MODULATION OF RESPIRATORY MUCOSAL IMMUNITY AGAINST PULMONARY TUBERCULOSIS

MODULATION OF RESPIRATORY MUCOSAL IMMUNITY AGAINST PULMONARY TUBERCULOSIS

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A Thesis Submitted to the School Of Graduate Studies in Partial Fulfillment of the

Requirements For The Degree Doctor Of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2014)

Medical Sciences

Hamilton, Ontