonucleic acid
RPMI media – (complete) Roswell Park Memorial Institute medium
SCID – severe combined immunodeficiency
SD – standard deviation
SEM – standard error of mean
Siglec – sialic acid-binding immunoglobulin-like lectin
TB – tuberculosis
Th1 – T helper 1
TLRs – toll-like receptors
TNF – tumour Necrosis Factor
Treg – regulatory T cell

CHAPTER 1: Introduction

[1.0] Research overview

Pulmonary Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M.tb*), is the leading cause of death due to infectious disease worldwide [1]. The critical role of adaptive immunity in anti-TB host defense has been firmly established [2]. Nonetheless, epidemiological data suggest that 20-25% of individuals who are chronically exposed to *M.tb* clear the infection without mounting conventional T cell responses, as these individuals remain Tuberculin Skin Test (TST) negative [3–5]. It is hypothesized that the innate immune system in these individuals is capable of clearing the bacilli before an infection is established. Interestingly, only study has shown that the protection in these individuals (who are exposed but remain uninfected and TST negative) is positively associated with their Bacillus Calmette–Guérin (BCG) vaccination status [4]. This suggests that BCG most likely alters lung and airway innate immune cells that go on to provide enhanced bacterial control during the early stages of *M.tb* infection.

BCG, which has been globally used as the only approved vaccine against TB, is also repeatedly documented to confer non-specific protection against unrelated pathogens [6–9]. The non-specific protective efficacy of BCG is attributed to its ability to induce memory-like characteristics in circulating monocytes [10] and bone marrow myeloid progenitors [11]. Additionally, epidemiological studies report the protective effects of BCG immunization against heterologous respiratory infections [8]. This suggests a plausible role for systemically administered BCG in modulating lung innate immune system.

Nonetheless, *it remains poorly understood whether parenteral BCG immunization induces memory-like characteristics in the lung innate immune cells, and if so, what is the contribution of these trained memory innate cells to anti-TB immunity*. It is speculated that the early clearance of *M.tb* in 25% of the exposed individuals is at least partially mediated by BCG-trained lung and airway innate immune cells which immediately eliminate the bacteria and exclude the need for adaptive immune responses.

[2.0] Tuberculosis

[2.1] Epidemiology and global health concerns

Pulmonary TB is the leading cause of death due to infectious disease worldwide, with an alarming rate of 1.8 million deaths and 10.4 million new cases of active disease in 2018 alone [1]. Moreover, it has been estimated that a quarter of the world's population is latently infected and 5-10% of these individuals will develop active TB sometime in their lifetime if not treated [12]. TB is especially a major cause of mortality and morbidity in countries located in South Africa and Southeast Asia [13]. While the incidence of TB in Canada is much lower than that of the South African and Southeast Asian countries, TB remains a major health concern in the Canada's Indigenous communities where infection rates are up to 300 times the rate of infection in non-indigenous Canadian-born population [14].

There are additional factors that contribute to the worsening of the TB epidemic, such as co-infections with human immunodeficiency virus (HIV) [15]. Many of the TB endemic regions coincide with those endemic to HIV/AIDS [15].

The ability of HIV/AIDS to dramatically suppress cellular immune responses has made co-infections with *M.tb* particularly deadly [15]. The increasing emergence of extensively drug-resistant and multi drug-resistant TB further contribute to the global TB problem [13]. Even in the case of drug-susceptible forms of TB, antibiotic treatment regimen requires an initial two-month therapy of rifampicin, isoniazid, ethambutol, and pyraminizide followed by at least a four-month continuation phase of the two former drugs [13]. The duration, cost and side-effects associated with antibiotic therapy often results in poor patience adherence, thus, disease relapse and emergence of antibiotic resistant TB [13]. In terms of prophylactic strategies, although vaccination with bacillus Calmette–Guérin (BCG) vaccine does offer some protection against pulmonary TB in younger age groups, its efficacy is highly variable between individuals [16]. Thus, there is an urgent need for the development of new vaccination strategies that can reliably provide protection against adult pulmonary TB, which is also the most prevalent form of TB in the world [17]. To do so, we must first develop a complete understanding of how vaccination with BCG modulates the immune system and how anti-TB immunity can be improved using new vaccination strategies that not only target the adaptive immune compartment, but also the innate immune compartment.

[2.2] BCG immunization

BCG, an attenuated form of *Mycobacterium bovis*, remains the only clinically-approved TB vaccine used to date. Developed more than 90 years ago, this vaccine has been globally administered intradermally shortly after birth in

countries where TB is prevalent [16]. There are over 4 billion people who are vaccinated with BCG worldwide [18]. Both Canada and the United States are among some developed countries where BCG is not a part of the national immunization program, although BCG is still offered to newborns in Canadian Aboriginal communities as well as adults travelling to TB endemic regions [19]. BCG has proven highly efficacious against severe, disseminated childhood forms of TB, nonetheless its protection against adult pulmonary TB ranges between 0-80% [16]. The variable efficacy of parenteral BCG vaccination is shown to be due to a multitude of factors including the waning of the vaccine-specific immune responses with age (10-15 years), varying doses and strains of the vaccine used worldwide, the age of vaccination, and previous exposure to environmental mycobacteria [16]. Despite its variable efficacy, BCG is still globally utilized and is deeply threaded into the WHO immunization program, partly because it does provide adequate protection against the non-pulmonary form of TB [16]. Thus, the field is primarily focused on understanding the immune mechanisms underlying BCG's failure to protect against pulmonary TB, and in turn, developing novel vaccination strategies that would boost the pre-existing BCG immune responses and enhance protection against pulmonary TB.

[2.3] Current TB vaccine initiatives

Replacing the BCG vaccine completely would be impractical partly due to the ethical issues of withholding a vaccine that is effective in preventing childhood disseminated TB [20]. Also, it has been repeatedly reported that BCG has non-

specific protective effects against various viral, fungal and bacterial infections, and in turn, BCG vaccination in children is linked to reduced mortality and morbidity during infancy [7,8,21,22]. Thus, regardless of being designed for prophylactic or therapeutic use, the current efforts in developing novel vaccination strategies are primarily focused on creating heterologous booster vaccines that could be used in conjunction to the BCG vaccine [12].

The current global TB vaccine pipeline consists of diverse vaccine platforms including whole cell vaccines, viral vectored vaccines, and subunit vaccines [23]. The goal of each of these novel vaccination strategies differs. For example, some are meant to be used prophylactically to protect against initial pulmonary TB infection in adults, while others are meant to be used therapeutically to shorten the antibiotic treatment duration or to prevent disease reoccurrence/relapse [24]. Some examples of vaccines currently in the global pipeline include: MVAAg85A, H56+IC31, recombinant BCG over expressing numerous *M.tb* antigens (VPM1002), *Mycobacterium vaccae*, AdHu5Ag85A, and Ad35 expressing several *M.tb* antigens [25].

Among the vaccine candidates in the global TB vaccine pipeline, the M72/AS01$_E$ (GlaxoSmithKline) vaccine has shown promising safety and efficacy when delivered to latently infected individuals [26]. This vaccine contains the M72 recombinant fusion protein that is derived from two immunogenic *M.tb* antigens, combined with the AS01 adjuvant [26]. This vaccine provided 54.0% protection in

latently infected adults against active pulmonary TB, without evident safety concerns [26]. The mechanism of protection is shown to be the induction of vaccine-specific Th1 CD4 T cell responses [27]. Besides T cell responses, innate immune responses generated by this vaccine should also be evaluated. It is possible that the gap in protection (partial protection) is due to the vaccine only targeting adaptive immune responses, with no efforts in modulating innate immune responses that are also critical in anti-TB immunity, particularly at early phases following infection.

[3.0] Outcomes following *M.tb* exposure

[3.1] Spectrum of TB infection & disease

There are three clinical outcomes following *M.tb* exposure in BCG vaccinated humans (Figure 1). Upon exposure, 5-10% of individuals develop active TB, whereas 60-70% become latently infected [13]. Interestingly, 20-25% of the exposed individuals successfully eliminate the bacteria without developing adaptive immune responses against *M.tb* [28], suggesting a protective role by innate immunity. Currently, it remains to be investigated how a quarter of the BCG vaccinated individuals that are exposed to *M.tb* are able to clear the infection in the absence of conventional T cell immune responses and what role the innate immune responses play in mediating such protection.

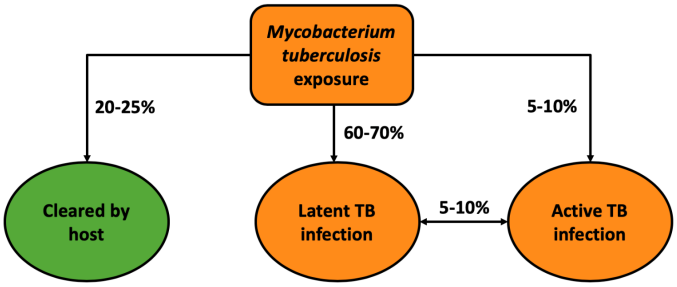


Figure 1. Possible outcomes following *M.tb* exposure in BCG vaccinated humans.

[3.2] Early clearance following *M.tb* exposure

Many epidemiological studies show that humans are capable of clearing *M.tb* infection shortly following exposure [3–5]. This phenomenon is termed as early clearance and is defined as elimination of bacilli prior to the generation of conventional T cell responses, as measured using interferon-gamma release assay (IGRA) and Tuberculin Skin Test (TST) [3–5]. Acquiring *M.tb* infection is a function of many factors such as duration of exposure, proximity to the source of infection, and the grade of sputum positivity, so the phenomenon of early clearance can be especially appreciated when these dimensions are at maximum. Many epidemiological studies have reported that a proportion of heavily exposed individuals do not develop active or latent *TB*, as these individuals remain negative on IGRA and TST (Figure 2). An example is the study from 1924 which reported that many of the heavily and persistently exposed nursing students remained TST negative [29]. More famously is the case of the U.S. naval ship outbreak where 66 sailors shared a cabin with 7 individuals with active TB and 13 out of 66 exposed individuals remained TST negative after 6 months of consistent exposure [30]. In such cases, it is highly likely that *M.tb* was inhaled, contained, and cleared before

the development of an adaptive immune response. Thus, early clearance can be considered a form of protective immunity against TB and studies investigating the underlying mechanisms of this phenomenon have the potential of advancing the development of vaccines and therapeutics against pulmonary TB.

Location of Study	Duration of Observation	%TST or IGRA-negative	Reference
Uganda	2 years	26.8	Bark CM, Manceur AM, Malone LL, *et al.*: **Identification of Host Proteins Predictive of Early Stage *Mycobacterium tuberculosis* Infection.** *EBioMedicine.* 2017; **21**: 150–7.
Uganda	2 years	9.9	Seshadri C, Sedaghat N, Campo M, *et al.*: **Transcriptional networks are associated with resistance to *Mycobacterium tuberculosis* infection.** *PLoS One.* 2017; **12**(4): e0175844.
Tanzania & Uganda	Up to 8 years	48	Fletcher HA, Snowden MA, Landry B, *et al.*: **T-cell activation is an immune correlate of risk in BCG vaccinated infants** *Nat Commun.* 2016; **7**: 11290.
Venezuela	3 years	18.6	Li HT, Zhang TT, Zhou YQ, *et al.*: **SLC11A1 (formerly *NRAMP1*) gene polymorphisms and tuberculosis susceptibility: a meta-analysis.** *Int J Tuberc Lung Dis.* 2006; **10**(1): 3–12.
Uganda	2 years	14.5	Hall NB, Igo RP Jr, Malone LL, *et al.*: **Polymorphisms in *TICAM2* and *IL1B* are associated with TB.** *Genes Immun.* 2015; **16**(2): 127–33.
The Gambia	6 months	60	Buchwald UK, Adetifa IM, Bottomley C, *et al.*: **Broad adaptive immune responses to *M. tuberculosis* antigens precede TST conversion in tuberculosis exposed household contacts in a TB-endemic setting.** *PLoS One.* 2014; **9**(12): e116268.
Uganda	2 years	11.7	Ma N, Zalwango S, Malone LL, *et al.*: **Clinical and epidemiological characteristics of individuals resistant to *M. tuberculosis* infection in a longitudinal TB household contact study in Kampala, Uganda.** *BMC Infect Dis.* 2014; **14**: 352.
Uganda	1-2 years	3.4	Mahan CS, Zalwango S, Thiel BA, *et al.*: **Innate and adaptive immune responses during acute *M. tuberculosis* infection in adult household contacts in Kampala, Uganda.** *Am J Trop Med Hyg.* 2012; **86**(4): 690–7.
Pakistan	2 years	25	Hussain R, Talat N, Shahid F, *et al.*: **Biomarker changes associated with Tuberculin Skin Test (TST) conversion: a two-year longitudinal follow-up study in exposed household contacts.** *PLoS One.* 2009; **4**(10): e7444.
Uganda	2 years	10.5	Stein CM, Zalwango S, Malone LL, *et al.*: **Genome scan of *M. tuberculosis* infection and disease in Ugandans.** *PLoS One.* 2008; **3**(12): e4094.
Brazil	8-12 weeks	30.2	Jones-López EC, Acuña-Villaorduña C, Fregona G, *et al.*: **Incident *Mycobacterium tuberculosis* infection in household contacts of infectious tuberculosis patients in Brazil.** *BMC Infect Dis.* 2017; **17**(1): 576.
USA	8-10 weeks	52	Horne DJ, Graustein AD, Shah JA, *et al.*: **Human ULK1 Variation and Susceptibility to *Mycobacterium tuberculosis* Infection.** *J Infect Dis.* 2016; **214**(8): 1260–7.
Brazil	1 year	26.5	Araujo LS, da Silva NBM, da Silva RJ, *et al.*: **Profile of interferon-gamma response to latency-associated and novel *in vivo* expressed antigens in a cohort of subjects recently exposed to *Mycobacterium tuberculosis*.** *Tuberculosis (Edinb).* 2015; **95**(6): 751–7.
Europe	2 years	58.2	Zellweger JP, Sotgiu G, Block M, *et al.*: **Risk Assessment of Tuberculosis in Contacts by IFN-γ Release Assays. A Tuberculosis Network European Trials Group Study.** *Am J Respir Crit Care Med.* 2015; **191**(10): 1176–84.

Figure 2. Studies reporting TST and IGRA-negative *M.tb* exposed individuals.

[3.3] Early clearance and its association with BCG immunization

There was a recent (unpublished) epidemiological study conducted on *M.tb* household contacts in Indonesia that defined and characterized the early clearers in an effort to understand the underlying mechanisms of innate-immune mediated early clearance in these individuals [4]. This study assessed the host factors associated with early clearance while accounting for the *M.tb* exposure levels. Individuals with active TB were recruited and their level of infection was assessed by measuring sputum bacillary burden and chest x-ray. These individuals were identified as index cases if they shared a household with others and if they were not treated for TB. The household contacts who lived with these infected individuals must have spent more than 5 hours a week with the index case and have no previous TB to be included in the study [4].

The household contacts were assessed using a questionnaire that obtained information about their demographic characteristics, smoking status, diabetes, HIV infection, TB symptoms, sleep proximity and hours spent with TB index case. The *M.tb* infection status (using IGRA) and BCG immunization status (vaccination scar) of the household contacts was also assessed. The IGRA assay was repeated again after 14 weeks [4].

At baseline, 58% of contacts were IGRA positive, 36% were IGRA negative, and 2% of contacts had TB [4]. These values are similar to the values reported by other epidemiological studies that have assessed outcomes following *M.tb* exposure (Figure 1). From the initially IGRA negative contacts cases, 71% of

the remained persistently IGRA negative over the duration of the study (as measured at 14 weeks post baseline). Thus, 25% of the total case contacts were initially IGRA negative and remained IGRA negative, and this population was defined as early clearers. The study indicated that BCG immunization in case contacts was associated with lower risk of positive IGRA at baseline (relative risk [RR]=0.90; 95% CI 0.83 - 0.99; p=0.03). Moreover, after accounting for exposure measures and all other variables such as age and sex, the risk of IGRA conversion among the individuals who were negative at baseline was determined to be lower in BCG immunized case contacts (RR=0.55; 95% CI 0.40 - 0.76; P<0.001). However, IGRA conversion risk was higher for smokers and case contacts with diabetes. Thus, this study claims that BCG immunization is much more protective than what has been reported by other studies in the field [4].

Characteristic	Baseline IGRA Positive n=780	Baseline IGRA Negative	
		IGRA Converter n=116	IGRA Persistently Negative n=317
Contacts			
Hours with the case median (IQR)	5·0 (2·0, 10·0)	5·0 (3·0, 8·0)	3·0 (1·0, 7·0)
Sleep proximity to index case			
Same room	235 (30·1)	22 (19·0)	64 (20·2)
Different room	545 (69·9)	94 (81·0)	253 (79·8)
Age (years) median (IQR)	30·6 (16·7, 46·7)	22·8 (14·6, 35·7)	22·2 (12·1, 39·8)
Female	452 (57·9)	59 (50·9)	170 (53·6)
BCG vaccination	607 (77·8)	86 (74·1)	276 (87·1)
Smoking history			
Never smoked	507 (65·0)	75 (64·7)	226 (71·3)
Quit >6 months ago	35 (4·5)	4 (3·4)	15 (4·7)
Current smoker	238 (30·5)	37 (31·9)	76 (24·0)
HIV infection, self-reported	2 (0·3)	0 (0·0)	0 (0·0)
Diabetes†			
No diabetes	704 (90·3)	103 (88·8)	292 (92·1)
Pre-diabetes	51 (6·5)	7 (6·0)	14 (4·4)
Diabetes	25 (3·2)	6 (5·2)	11 (3·5)

Adapted from Verrall et al, 2018 – unpublished

Figure 3. Characteristics of the household contacts from the Indonesian study.

[4.0] Host defense against TB

[4.1] Pathogenesis of *M.tb* infection

M.tb is a facultative intracellular pathogen that preferentially establishes an infection within the lung [31]. The pathogen is spread person-to-person through infected aerosols generated by coughing or sneezing. The bacteria are deposited within the lung alveoli of the new host [31]. In the newly infected host if the innate immune cells cannot immediately clear the bacilli, there is an eventual formation of granuloma structures within the lung [31]. Initially, the granulomas are composed of primarily of *M.tb*-infected macrophages; however, antigen-specific CD4 and CD8 T cells eventually arrive at the lung 3 weeks following infection and surround the infected macrophages [32]. The secretion of activating cytokines such as IFN-γ by antigen-specific T cells causes a morphological change in the *M.tb*-infected macrophages, making these cells epithelial-like [32]. Also, infected macrophages can become giant multinucleated cells following fusion with each other [32]. As discussed above, in most cases (60-70% of exposed individuals), *M.tb* is sequestered within the granulomas. However, in 5-10% of individuals disruption of these structures occurs (usually when individuals become immune-compromised) and these individuals develop active TB. Disease re-activation occurs when the centre of the granuloma develops a necrotic core as a result of the macrophages being unable to contain the infection and subsequently undergo necrosis [32].

For many years, it has been believed that the generation of adaptive T-helper 1 (Th1) lymphocytic responses are key for protection against *M.tb* [2]. Specifically, IFN-γ producing CD4 T cells were identified to be the primary mediators of anti-TB immunity in animal models [2], and the induction of antigen-specific CD4 T cells by BCG was identified as the mechanism of protection provided by BCG against *M.tb* [33]. However, it is becoming more evident that the capacity of innate immune cells to control bacterial growth at the early stages following infection is very important in disease outcome [34]. Innate immune cells play important roles both in the prevention of *M.tb* infection and also in containing the infection in latently and actively infected individuals [35].

[4.2] Importance of innate immunity against TB

Since the main route of pathogen-entry is through the respiratory tract, the innate immune-mediated protection against TB is comprised of a variety of lung and airway innate immune cell types, both tissue-resident and recruited defenders [35]. Among the major innate immune cells in the lung and airways are the phagocytic macrophages which include alveolar macrophages (AM), interstitial macrophages (IM) and monocyte-derived macrophages (MDM) [35]. AM are the airway resident macrophages, mainly situated on the mucosal surfaces of the airways and thus the primary cells to become infected with *M.tb*. In fact, in steady state, AM constitute 90-95% of the cell population within the alveolar space [36]. AM are of embryonic origin and long-lived with a slow turnover rate [36]. During homeostatic conditions, AM undergo self-renewal, without any contribution from

the circulatory monocytes or other lung macrophages [36]. However, upon infection, AM can be replaced by bone marrow-derived macrophages and/or circulating monocytes [37]. During this process, lung macrophages (MDMs & IMs) serve as an obligatory intermediate between circulating monocytes and AM [38]. Other important lung tissue resident and recruited cells such as innate dendritic cells (DC), Natural Killer (NK) cells and neutrophils are also crucial in early anti-TB immunity but are mostly located within the lung interstitium and are not as acquiescent to *M.tb* infection [31]. Repeatedly it has been shown that the fate of *M.tb* infection is primarily determined by the effectiveness of innate phagocytic cells to inhibit bacterial growth at the early stages of infection [39]. Thus, the functions of AM and other lung macrophages upon *M.tb* infection must be carefully studied as they may play a critical role in early anti-TB immunity, particularly before the arrival of adaptive immunity in the lung [39].

[4.3] Early immune events following *M.tb* infection

Infection of the AM occurs through receptor-mediated phagocytosis [40,41]. There are a variety of receptors on the AM that are involved in phagocytosis of *M.tb* including complement receptors (CR3 and CR4), the mannose binding receptor, and surfactant molecules [40,41]. Upon entry, recognition of *M.tb* is mediated through the engagement of pattern recognition receptors (PRRs) such as Toll Like Receptor (TLR)-2, 4, 9, and NOD2 [40,41]. Following recognition of *M.tb*, the infected AM attempt to destroy the bacilli through activation of multiple antimicrobial mechanisms such as phagolysosome formation and the generation of

reactive oxygen and nitrogen species which damage the integrity of the bacterial cell wall [41]. Moreover, the interaction of the *M.tb* with PRRs on AM initiates a signalling cascade through the innate immune signal transduction adaptor molecule, MyD88, that activates the transcription factor NF-κB [41]. This transcription factor is responsible for the production of inflammatory cytokines such as TNF-α, IL-1β, IL-12 and IL-6 [31]. Thus, through the production of such inflammatory cytokines, AM also signal to various Antigen Presenting Cells (APCs) to migrate to the lung, acquire *M.tb,* and return to the mediastinal lymph nodes to prime naïve antigen-specific T cells [41]. Further bacterial control is then mediated by the migration of antigen-specific T cells to the lung. Both CD4 and CD8 T cells play major roles in protection against *M.tb*. For instance, Th1 CD4 T cells produce pro-inflammatory cytokines such as IFN-γ and TNF-α that further activate antimicrobial pathways of AM and allow the macrophages to produce nitric oxide (NO) [42]. Moreover, cytotoxic CD8 T cells can induce apoptosis in infected macrophages through the release of cytotoxic granules such as perforin and granzyme. Moreover, CD8 T cells can also induce apoptosis in infected macrophages through FasL/Fas and TNF [17].

[4.4] Delayed immune responses following *M.tb* infection

M.tb infection can be cleared by the innate immune system before the initiation of an adaptive immune response. However, *M.tb* has evolved strategies to circumvent and suppress the early innate immune responses required for the elimination of bacilli [31]. As stated previously, AM are the primary host cells for *M.tb* infection and therefore are fundamental targets of suppression [43]. Upon

infection, a major cell wall component of *M.tb*, lipoarabinomannan (LAM) signals through the mannose receptor on the AM, inducing an anti-inflammatory responses [44]. This impairs the secretion of pro-inflammatory cytokines (TNF-α and IL-1β) and chemokines (MCP-1 & IP-10), resulting in delayed recruitment of other innate and adaptive immune cells to the lung. *M.tb* also interacts with the airway epithelial cells to induce the production of MMP-9, further attracting macrophages to the site of infection and facilitating its own propagation [41,45]. When inside the macrophage, *M.tb* utilizes various mechanisms such as blocking of phagolysosome formation and detoxifying of oxygen and nitrogen radicals to down-modulate innate responses, allowing it to generate an environment favoring its survival and replication [46]. Moreover, the virulence factors of *M.tb* actively suppress macrophage activation through the down regulation of the expression of TLRs, MHCII, and co-stimulatory molecules on AM [35]. It is due to this active suppression of innate immunity that the adaptive immune responses are also delayed and *M.tb* is able to establish a foothold in the lung [47] (Figure 4).

Following pulmonary *M.tb* infection, CD103+ DCs counter-regulate CD11b+ DC that are important in Th1 activation by producing IL-10 [48]. This delays the migration of antigen-bearing DCs to the local draining lymph nodes by approximately 8-9 days [48] (Figure 4). Eventually, the antigen-bearing DCs arrive at the draining mediastinal lymph nodes (MLN) and they present antigens to antigen-specific T cells [41,47,48]. However, due to the delay in the initial innate responses and the migration of DCs to MLN, the adaptive immune responses are

also delayed. Approximately 18-21 days following infection, effector T cells finally migrate back to the lungs [31] (Figure 4). The delay in the migrations of DCs to the MLN and the priming of T cells allows *M.tb* to replicate to approximately 5 logs by 20 days following infection and establish a foothold in the lung [48]. Thus, it is important to have effective innate immune responses early following infection in order to control bacterial growth prior to the arrival of adaptive immunity to the lung.

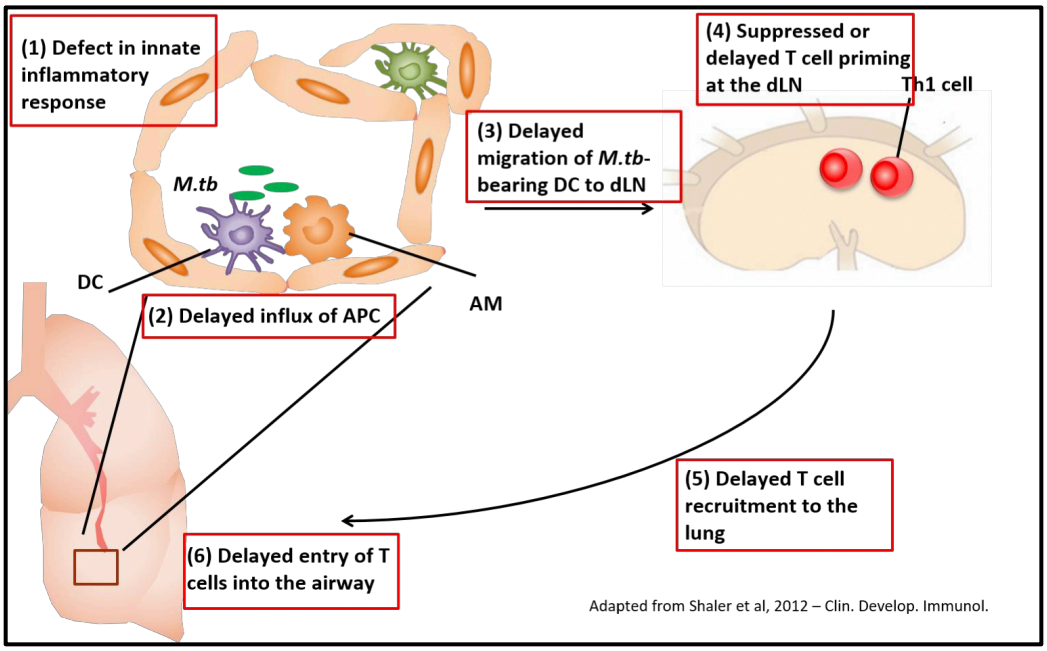


Figure 4. Delays in anti-TB immunity due to immunoevasion by *M.tb*.

[5.0] Immune responses following BCG Immunization

[5.1] Systemic immune responses to BCG

Parenteral BCG immunization triggers systemic and site-specific immune responses [16]. Upon immunization, the attenuated *Mycobacterium bovis* bacilli infect the skin resident macrophages and are also picked up by Langerhans cells that reside in the skin. Langerhans cells that carry the BCG antigens eventually migrate to the local draining lymph nodes to prime naïve T cells [49]. Following priming, Antigen-specific T cells differentiate into IFN-γ producing effector T cells that proliferate and return to the site of vaccination [31]. Upon arrival at the site of vaccine injection, the effector CD4 T cells activate infected skin macrophages through secretion of IFN-γ, allowing macrophages to kill the bacilli [31]. In addition to priming CD4 T cells, parenteral immunization with BCG also generates a small subset of effector CD8 T cells that provide bacterial control through production of cytotoxic granules that induce apoptosis in the BCG-infected skin macrophages [31]. Finally, a pool of memory T cells is generated that reside mainly in the spleen, skin, gut, lymph nodes, and lung interstitium [50].

[5.2] Pulmonary immune responses to BCG

Protective immunity against *M.tb* infection requires the presence of antigen-specific adaptive immune responses in the airways [12]. Even though parenteral BCG immunization in mice induces significant T cell responses in the lung interstitium, it fails to elicit airway luminal T cells until 14 days following *M.tb* challenge [51]. A previous study in mice has shown that when T cells are recruited

to the airways by intranasal delivery of *M.tb* culture-filtrate prior to *M.tb* challenge, protection is enhanced in the early phases of *M.tb* infection [51].

Even though T cell responses following parenteral BCG immunization and their role in anti-TB are thoroughly investigated, the impact of BCG on the innate immune system and its role in early anti-TB immunity remains largely unknown. There is a plethora of epidemiological data suggesting that BCG is able to induce long-lasting changes in circulating and tissue-specific innate immune cells [8,10,11,52].

[6.0] Heterologous protection provided by BCG immunization

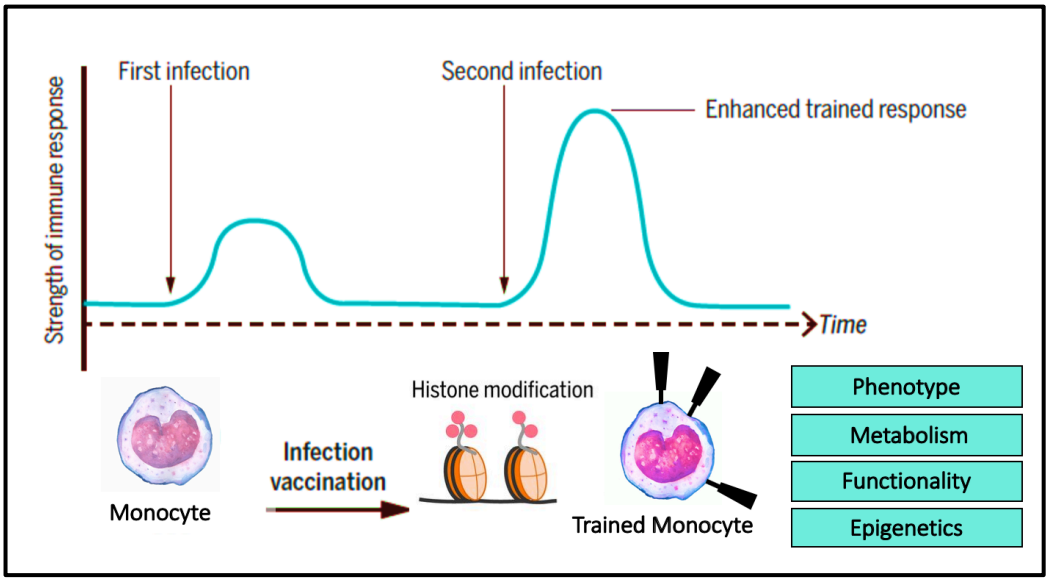
Although BCG is intended to protect against TB, its nonspecific beneficial effects against other infections and conditions have been described for many decades[7–9,22]. Beneficial effects of BCG have been reported in many conditions such as leprosy, asthma, yellow fever, childhood pneumonia and all-cause infant mortality [8]. The non-specific protective effects of BCG have been attributed to the imprinting of monocytes and NK cells following immunization [52]. It has been shown that epigenetic reprogramming of immune-inflammatory genes in the circulating monocytes is responsible for the conservation of memory-like characteristics in these cells following BCG immunization [10]. The imprinted monocytes have an altered functionality and produce higher levels of inflammatory innate immune cytokines such as IL-1ß upon restimulation with heterologous pathogens [53]. In a recent experimental study in humans, it has been shown that these BCG-induced changes correlate with protection against an experimental viral

infection [54]. In these individuals, viremia reduction correlates with increased expression of IL-1ß, indicative of trained innate immunity [54]. Moreover, this study shows that SNPs in IL-1ß gene affect the induction of trained innate immunity by BCG [54]. Another study shows that intradermally administered BCG immunization alters the bone marrow cellular profile in humans, reprogramming the hematopoietic stem cells and enhancing myelopoiesis [11]. This is suggested as a mechanism of maintenance for long-lasting imprinted monocytes in circulation.

[7.0] Innate Immune Memory

Recently, the traditional immunological paradigm that innate immune cells cannot display memory-like characteristics has been challenged. Both experimental and epidemiological studies have discovered that following exposure to certain stimuli, innate immune cells undergo metabolic, epigenetic and transcriptional reprogramming such that upon secondary exposure to the same or heterologous stimuli, these cells respond differently (Figure 5) [53,55–57]. This phenomenon has been termed as *innate immune memory*. Innate immune memory has been observed in many innate cell types including macrophages, monocytes, NK cells, innate lymphoid cells, and even myeloid progenitors [53,55–59]. Moreover, innate immune memory is shown to be compartmentalized within the body, with only innate immune cells residing in specific tissues becoming imprinted following vaccination or exposure to pathogens. A recent study showed that following intranasal administration of a viral-vectored vaccine, airway and lung AM are imprinted while the circulatory monocytes remain unaltered [60]. This shows a

great potential for innate immune cells to become targets of novel vaccination strategies, since vaccines often aim to create protective immunity at specific tissue sites.



Adapted from Netea et al, 2016 – Science

Figure 5. Trained innate immunity describes adaptive-like characteristics for innate immune cells. Trained immune cells are described to have altered phenotype, metabolism, functionality and epigenetic profile.

[7.1] Trained innate immunity and innate immune tolerance

Innate immune memory has two arms: *trained innate immunity* and *innate immune tolerance* [56,57,61,62]. Trained innate immunity (TII) refers to the long-term enhancement of anti-pathogenic functions of the innate immune host defense after vaccination or primary infection (Figure 6) [63]. It has been shown that imprinted innate cells can confer nonspecific protection against secondary infections [7,9,64]. However, innate immune memory can also have detrimental outcomes for secondary infections in the context of innate immune tolerance, where

the initial priming results in the downregulation of immune responses to subsequent infections (Figure 6). Thus, innate immune memory induced by vaccination or primary infection may determine the effectiveness of subsequent innate immune responses. Nonetheless, innate immune memory is shown to be reversible as shown in a recent study where LPS-tolerized monocytes were activated by subsequent stimulation with β-glucan [62].

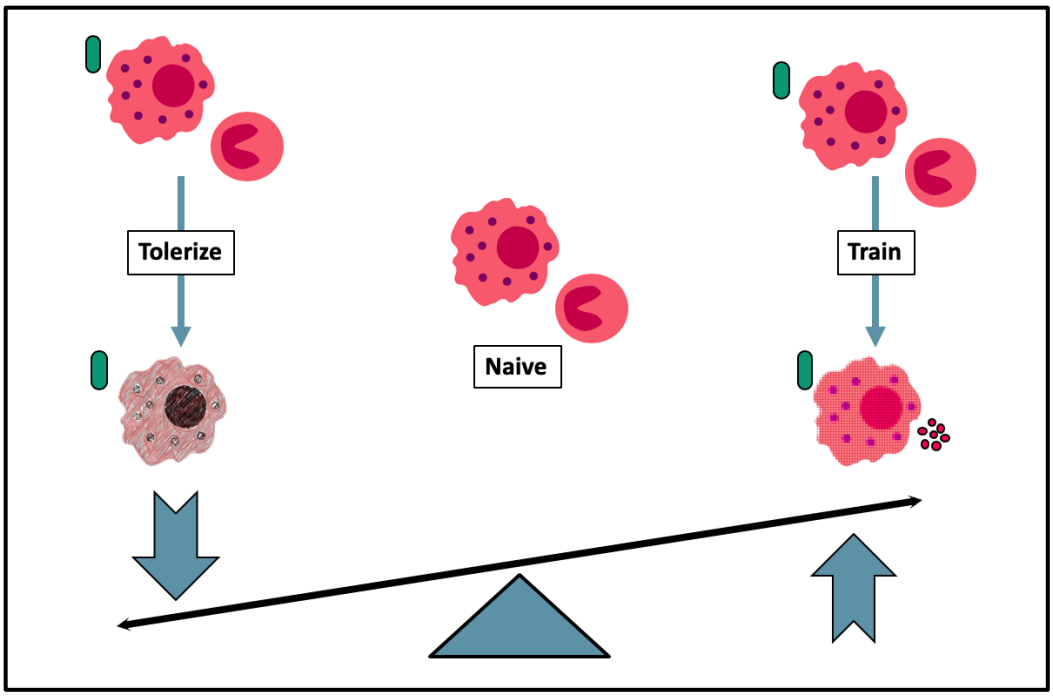
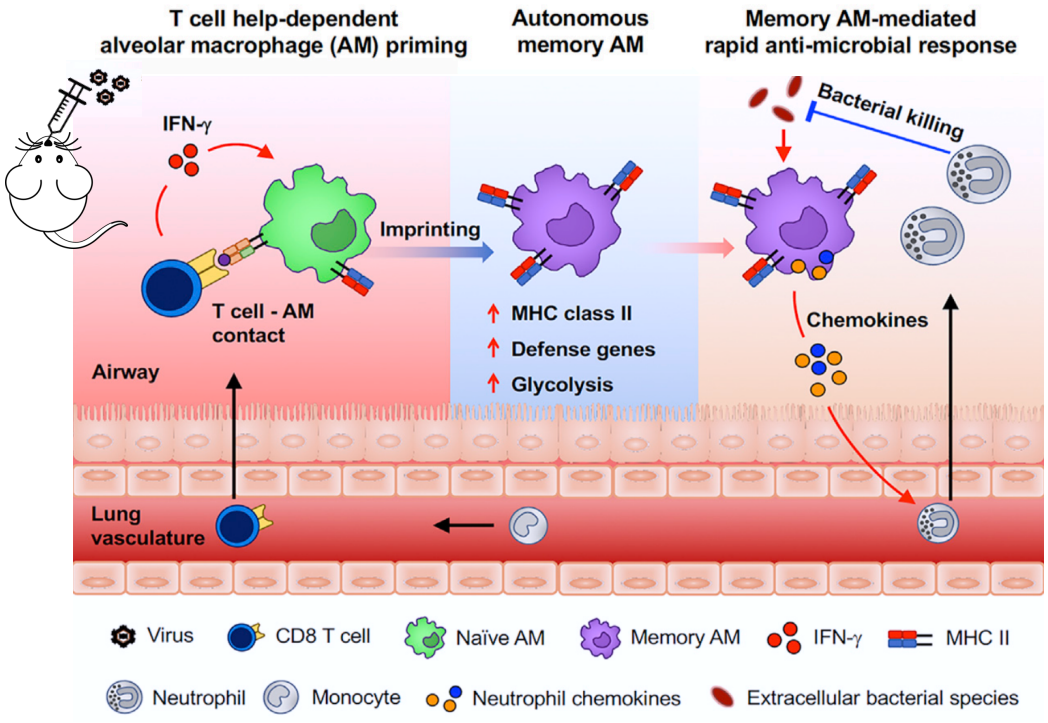


Figure 6. Trained innate immunity and innate immune tolerance are two opposing phenomena related to innate immune memory. Tolerance makes innate immune cells hypo-responsive, while training makes them hyper-responsive.

[7.2] Evidence of memory alveolar macrophages

In a recent study, Yao et al. showed that AM are imprinted following intranasal administration of a viral-vectored TB vaccine. In this study, memory-AM were characterized as expressing high levels of MHCII (an activation marker), shifting towards glycolytic metabolism and producing higher levels of chemokines

upon re-stimulation [62]. Interestingly, the imprinted AM provided heterologous protection against an unrelated bacterial infection in the lung (Figure 7). Nonetheless, it remains to be investigated whether AM can be imprinted following systemic vaccination strategies and subsequently provide protection against local respiratory infections [62].



Adapted from Yao et al, 2018 – Cell

Figure 7. Intranasal immunization with an adenoviral-vectored TB vaccine imprints bona fide resident AM. Imprinted AM enhance protection against an extracellular bacterial infection by producing higher levels of neutrophil chemoattractant molecules and enhancing the recruitment of neutrophils to the lung.

[8.0] BCG-induced TII in the lung and its role in anti-TB immunity

Epidemiological and experimental studies show that systemically administered BCG augments lung and airway innate immune responses [8,9,65–

67]. BCG has been reported to reduce the risk of pneumonia-caused death by 50% and also protect against a range of viral and bacterial respiratory infections [8,68]. Furthermore, BCG is shown to modulate IL-8 mRNA expression and protein production in AM of guinea pigs infected with *M.tb* [69]. As reported, AM isolated from BCG-immunized host produce significantly higher levels of IL-8 in comparison to AM isolated from unimmunized counterparts upon *ex vivo* challenge with *M.tb*. The significant increase in IL-8 production may aid in the recruitment of inflammatory cells to the lung, contributing positively to anti-TB immunity at early phases of infection [69]. Together, these studies suggest that the local lung immune system is impacted following parenteral BCG immunization. Nonetheless, it still remains to be investigated whether BCG is able to imprint AM in a long-lasting manner (Figure 8). If so, the changes in AM have to be fully characterized and the underlying mechanism(s) of imprinting have to be elucidated.

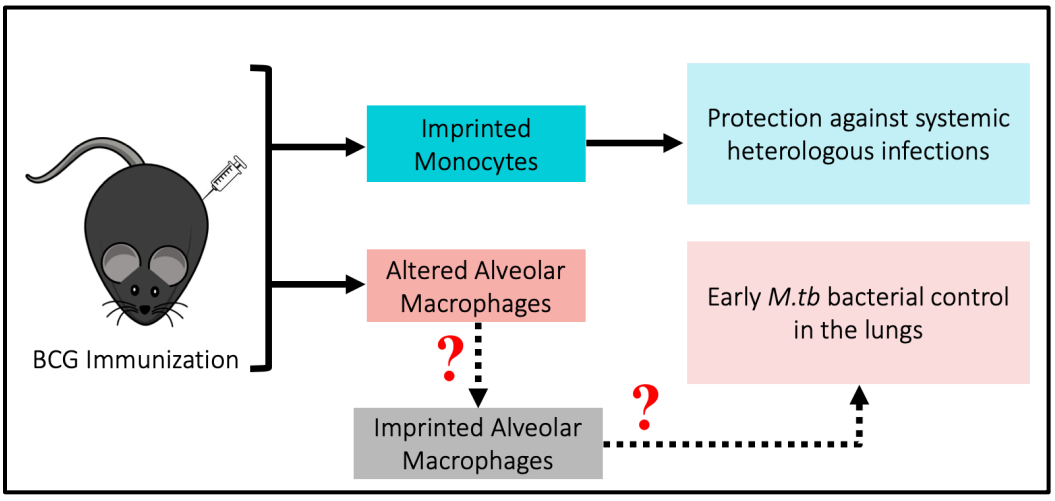


Figure 8. Outstanding questions related to the imprinting of AM post parenteral BCG immunization and their potential contribution to anti-TB immunity.

[9.0] Central question, hypothesis and research aims

Current efforts remain focused on understanding the role of T cells in natural and BCG-mediated immunity against pulmonary TB. However, these studies largely disregard the potential contribution of natural and BCG-modulated innate immune cells in early protection against TB.

The limited number of studies which have addressed BCG's impact on the innate immune cells have mostly focused on its effects on systemic/circulating cell populations. Thus, the impact of parenteral BCG immunization on the lung and airway innate immune responses and the contribution of BCG-modulated innate immune cells in anti-TB immunity remains poorly understood, especially in the early stages following infection. *We hypothesize that parenteral BCG immunization induces memory-like characteristics in alveolar macrophages, allowing them to control M.tb in the early phase of infection.* In this study, using a murine model, we aim to:

Aim 1 – Characterize the effect of parenteral BCG immunization on alveolar macrophages.

Aim 2 – Evaluate the contribution of BCG-trained alveolar macrophages to early protection against pulmonary TB.

Aim 3 – Investigate the mechanism(s) of BCG-induced memory in alveolar macrophages following parenteral immunization.

CHAPTER 2: Experimental Methods

[2.1] Ethic statement. All animal experiments and procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care. This study was approved by the Animal Research Ethics Board of McMaster University under the animal utilization protocol number 17-06-29.

[2.2] Mice. Wild-type female 6-8 weeks old C57BL/6 mice were purchased from Charles River Laboratories (Saint Constant, QC, Canada). Female Chemokine (C-C motif) receptor 2 (CCR2) knockout mice (B6.129S4-*Ccr2*^{tm1Ifc}/J and P25 TCR-Tg transgenic mice containing CD4 T cells expressing Ag85B receptor (C57BL/6-Tg(H2-K^{b}-Tcra,-Tcrb)P25Ktk/J) on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free level B facility at McMaster University or at the Biosafety Level 3 facility at McMaster University Health Sciences Center.

[2.3] Mycobacterial preparation for immunization and infection. *M. Bovis* BCG (Pasteur Strain), virulent *M.tb* (H37RV;ATCC 27,294) and attenuated *M.tb* (H37Ra; ATCC 25,177) were grown in Middlebrook 7H9 broth supplemented with Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Invitrogen Life Technologies, Carlsbad, CA), 0.002% glycerol, 0.05% Tween 80 for < 10-15 days, then aliquoted and stored in -70°C until needed. Prior to each use, bacilli were washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 80 and passed through a 27-gauge needle 10 times to disperse clumps.

[2.4] Mycobacterial challenge and immunization. Mice were initially anesthetized by Isoflurane exposure. Mice were then challenged intratracheally (i.t.) with a dose of $0.5x10^6$ CFU/mouse of *M.tb* $H_{37}Ra$ in 40 µl of PBS or intranasally (i.n.) with a dose of $1x10^4$ CFU/mouse of *M.tb* $H_{37}Rv$ in 25 µl of PBS. For parenteral immunization, mice were immunized subcutaneously (s.c.) with 1-$5x10^4$ CFU/mouse of BCG in 100 µl of PBS. The exact dose of infection and immunization was verified each time by tittering the inoculum using Colony Forming Unit (CFU) assay.

[2.5] Bacterial enumeration following mycobacterial challenge. The level of bacterial burden in the lung was enumerated at various timepoints post infection by plating neat and serial dilutions of lung homogenates in triplicates onto Middlebrook 7H10 agar plates supplemented with Middlebrook OADC enrichment (Invitrogen Life Technologies, Carlsbad, CA), 0.002% glycerol, 0.05% Tween 80, 50 mg/ml of Cycloheximide, and 5 mg/ml of Rifampicin. Plates were sealed in plastic bags and incubated at 37°C for 15-21 days until colonies were visible. Colonies were then enumerated and expressed as $\log_{10}$ CFU per lung.

[2.6] Cell isolation from peripheral blood, bone marrow, airway lumen, and lung interstitium. Mice were initially anesthetized by Isoflurane exposure and euthanized by exsanguination. Cells from peripheral blood, bronchoalveolar lavage, and lung tissue were isolated as previously described [24,47,51,70,71]. Briefly, the lung was lavaged five times to a volume of 1.35 ml of PBS by

cannulating the trachea using polyethylene tubing attached to 23-gauge needle and syringe. Following exhaustive bronchoalveolar lavage [70], lungs were cut into small pieces and digested in RPMI solution containing collagenase type 1 (ThermoFisher Scientific Waltham, MA) for 1 hour at 37°C in an agitating incubator. Single-cell suspension was obtained by crushing the digested lung tissue through a 100 μm basket filter (BD Biosciences, San Jose, CA) and subsequent lysis of RBCs by resuspending the cell pellet in Ammonium-Chloride-Potassium (ACK) lysing buffer for 2 min. Peripheral blood was processed as previously described [60]. Briefly, heparinized blood was mixed with 10 x volume of ACK lysing buffer (Life Technologies, Grand Island, NY) and incubated at room temperature for 5 min prior to the removal of the lysed RBCs by centrifugation. An additional round of ACK treatment was carried out by resuspending the pellet in 2 mL of ACK lysing buffer. Blood leukocytes were then washed in PBS. To obtain hematopoietic stem cells and myeloid cell progenitors, the spine, femur and tibia from mice were crushed using a mortar and pestle in PBS [60]. Bone marrow cells were filtered through a 40 μm basket filter (BD Biosciences, San Jose, CA) and mixed with 2ml ACK lysis buffer and incubated at room temperature for 2 min. Bone marrow cells were then washed in PBS [60]. Isolated cells from all tissues described were then resuspended in either complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 10 mM HEPES, 0.5mM Na pyruvate, 55 μM 2-Mercaptoethanol, 0.1mM NEAA, and with or without 1% penicillin/streptomycin) for *ex vivo* culture or in PBS for flow cytometry staining.

[2.7] Immunostaining for cell phenotype characterization using flow cytometry. Cell immunostaining and flow cytometry were performed as previously described [72]. Briefly, mononuclear cells from bronchoalveolar lavage (BAL), the lung, and peripheral blood were plated in U-bottom 96-well plates at a concentration of 20 million cells/ml in PBS. Following staining with Aqua dead cell staining kit (ThermoFisher Scientific Waltham, MA) at room temperature for 20-30 min, cells were washed and blocked to avoid non-specific staining with anti-CD16/CD32 (clone 2.4G2) in 0.5% BSA-PBS (FACS buffer) for 15 min on ice and then stained with fluorochrome-labeled mAbs for 20-30 min on ice [60]. Fluorochrome-labeled mAbs used for staining myeloid cells including alveolar macrophages, interstitial macrophages, monocyte-derived macrophages, monocytes, dendritic cells and neutrophils were anti-CD45-APC-Cy7 (clone 30-F11), anti-CD11b-PE-Cy7 (clone M1/70), anti-CD11c-APC (clone HL3), anti-MHC II-Alexa Flour 700 (clone M5/114.15.2), anti-CD3-V450 (clone 17A2), anti-CD45R-V450 (clone RA3-6B2), anti-Ly6C-Biotin (clone HK1.4), Streptavidin-Qdot800, anti-CD24-BV650 (clone M1/69), anti-CD64-PE (clone X54-5/7.1), anti-Ly6G-BV605 (clone 1A8), anti-Siglec-F-PE- CF594 (clone E50-2440), anti-CD80-PerCP-Cy5.5 (clone 16-10A1), anti-CD86-V450 (clone GL1), anti-CD284(TLR4)-APC (clone SA15-21), anti-CD11c-BV711 (clone HL3), anti-CD282(TLR2)-BV421 (clone CB225), anti-CD192(CCR2)-BV421 (clone SA203G11), anti-CD45.2-PerCP-Cy5.5 (clone 104). As indicated, some cells were further stained with anti-AnnexinV- APC. Fluorochrome-labeled mAbs used for T cell surface and

intracellular cytokine staining were anti-CD3-V450 (clone 17A2), anti-CD4-PE-Cy7 (clone RM4-5), anti-CD8-APC-Cy7 (clone 53-6.7), anti-IFN-g-APC (clone XMG1.2) and anti-CD44-BV650. For intracellular cytokine staining in T cells, lung cells were cultured in the presence of GolgiPlug (5 mg/ml brefeldin A; BD PharMingen) with or without stimulation for 24 hours with a mixture of crude BCG (50,000 CFU/well) and *M.tb* culture filtrate proteins (2µg/well). Stimulated cells were stained with cell surface antibodies, followed by fixation/permeabilization by using Fixation/Permeabilization Solution Kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions [60]. Cells were then stained with anti-IFN-g-APC mAb in Perm/Wash buffer (BD Biosciences, San Jose, CA) for 30 min on ice. Immediately upon completion of the staining procedure, data on immunostained cells was collected with the LSRII flow cytometer (BD Pharmingen) using FACSDiva software (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

[2.8] *Ex vivo* stimulations for cytokine and chemokine measurements. Isolated alveolar macrophages were resuspended in complete RPMI 1640 medium and plated at $1x10^5$ cells/well in 96-well flat-bottom plates (250 µl/well). Cells were incubated in 37°C 5% CO_2 cell-culture incubator overnight. Following incubation, cells were washed twice with 200 µl of warm PBS twice and fresh complete RPMI was added to the wells (250 µl per well). Control wells (unstimulated) received no further treatment. Stimulated wells either received 5:1 MOI of *M. Bovis* BCG

(prepared as explained above) or 0.4 μg/ml of *M.tb* whole cell lysate. Cells were incubated with the stimuli for an additional 24 hours in 37°C 5% CO_2 cell-culture incubator. Following overnight incubation, culture supernatants were collected and stored in -20°C freezer for chemokine and cytokine measurements. Chemokines and cytokines were measured using MCYTOMAG-70K mouse chemokine and cytokine detection kit (Millipore Sigma, Etobicoke, ON) according to the manufacturer's instructions. Plates were read on a MagPix reader (ThermoFisher Scientific Waltham, MA) using a xPONENT software (ThermoFisher Scientific Waltham, MA).

[2.9] *In vivo* depletion of T cells. To deplete CD4 T and CD8 T cells *in vivo*, mice were injected i.p. with 200 mg of anti-CD4 mAb (clone GK1.5) and 200 mg of anti-CD8 mAb (clone 2.43). To achieve continuous T cell depletion, two days following the initial injection repeated doses of 100 mg of anti-CD4 mAb and 100 mg of anti-CD8 mAb were administered i.p. at a 7-day interval as needed.

[2.10] *Ex vivo M.tb* phagocytosis and killing assays. Isolated alveolar were resuspended in complete RPMI 1640 medium without streptomycin and plated at $2x10^5$ cells/well in 24-well plates (500 ml/well). Cells were incubated in a 37°C 5% CO_2 cell-culture incubator for 4 hours with 5:1 MOI of *M.tb* H37Ra (grown and prepared as described above) and 10% naïve C57BL/6 mouse serum (made in house). After 4 hours, cells were then washed twice with warm PBS to remove extracellular bacteria that were not phagocytosed by the cells. Cells were supplied

with fresh complete RPMI without streptomycin and IFN-γ (0.4 ng ml^{-1}) and incubated for an additional 24 or 48 hours. At the end of 24 or 48 hours, bacterial-stimulated alveolar macrophages were lysed by adding 1 mL per well of autoclaved distilled water. Cell lysates were diluted by 1:10 serial dilutions in PBS and plated on Middlebrook 7H10 agar plates (not containing antibiotics) and cultured for 14-21 days. Bacterial phagocytosis and killing were calculated based on the CFUs in the culture plates. In brief, bacterial CFUs at 4 hours post bacterial stimulation were considered as phagocytosis and bacterial CFUs at 24 and 48 hours post bacterial stimulation were compared with that at 4 hours to show the percentage killing of phagocytosed bacteria. Moreover, the phagocytosis and killing assay was repeated with dsRed-BCG (red fluorescent) obtained from Dr. Zakaria Hmama's lab at University of British Columbia [73]. The cells were cultured in a similar manner as above with 30:1 ratio of fluorescent BCG and at 4 and 24 hours post infection, cells were analyzed for mycobacterial burden using flow cytometry.

[2.11] Metabolic assay of alveolar macrophages. Real-time cell metabolism of alveolar macrophages was determined by using the Seahorse XF Glycolysis Stress Test Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Alveolar macrophages were obtained from the BAL and seeded onto a 24-well microplate (Agilent Technologies, Santa Clara, CA) at a density of $2x10^5$ cells/well [60]. The plate was incubated for 2 h in a 37°C 5% CO_2 cell culture incubator and washed twice with culture media to remove non-adhering cells. Alveolar macrophages were then incubated overnight in complete RPMI 1640

supplemented with 10 mM HEPES, 0.5 mM Na pyruvate, 55 μM 2-Mercaptoethanol, 0.1mM NEAA in a 37°C 5% CO_2 cell culture incubator [60]. On the following day, alveolar macrophages were washed twice and cultured with Seahorse XF base medium supplemented with 2 mM L-glutamine (Agilent Technologies, Santa Clara, CA) for the duration of the assay. Extracellular Acidification Rates (ECAR) was assessed using a Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA). Glycolysis was represented by ECAR after the addition of 10 mM glucose. Glycolytic capacity was represented by maximum ECAR following the addition of 1 μM oligomycin. Glycolytic reserve was represented by the difference between glycolytic capacity and glycolysis. Data were analyzed using Wave Desktop software version 2.6 (Agilent Technologies, Santa Clara, CA) and normalized to protein [60].

[2.12] Transgenic T-cell purification, CFSE labelling, and *ex vivo* antigen presentation assay. CD4 T cells were purified from the mononuclear cells isolated from the spleen and lymph nodes of P25-Tg mice using a Mouse CD4 T cell negative selection kit according to manufacturer's instructions (StemCell Technologies, Vancouver, CA). Purity (>90%) was verified by flow cytometry on the LSRII using FACSDiva Software from BD Biosciences (San Jose, CA, USA). CD4 T cells were labelled with Carboxyfluorescein succinimidyl ester (CFSE) from Invitrogen (Burlington, ON, Canada). Following CFSE labelling, cells were washed twice with 5% FBS/PBS to remove excess CFSE, after which they were resuspended in PBS. Cells were then used in the antigen presentation assay. For

antigen presentation assay, CFSE labelled CD4 T cells were used to assess antigen presentation capabilities alveolar macrophages isolated from naïve or immunized mice. Alveolar macrophages were isolated as described previously and paired with P25-Tg CD4 T cells at a 1:2 ratio of alveolar macrophages to T cells ($1x10^5$ AM to $2x10^5$ T cells) for 96 hours at 37°C [48]. To assess the priming potential of alveolar macrophages, proliferation of T cells was assessed using LSRII flow cytometer (BD Pharmingen) and analyzed with FlowJo software (Tree Star, Ashland, OR).

[2.13] Intravascular immunostaining to distinguish T cell populations in lung vasculature and interstitium. Intravascular immunostaining was carried out as previously described [74]. Briefly, monoclonal anti-CD45.2-PerCP-Cy5.5 (clone 104) (BD Pharmingen, San Jose, CA) was prepared at 1 µg per mouse in 250 µl concentration and injected intravenously via tail vein. Within three minutes following injection, animals were sacrificed tissues were obtained for analysis.

[2.14] RNA isolation and sequencing. Alveolar macrophages from unimmunized or 4-week BCG immunized mice were purified from the BAL as described above. To ensure sufficient RNA for sequencing, 2 mice were pooled per sample. Triplicate samples were set up per group/condition. Unstimulated and stimulated samples were paired. Isolated alveolar macrophages were resuspended in complete RPMI 1640 medium and plated at $2x10^5$ cells/well in 48-well flat-bottom plates (300 µl/well). Cells were incubated in 37°C 5% CO_2 cell-culture incubator overnight. Following incubation, cells were washed twice with 300 µl of warm PBS

twice and fresh complete RPMI was added to the wells (300 μl per well). Control wells (unstimulated) received no further treatment. Stimulated wells received 0.4 μg/ml of *M.tb* whole cell lysate. Following incubation, total cellular RNA was isolated using a RNeasy mini kit (QIAGEN, Germantown, MD) that contained RNase-free DNase kit. Disrupted cells in RLT buffer were processed according to the manufacturer's instructions. RNA was eluted using 14μl of RNase-free water. RNA samples were stored at -80°C until use. Quality of RNA and subsequent RNA sequencing was carried out by Farncombe Metagenomic Facility. In the Farncombe Metagenomic Facility, RNA integrity was checked using the Agilent bioanalyzer. It was ensured that all of the RNA samples have a RIN (RNA integrity number) of 7.0 or greater for the best quality libraries. Then, the RNA was put through a polyA bead enrichment (NEBNext_PolyA_mRNA) process which enriched for mRNA with polyA tail, ensuring that high quality RNA was obtained as any degraded transcripts would not be sequestered at this point. This step also ensured the removal of ribosomal RNA. The isolated mRNA was then converted to cDNA and made into a library containing adaptors and unique indexes using a ligation-based library prep kit (NEBNext_Ultra_II_Directional_RNA). The libraries were run on the bioanalyzer for a check of size and distribution and the concentration was checked using qPCR. The libraries were then pooled and run on two lanes of an Illumina HiSeq 1500 using on-board clustering in Rapid Mode. 25M clusters were obtained per sample.

[2.15] Quantification and statistical analysis. Statistical parameters including the exact value of n and statistical significance are reported in Figures and Figure Legends. A p value of < 0.05 was considered significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). A two-tailed Student t test was performed for pairwise comparisons. One-way ANOVA followed by a Tukey test was performed to compare more than two groups. All analyses were performed by using GraphPad Prism software (Version 6, GraphPad Software, La Jolla, CA).

CHAPTER 3: Results

[3.1.1] Parenteral BCG immunization enhances early *M.tb* clearance in the lung following pulmonary infection

Subcutaneous (s.c.) BCG immunization in BALB/c mice enhances bacterial control in the lung and reduces pulmonary bacterial burden 4 weeks following intranasal (i.n.) *M.tb* challenge [51]. This protection correlates with the arrival of antigen-specific T cells to the airway lumen [51]; however, only a limited number of studies to date have addressed BCG-mediated protection against pulmonary *M.tb* infection at earlier time points in the C57BL/6 model. Yet, it is becoming more evident that understanding the kinetics of infection at early time points is crucial, as early immune events dictate disease progression and outcome. Previous studies have documented that the fate of *M.tb* infection is primarily determined by the effectiveness of the innate immune responses that inhibit and control bacterial growth at early stages of infection [5,34,39,75]. As such, we sought to examine the BCG-mediated protection against pulmonary *M.tb* infection at 7 and 14 days post pulmonary *M.tb* challenge.

To examine early bacterial control in the lungs, 6-8 weeks old, female C57BL/6 mice were subcutaneously (s.c.) immunized with 20,000 CFU/mouse of BCG and 4 weeks following immunization, mice were intranasally (i.n.) challenged with 10,000 CFU/mouse *M.tb* H37Rv (Figure 1A). Bacterial burden in the lung was assessed 7 and 14 days post pulmonary challenge, using a colony forming unit (CFU) assay (Figure 1A). Pulmonary bacterial burden was significantly reduced in

the lungs of BCG immunized mice in comparison to unimmunized controls at both 7 and 14 days post pulmonary challenge (Figure 1B). This suggests that parenteral BCG immunization enhances protection against *M.tb* in C57BL/6 mice at early time points (7 and 14 days) following pulmonary challenge.

[3.1.2] Parenteral BCG immunization-mediated early anti-TB immunity in the lung is independent of CD4 and CD8 T cells

T-cell mediated immunity is known to be critical to anti-TB immunity [2] and the role of T cells in anti-TB immunity is extensively studied [17]. It has been previously shown in mice that the arrival of antigen-specific T cells to the airway lumen correlates with the reduction of *M.tb* bacterial burden in the lungs [51]. Also, it has been shown that parenteral BCG immunization accelerates T cell recruitment to the lung by approximately 5 days following *M.tb* infection in the BALB/c model [51]. Nonetheless, there are no antigen-specific T cells in the airways until 14 days following *M.tb* challenge in BCG immunized BALB/c mice [51]. As shown above, in the C57BL/6 model, we have seen enhanced protection in vaccinated mice as early as 7 days post pulmonary challenge (Figure 1B). We sought to examine the kinetics of T cell responses starting early timepoints post *M.tb* challenge in our model to obtain information regarding the contribution of antigen-specific and activated T cells in the enhanced early protection in the C57BL/6 model.

To assess the kinetics of T cell responses, 6-8 weeks old, female C57BL/6 mice were subcutaneously (s.c.) immunized with 20,000 CFU/mouse of BCG and 4 weeks following immunization, mice were intratracheally (i.t.) challenged with

500,000 CFU/mouse *M.tb* H37Ra (Figure 1E). The H37Ra strain was used because we could not carry out intravascular (i.v.) staining to distinguish various lung compartments in the BSL3 facility. 0, 3, 7 and 14 days following i.t. challenge, antigen-specific and total T cell responses were assessed in the lung parenchyma (LP) (Figure 1E). Data indicates that there is a lack of vaccine-induced antigen antigen-specific (tet^+) CD4 T cells and total activated ($CD44^+$) T cells in the lung prior to challenge and following *M.tb* exposure, T cells remain absent from LP until 7 days (Figure 1F & H).

Considering that T cells begin to enter the LP of BCG immunized mice at 7 days post challenge, we sought to deplete the T cells and assess the BCG-mediated protection in mice in the complete absence of CD4 and CD8 T cells. BCG immunized mice were depleted of CD4 and CD8 T cells prior to challenge using two injections of depleting antibodies and pulmonary bacterial burden was assessed at 7 days post *M.tb* challenge (Figure 1C). It was observed that lung bacterial burden was significantly reduced in the lungs of BCG immunized mice in comparison to unimmunized controls, even in the absence of CD4 and CD8 T cells (Figure 1D). This suggests that parenteral BCG immunization provides enhanced early bacterial control independent of T cell responses, and it suggests a potential role for the innate immune cells in early anti-TB immunity.

[3.2.1] Parenteral BCG immunization induces long-lasting changes in the phenotype and activation state of alveolar macrophages

Since previously we indicated that BCG-mediated enhanced anti-TB immunity in C57BL/6 mice is independent of CD4 and CD8 immune responses, next we sought to assess the impact of parenteral BCG immunization on the innate immune cells of the lung and airways prior and post pulmonary *M.tb* challenge. We particularly focused on understanding the impact of BCG on alveolar macrophages (AM), since it has been previously shown that alveolar macrophages are among the major lung and airway phagocytic populations which primarily encounter and eliminate *M.tb* [31]. A recent study by Cohen et al. showed that in murine *M.tb* infection model, productive infection takes place almost exclusively within resident alveolar macrophages in the airways [43]. Thereafter, infected alveolar macrophages migrate to the lung interstitium and they are eventually taken up by recruited monocyte-derived macrophages and neutrophils [43]. Thus, it is important to understand how and if alveolar macrophages can be altered by BCG immunization and respond differently to *M.tb* infection.

Thus, initially we sought to characterize the phenotype of alveolar macrophages 4 weeks following subcutaneous (s.c.) BCG immunization and 3 days following *M.tb* challenge. To assess the activation and phenotype of alveolar macrophages, 6-8 weeks old, female C57BL/6 mice were subcutaneously (s.c.) immunized with 20,000 CFU/mouse of BCG and 4 weeks following immunization, mice were intratracheally (i.t.) challenged with 500,000 CFU/mouse *M.tb* H37Ra

(Figure 2A). An extensive gating strategy was used to confidently distinguish alveolar macrophages from other lung macrophage populations (Figure 8). The expression of MHCII (an activation marker) on alveolar macrophages was assessed in unimmunized and immunized mice both prior to and following pulmonary challenge. Compared to their unimmunized counterparts, alveolar macrophages of BCG-immunized mice showed an enhanced activated phenotype expressing higher levels of MHCII (Figure 2B & C). MHCII expression in the BCG-immunized group was further increased following pulmonary challenge (Figure 2B & C).

In order to understand the kinetics of phenotypic changes to alveolar macrophages following BCG immunization. Thus, we assessed the expression of MHCII on alveolar macrophages 2, 4, and 8 weeks post BCG immunization (Figure 2D). We observed that at 2 weeks post BCG immunization, MHCII expression on AM was significantly reduced; however, the expression of MHCII began increasing at 4 weeks following immunization and continued to further increase up to 8 weeks post immunization (Figure 2E). Thus, we concluded that even in the absence of infection, alveolar macrophages have increased expression of MHCII at least up to 8 weeks post immunization. The kinetics of phenotypic changes provided us with insights related to potential mechanism(s) of BCG-induced imprinting of alveolar macrophages.

[3.2.2] Parenteral BCG immunization induces long-lasting changes in the metabolism of alveolar macrophages

As it has been previously shown that BCG-imprinted monocytes shift towards glycolytic metabolism [76], thus, we also wanted to assess the potential changes to the glucose metabolism of alveolar macrophages following BCG immunization. To do so, we measured the basal glycolysis and glycolytic capacity of cells obtained from mice at 2- and 8-weeks post immunization (Figure 3A) using Glycolysis Stress Test. Interestingly, the changes to glycolytic capacity of alveolar macrophages follows the same trend as changes to their MHCII surface marker expression. In comparison to alveolar macrophages obtained from unimmunized controls, alveolar macrophages from immunized mice showed significantly reduced glycolytic capacity at 2 weeks post immunization; however, their glycolytic capacity began increasing at 4 weeks (data not shown) and at 8 weeks post immunization, BCG-trained alveolar macrophages had significantly enhanced glycolysis metabolism in comparison to the unimmunized controls (Figure 3B).

[3.2.3] Parenteral BCG immunization changes the transcriptional profile of alveolar macrophages

As previously described in a study by Yao et al., autonomous memory alveolar macrophages that have been trained by an intranasally delivered adenoviral vaccine, exhibit major differences in transcriptional profiles in comparison to the alveolar macrophages obtained from unimmunized mice [60]. Interestingly,

immunization alters the majority of the genes related to host defense against an extracellular bacterial infection [60]. In this mucosal vaccination model, changes to the transcriptional profile of pulmonary cells are expected; however, it has been previously shown that parenteral BCG immunization (2×10^5 CFU/mouse) in BALB/c mice induces specific pulmonary transcriptome biosignatures [65]. As such, we sought to assess the changes to the transcriptional profile of alveolar macrophages following BCG immunization. This would not only allow us to better define and characterize the BCG-trained alveolar macrophages and identify genes and pathways that are differentially regulated following immunization, but it would also provide information about the origin of imprinted alveolar macrophages (embryonic vs monocyte-derived) and the mechanism(s) of imprinting.

To do so, we obtained alveolar macrophages from unimmunized and BCG immunized mice at 5 weeks following immunization. Half of the cells were stimulated for 24 hours with *M.tb* whole cell lysate and the other half was cultured without stimulation. After 24 hours, RNA was isolated from unstimulated and stimulated alveolar macrophages and sent for RNA sequencing to McMaster Genome Facility (Figure 4A). Initial Principal Component Analysis (PCA) to identify clustering of the gene expression showed a strong and clear effect of BCG immunization on unstimulated alveolar macrophages. However, the effect of BCG on stimulated alveolar macrophages seemed slightly weaker (Figure 4B). Further analysis is being conducted on this data.

[3.2.4] BCG-trained memory alveolar macrophages produce higher levels of cytokines and chemokines upon secondary stimulation

It has been previously shown that following parenteral BCG immunization, monocytes are imprinted and show heightened response to secondary stimuli *ex vivo* in both humans and murine models [10,52,64,77]. As this is an established approach in the field of trained innate immunity to assess the imprinting of innate immune cells, we set out to assess whether alveolar macrophages obtained from BCG-immunized mice also respond differently to secondary stimulations *ex vivo.* As such, mice were subcutaneously immunized with BCG and 4 weeks post immunization, alveolar macrophages were obtained and stimulated with either *M.tb* lysate or live BCG (Moi 5:1) (Figure 5A). Unstimulated controls were also plated. 24 hours following *ex vivo* stimulation, supernatants were obtained, and chemokine/cytokine levels were measured using Lumiex assay. Compared to their unimmunized counterparts, alveolar macrophages of BCG-immunized mice showed significantly enhanced production of IP-10 and MIG following secondary stimulation with live BCG and significantly enhanced production of IL-1ß, IL-6, IFN-γ, IL-12, IP-10, MIG and MIP-1α following secondary stimulation with *M.tb* lysate (Figure 5B & C). Interestingly, alveolar macrophages of BCG-immunized mice also showed significantly reduced production of MCP-1 following secondary stimulation with *M.tb* lysate (Figure 5B).

[3.2.5] BCG-trained memory alveolar macrophages have enhanced anti-mycobacterial and antigen-presentation capacities

Effective control of *M.tb* infection requires responsive innate phagocytic functions [34]. It has been previously documented that the fate of *M.tb* infection is primarily determined by the effectiveness of the alveolar macrophages to eliminate bacilli at early stages of infection [34]. The effectiveness of the macrophages to clear bacterial infections has also been previously shown to be modulated by parenteral BCG immunization. A previous study done by Ratzan et al shows that peritoneal macrophages isolated from BCG-immunized mice ingest significantly more *Listeria monocytogenes* bacilli and are able to inhibit bacterial growth significantly better than the unimmunized mice [78] . This indicates that macrophages have the ability to be modulated by BCG immunization and provide better protection against subsequent bacterial infections.

As alveolar macrophages were phenotypically altered at 4 weeks post BCG immunization and they began exhibiting an enhanced activated state, we next sought out to evaluate the changes to their anti-mycobacterial killing capacity at this time point. This would also give us an indirect measure of how well these alveolar macrophages are able to control infection in an *in vivo* setting, in the absence of any other cells, in particular CD4 and CD8 T cells. To do so, alveolar macrophages were obtained from BCG-immunized and unimmunized mice and infected with H37Ra *M.tb* expressing BCG *ex vivo*, supplemented with mouse

serum and IFN- γ. The anti- mycobacterial activity of these cells was then measured using CFU assay at 4, 24- and 48-hours post-infection (Figure 6A). It was observed that alveolar macrophages from immunized mice showed significantly higher anti-mycobacterial activity in comparison to the unimmunized controls as they were able to kill a significantly higher percentage of the phagocytosed bacteria (Figure 6B). Moreover, the killing and phagocytosis assay was repeated with dsRed expressing red fluorescent BCG. Similarly, at 4 hours and 24 hours following infection, bacterial burden/infection rate in the cells was assessed using flow cytometry (Figure 6C). At 4 hours post infection, alveolar macrophages from immunized and unimmunized mice expressed comparable MFI of dsRed; however, 24 hours post infection, alveolar macrophages from immunized mice showed a significantly lower MFI of dsRed in comparison to cells obtained from their unimmunized counterparts (Figure 6D). This suggests that parenteral BCG immunization augments the functionality of alveolar macrophages enhances their anti-mycobacterial capacity.

As an additional functional assay, we investigated the antigen presentation ability of alveolar macrophages at 4 weeks post BCG immunization. Cells were obtained from BCG immunized and unimmunized mice and were co-cultured with CFSE-labelled antigen specific T cells and peptide antigen *ex vivo* for 4 days (Figure 6E). Alveolar macrophages obtained from BCG immunized mice were better at priming antigen-specific T cells, as T cells co-cultured with BCG-imprinted alveolar macrophages proliferated significantly more (Figure 6F).

[3.3.1] BCG-trained memory alveolar macrophages likely develop independently of circulating monocytes

As discussed previously, during homeostatic conditions, alveolar macrophages undergo self-renewal without any contribution from the circulatory monocytes or other lung macrophages [37]. Upon infection, alveolar macrophages can be replaced by bone marrow-derived macrophages and/or circulating monocytes [37]. Since it has been previously shown that parenteral BCG immunization imprints circulating monocytes [10] and myeloid progenitors in the bone marrow [11], it is possible that imprinted monocytes could be contributing to the population of BCG-trained memory alveolar macrophages in the airway.

We first sought to verify the imprinting of circulating monocytes and the changes that occur to myeloid progenitors in the bone marrow following BCG immunization in our model. Also, it is important to point out that our dose was significantly lower ($4x10^4$ CFU/mouse) than the previous studies that have assessed imprinting of bone marrow myeloid progenitors and monocytes ($1x10^6$ CFU/mouse). To assess the imprinting of circulating monocytes, we obtained whole blood from BCG immunized and unimmunized mice and processed, stained and assessed them for the expression of TLR2, TLR4, CD206 and MHCII (Figure 7A). The data indicated that monocytes expressed higher levels of TLR4 and MHCII following immunization, while they had lower expression of TLR2 and CD206 (Figure 7B). In line with previous research, our data indicates that

circulating monocytes are imprinted following parenteral BCG immunization and they express differential levels of various surface markers.

To assess the effect of parenteral BCG immunization on the bone marrow immune cell progenitors, we obtained total bone marrow cells from femur, tibia and spine of unimmunized and BCG immunized mice and stained them with cell surface markers for flow cytometry (Figure 7C). Data indicated that the stem cell progenitors of BCG immunized mice shifted significantly towards myelopoiesis when compared to unimmunized controls (Figure 7D). Gating strategy for hematopoietic stem cells was adapted from Kaufmann et al. [11]. This shows that innate immune cells are altered by BCG immunization at the progenitor level.

As we indicated that myeloid progenitors and circulating monocytes are imprinted following parenteral BCG immunization, we sought to investigate whether the BCG-trained memory alveolar macrophages in the airways are from the embryonic origin and they are imprinted at the tissue site, or are they derived from the circulating monocytes that have migrated to the lung and airway following immunization. To do so, we used $CCR2^{-/-}$ mice lacking classical Ly6-C^{hi} monocytes, a major contributor to tissue monocyte-derived macrophages (Figure 7E). In the absence of such monocytes, alveolar macrophages obtained from the airways and lungs of BCG immunized wild type (WT) mice and BCG immunized $CCR2^{-/-}$ mice (KO) had comparable levels of MHCII expression 4 weeks following immunization (Figure 7F). To support this data, we also looked at the expression

of CD11b, Ly6C (monocyte markers), and Siglec F (alveolar macrophage marker) on alveolar macrophages at 2, 4- and 8-weeks post BCG immunization. Data indicated that the alveolar macrophages did not express CD11b and Ly6C at any of the time points post BCG immunization, and they maintained their high level of Siglec F expression over time (data not shown). These two experiments highly suggest that the BCG-trained memory alveolar macrophages in the airways are from the embryonic origin, and it is unlikely that the BCG-trained circulating monocytes contributed to this population after immunization.

CHAPTER 4: Discussion, conclusion and significance

Discussion

The traditional paradigm of immunological memory states that protection from re-infection with the same pathogen is mediated by T cells and B cells which are capable of acquiring immunological memory to specific pathogens [79]. Similarly, the goal of vaccines have been traditionally to produce adaptive immunological memory against specific pathogens [80]. Nonetheless, it is becoming more evident that exposure to pathogens and vaccines produces not only adaptive immune memory but also memory-like characteristics in the innate immune cells [7,9,22,53,81].

The recently emerging concept of innate immune memory describes that innate immune cells can be reset from their homeostatic state following the resolution of a primary infection, and as such, they can respond to differently to subsequent infections with the same or an unrelated pathogen [55,82,83]. Much of the studies that have investigated the prevalence and mechanisms of innate immune memory have made use of the BCG vaccine model [53,63,81,84]. This is partly because BCG has been widely used across the globe for decades [16] and this has allowed for the generation of a plethora of evidence supporting the concept of innate immune memory. Many of these studies have reported that BCG confers non-specific protection against various non-mycobacterial infections, malignancies (bladder cancer) and autoimmune conditions such as asthma [66]. Nonetheless, there has been skepticism towards the findings of these studies due to the lack of

scientific and biological explanation(s) for this phenomenon [63]. Fortunately, in the past few years there have been great advances into understanding the biological basis for BCG's non-specific effects [10,59,83].

One of the mechanisms underlying BCG's heterologous protective capacity is described to be the long-term imprinting of innate immune cells [64]. Nonetheless, the concept of trained innate immunity in the context of BCG immunization has been investigated only in the case of heterologous infections unrelated to *M.tb* and have mainly focused on the circulating monocytes and NK cells [6,8,9,52]. From these studies, a few have pointed to the BCG's ability to impact the respiratory innate immune system, specifically, altering the responsiveness of innate immune cells residing in the lung and airways to heterologous infections. Namely, epidemiological studies in Guinea-Bissau [85] and Brazil [67] have reported that the rate of pneumonia is lower in children vaccinated with BCG. Moreover, another study based on international demographic and health surveys of more than 150,000 children from various countries around the world has shown that BCG vaccination in children is associated with the reduced risk of infection with acute lower respiratory infections [86].

In line with the studies that show BCG's protective effects against respiratory infections, there is an unpublished epidemiological study that has indicated an associative link between parenteral BCG immunization in humans and the phenomenon of early clearance following chronic pulmonary exposure to *M.tb*

[4]. All these studies point to the possibility of innate immune imprinting that could have taken place in the lung and airways following BCG immunization. Nonetheless, the impact of BCG immunization on the lung innate immune system and its relative contribution towards protection against pulmonary TB has remained largely uninvestigated [63]. In order to conclusively demonstrate that a part of BCG's ability to enhance protection against pulmonary TB and other respiratory infections is mediated by innate immune mechanisms, experimental studies have to be conducted.

In this study, we have investigated the impact of parenteral BCG immunization on the lung innate immune system and its relative contribution to anti-TB immunity using an experimental murine model. Here, we show that parenteral BCG immunization enhances early protection against pulmonary *M.tb* in a T cell-independent manner. This suggests an important role for the lung innate immune cells, in particular alveolar macrophages, in early protection against *M.tb*. In parallel, we have extensively shown that parenteral BCG immunization induces memory alveolar macrophages with sustained changes in surface markers, gene expression, metabolism, cytokine/chemokine production, antigen presentation and anti-mycobacterial capacities. We can confidently call this alveolar macrophage population in the airways as "memory alveolar macrophages", as this population has the characteristics that have been described in a recent study that defines autonomous alveolar macrophages in the airway following intranasal adenoviral infection [60].

Next steps

We believe that the population of memory alveolar macrophages produced following parenteral BCG immunization is likely imprinted locally and is independent of replenishment from BCG-trained circulatory monocytes, under homeostatic conditions. Nonetheless, additional experiments must be conducted to confidently rule out the contribution of circulating monocytes to this memory alveolar macrophage population in the line. One of the ways we would like to confirm this in the future is by using a staining approach in which a florescent dye (PKH-26) is intranasally delivered to the mice. Resident bona fide alveolar macrophages will phagocytose the dye and thus can be detected using flow cytometry in conjunction with alveolar macrophage markers. 1 day after the delivery of PKH-26, mice will be immunized with BCG. BAL cells will be obtained from mice at various timepoints post BCG immunization and will be analyzed for fluorescent intensity. The alveolar macrophages from the monocytic origin may be positive for alveolar macrophage markers, but they will lack the expression of PKH-26. As such, the contribution of monocytes to the imprinted alveolar macrophage population would be identified. Additionally, we will be using data obtained from RNA sequencing to compare the embryonic signature genes in alveolar macrophages obtained from unimmunized versus BCG-immunized mice. If BCG-trained alveolar macrophages are from the monocytic origin, they should express higher levels of monocytic genes in comparison to alveolar macrophages obtained from unimmunized mice.

Regardless of the mechanisms of generation of BCG-trained memory alveolar macrophages, we speculate that the BCG-mediated early protection is primarily mediated by BCG-trained alveolar macrophages that are functionally and phenotypically augmented. Nonetheless, further experiments are necessary to firmly establish a causative role between BCG mediated innate immune training and enhanced early protection against *M.tb*. One such experiment is to look at the bacterial burden within alveolar macrophages and airways at very early timepoints (day 3 and 7) following pulmonary challenge with *M.tb* using CFU assay. This approach is warranted because it has been previously shown that alveolar macrophages are the primary cells to carry mycobacteria, until at least up to 8 days following pulmonary infection [43]. If memory alveolar macrophages are better at clearing/controlling infection, we expect to see lower bacterial burden both within the cells and also in the extracellular space (airway lumen). If this is the case, we speculate that memory alveolar macrophages are better at phagocytosing and killing bacteria *in vivo* (Figure 9). However, the mechanism(s) underlying such protection should also be investigated.

Another way that trained alveolar macrophages could be providing better protection against TB at early phases post infection is through faster recruitment and better priming of T cells (Figure 9). As we have shown, BCG-trained memory alveolar macrophages not only have a higher expression of MHCII, but they also produce higher amounts of T cell recruiting chemokines such as MIG and IP-10 following secondary stimulation with live mycobacteria and mycobacteria cell wall

components. Moreover, when these cells are co-cultured with antigen specific T cells, they drive enhanced proliferation in these antigen-specific CD4 T cells.

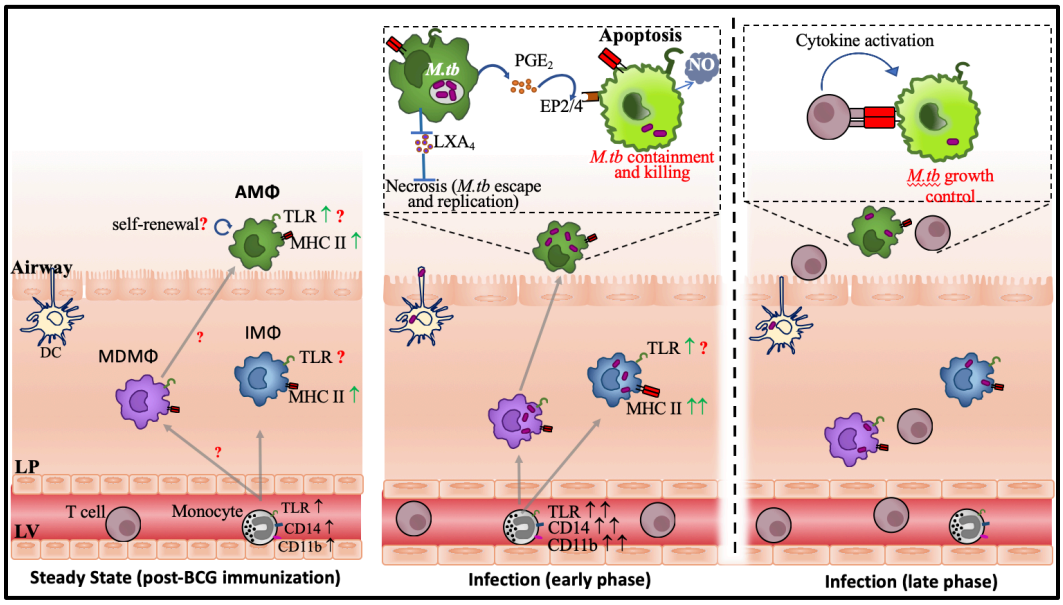


Figure 9. We speculate that BCG-trained memory alveolar macrophages are able to provide enhanced protection independent of T cell immunity by inducing macrophage apoptosis in early phases of *M.tb* infection in the lung while they enhance T cell-mediated immunity in later phases of infection.

Lastly, we would like to assess the role of genetics in imprinting of alveolar macrophages by parenteral BCG immunization. Interestingly, the early enhanced protection in BCG-vaccinated C57BL/6 mice is not observed in BCG immunized BALB/c mice which are not any better protected than unimmunized controls in the first 7 or 14 days post-*M.tb* challenge [51]. Our speculation is that mice of varying genetic backgrounds develop differential levels of BCG-induced alveolar macrophage memory in their lung and airways, which likely contributes to differences in their protection against TB at early stages following pulmonary

challenge. This is a clinically relevant question to address, since only a quarter of the exposed individuals are able to clear the infection prior to developing conventional T cell responses [12]. While there are many factors such as malnutrition, smoking, diabetes and co-infections with HIV/AIDS that could contribute to the risk of developing active or latent TB, genetics also plays a role [87]. Interestingly, previous studies have reported polymorphisms in host innate immune genes that are associated with TB pathogenesis and susceptibility to TB in various human populations [87]. Thus, it is important for us to understand the role of genetics in imprinting of innate immune cells following BCG immunization.

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Conclusion and significance

In conclusion, together the findings of our study provide new insights into how systemically administered BCG immunization can modulate local lung innate immune responses and point to the importance of the lung innate immune responses in the early phases of pulmonary *M.tb* infection.

Our data has important implications in developing novel vaccination strategies against TB, especially since we have shown here that a of the protective effects of BCG against tuberculosis are due to trained innate immunity. To this day, vast efforts have been expended to develop more effective vaccination strategies against pulmonary TB, but the most promising vaccine candidate ($M72/AS01_E$) has only shown partial protection against active TB. One of the most important lessons from this trial is that we have an incomplete understanding of the correlates of protection of TB vaccines. Most of these vaccination strategies have focused primarily on the induction of antigen-specific T cells in the site of infection and have completely left the innate immune compartment out of the picture. However, now we are starting to understand that trained immunity plays an important role in infection prevention, even in the case of TB. Thus, efforts aimed at boosting both T cell-dependent adaptive immunity and trained innate immunity in designing novel vaccination strategies is well warranted.

CHAPTER 5: References

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CHAPTER 6: Data figures

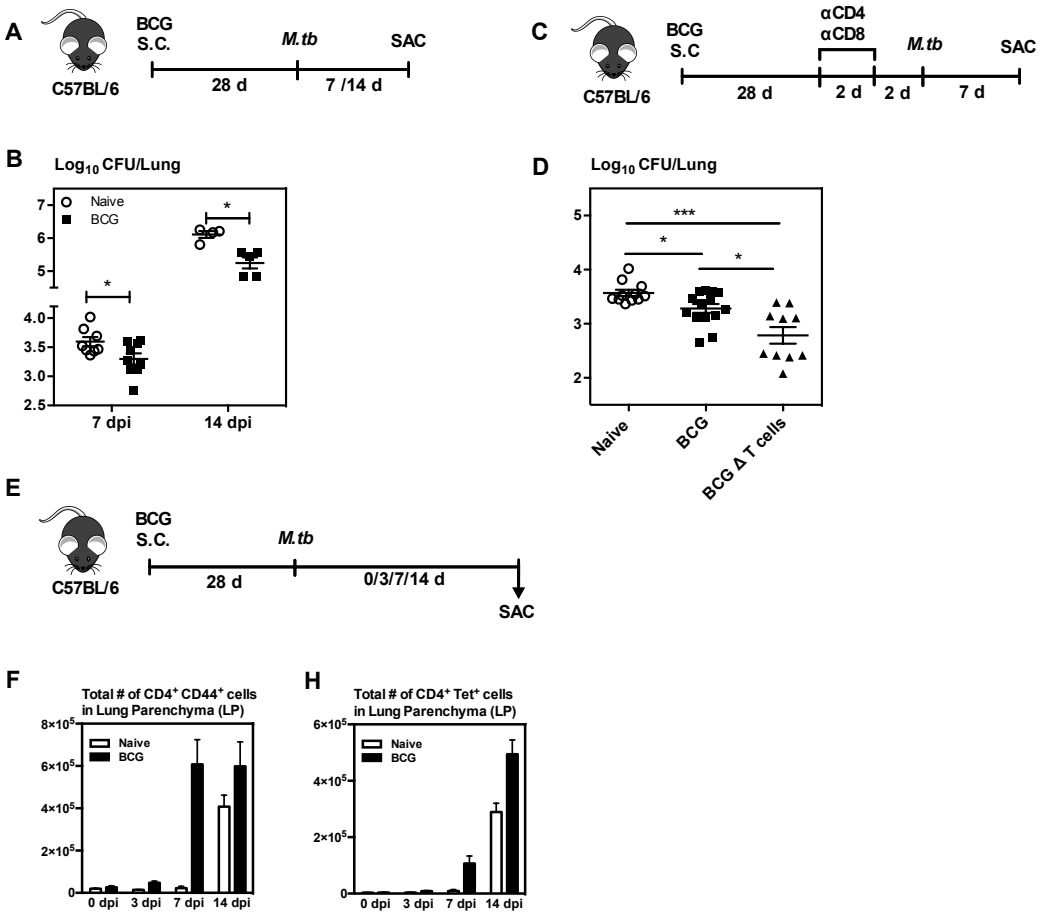

Figure 1. (A) Schematic representation of experimental outline. (B) BCG immunized, and unimmunized mice were infected with *M.tb*. 7- and 14-days post-infection, lungs were assessed for bacterial burden (day 7 data is pooled from 2 experimental repeats). (C) Schematic representation of experimental outline. (D) BCG immunized, and unimmunized mice depleted of CD8 and CD4 T cells were infected with *M.tb* and 7 days post-infection, lungs were assessed for bacterial burden (data is pooled from 3 experimental repeats). Data are expressed as mean ±SEM of pooled experiments. *$p<0.05$ (two-tailed t-test (B) and One-way ANOVA (D)). (E) Schematic representation of experimental outline. (F & H) Kinetics of antigen-specific and activated T cell responses were assessed 4 weeks following BCG immunization and at 3, 7- and 14-days post *M.tb* infection. Data are expressed as mean ±SEM of 3 samples per group (Data is representative of 2 repeated experiments).

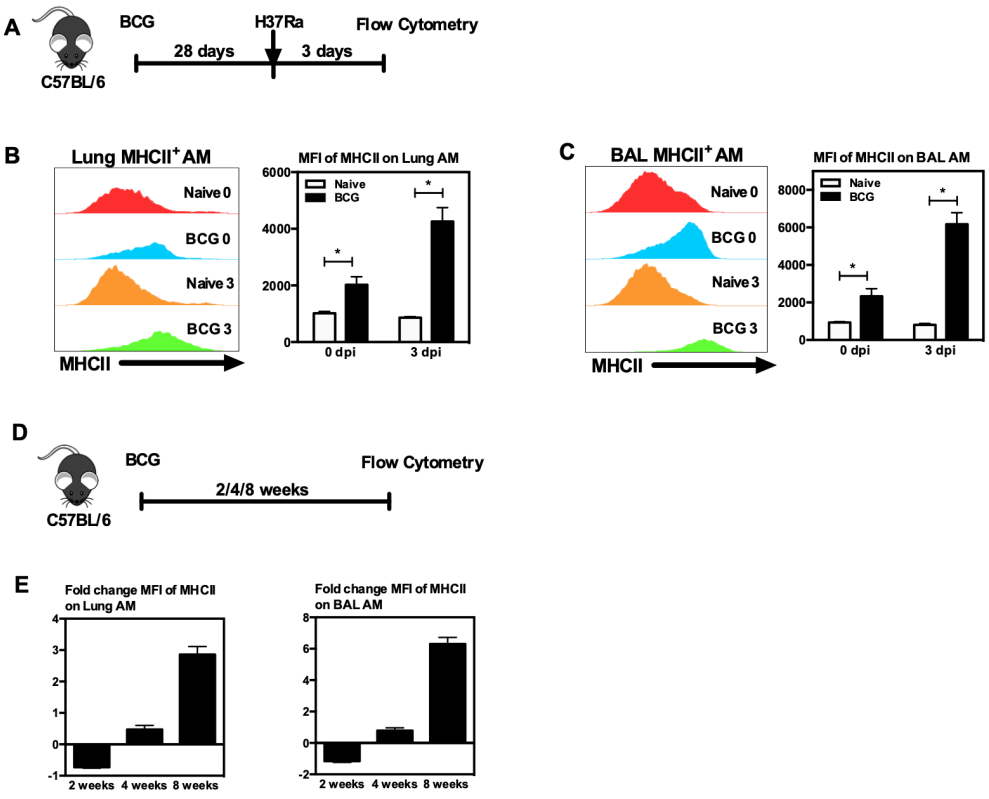

Figure 2. (A) Schematic representation of experimental outline. (B & C) The expression of MHC II surface marker was assessed on lung and airway alveolar macrophages (Data is representative of 6 repeated experiments). Data are expressed as mean ±SEM of 3 mice per group. *$p<0.05$ (two-tailed t-test). (D) Schematic representation of experimental outline. (E) The expression of MHC II surface marker was assessed in lung and airway alveolar macrophages. Data are expressed as mean ±SEM fold change of MHCII expression of immunized mice relative to Naive controls (Data is representative of 2 repeated experiments).

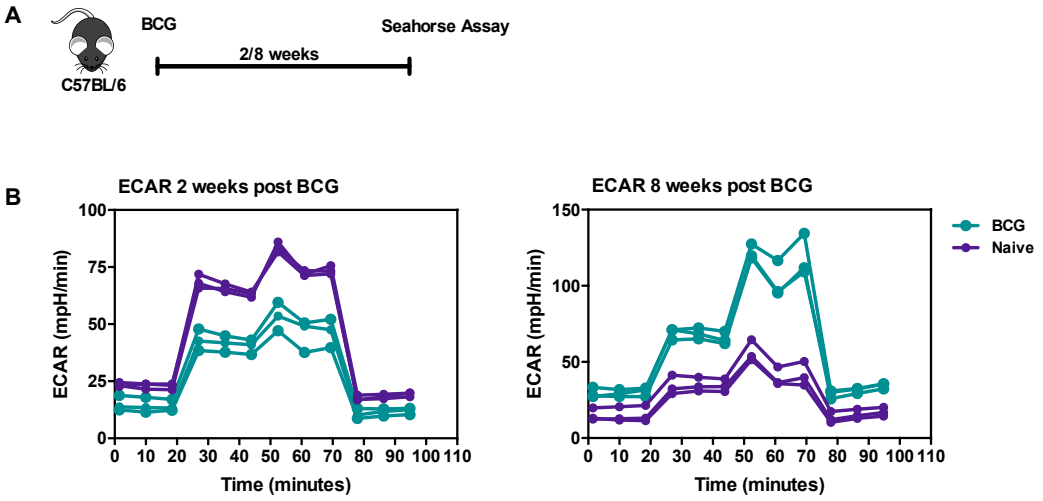

Figure 3. (A) Schematic representation of experimental outline. (B) Glycolytic capacity of AM was measured using glycolysis stress test at 2- and 8-weeks post BCG immunization. Extracellular Acidification Rate (ECAR) is an indication of glycolytic activity (n=3 per group, data is representative of one experiment).

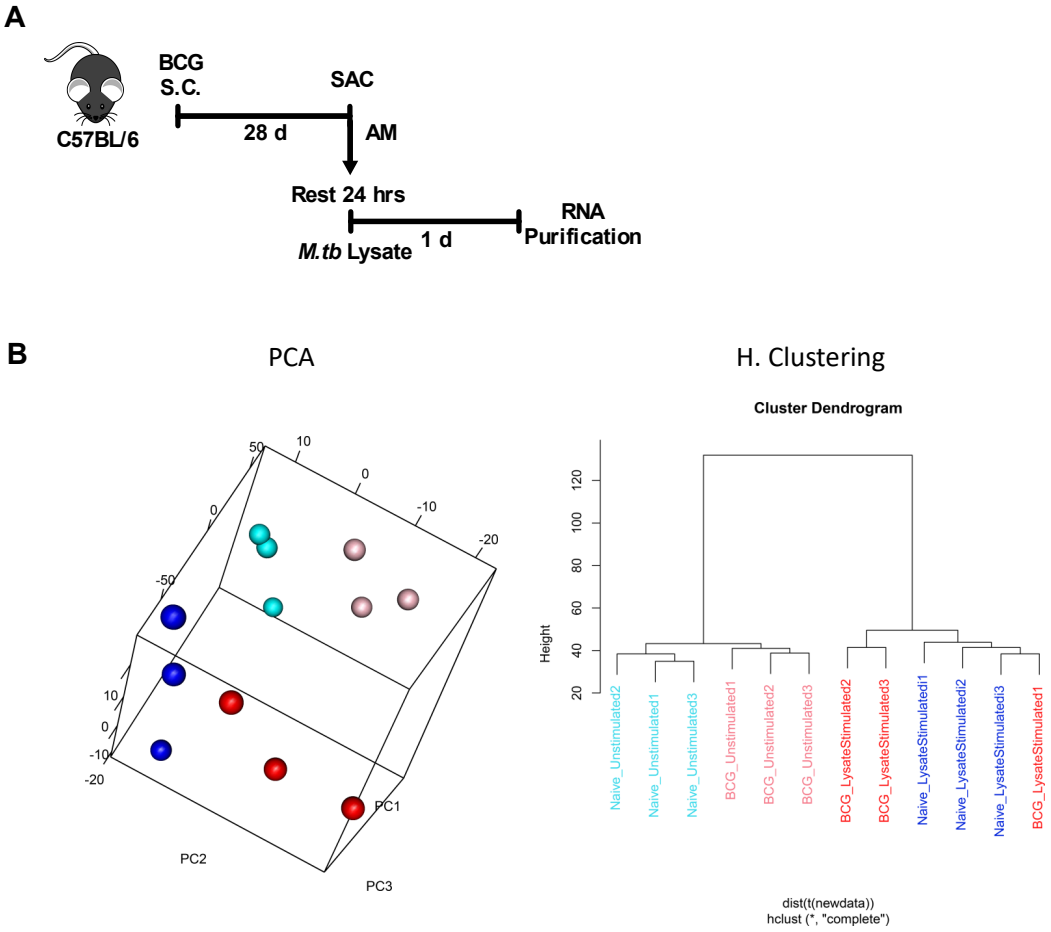

Figure 4. (A) Schematic representation of experimental outline. (B) RNA sequencing was performed on RNA obtained from AM of BCG immunized (BCG) and unimmunized (Naïve) prior to and post *M.tb* lysate stimulation (n=3 per group, data is representative of one experiment).

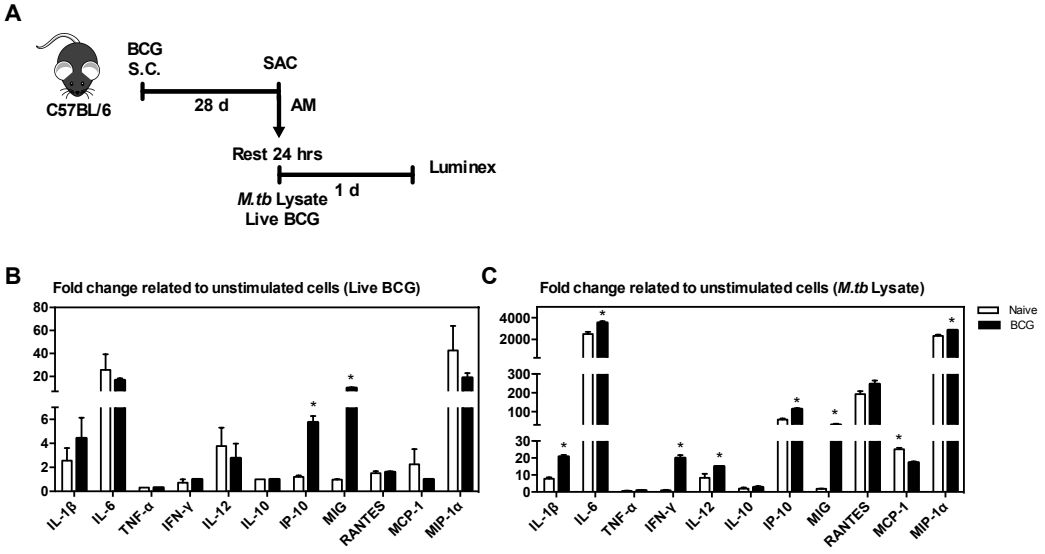

Figure 5. (A) Schematic representation of experimental outline. (B) Cytokine/chemokine production of AM harvested from immunized and unimmunized host was assessed 24 hours following *ex vivo* stimulation with *M.tb* lysate and live BCG. Data are expressed as mean ±SEM of triplicates per condition. Fold change is calculated based on comparison to unstimulated cells. *$p<0.05$ (two-tailed t-test, comparing unimmunized (Naïve) to immunized (BCG).

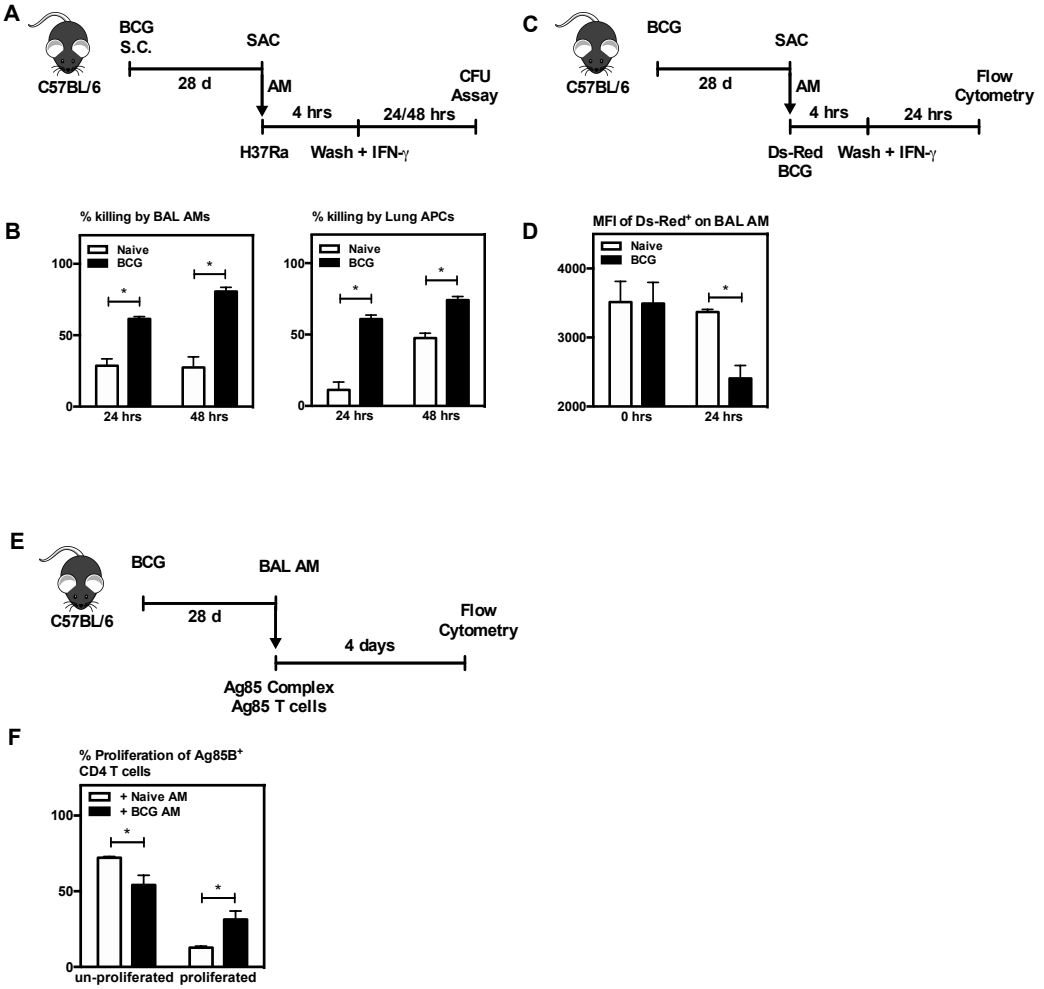

Figure 6. (A) Schematic representation of experimental outline. (B) Percent killing of *M.tb* by AM harvested from immunized (BCG) and unimmunized (Naïve) host was assessed 24- and 48-hours following *ex vivo* infection using CFU assay (n=3 per group, data representative of one experiment). (C) Schematic representation of experimental outline. (D) Bacterial burden in AM harvested from immunized and unimmunized host was assessed at 0 (4) and 24 hours following *ex vivo* infection with dsRed BCG using flow cytometry (n=3 per group, data representative of one experiment). (E) Schematic representation of experimental outline. (F) T cell proliferation was assessed using flow cytometry. CFSE-labelled, antigen-specific T cells were co-cultured with AM harvested from either Naive or BCG immunized host (n=3 per group, data representative of one experiment). Data are expressed as mean ±SEM. *$p<0.05$ (two-tailed t-test, comparing unimmunized (naïve) to immunized (BCG).

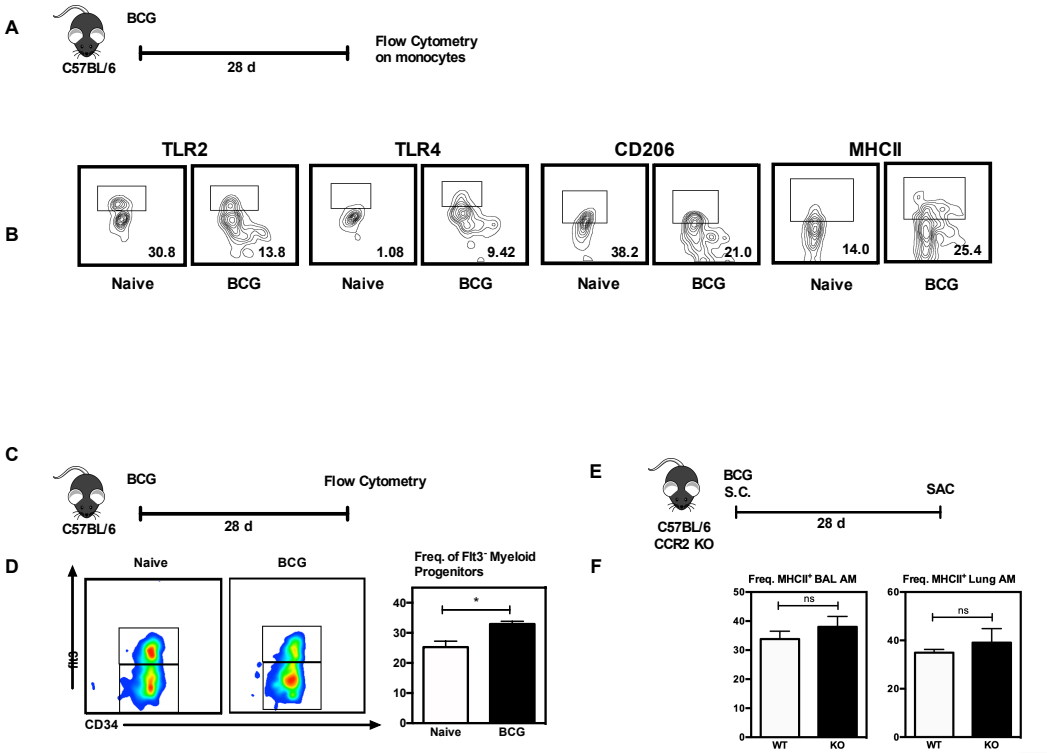

Figure 7. (A) Schematic representation of experimental outline. (B) The expression of various surface markers was assessed on monocytes obtained from whole blood at 4 weeks post immunization. This data is qualitative (n=1). (C) Schematic representation of experimental outline. (D) Myelopoiesis was assessed on hematopoietic stem cells obtained from femur, tibia and spine of unimmunized and immunized mice. (E) Schematic representation of experimental outline. (F) MHCII expression was assessed using flow cytometry on AM obtained from WT and CCR2 KO mice 4 weeks following BCG immunization. Data are expressed as mean ±SEM of 3 samples per condition. *$p<0.05$ (two-tailed t-test, comparing unimmunized (naïve) to immunized (BCG). All data representative of one experiment.

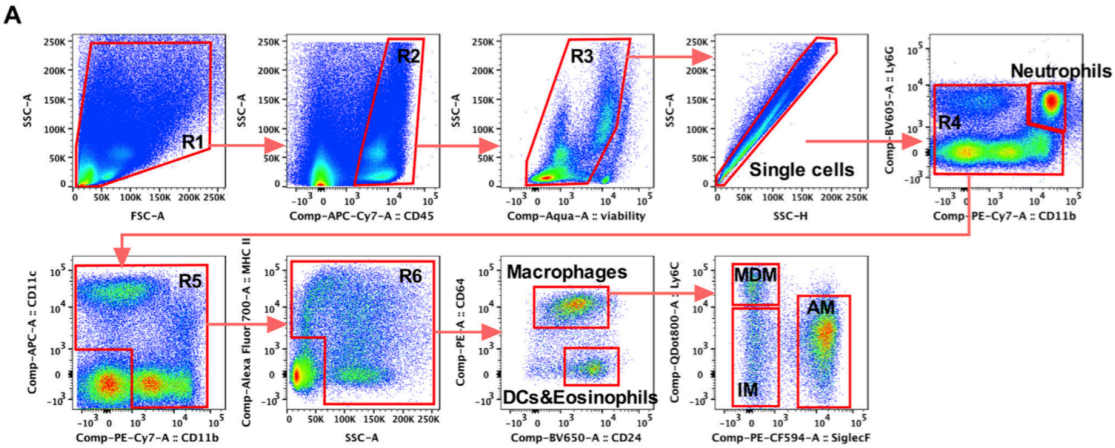

Figure 8. Gating strategy for the lung and BAL [60].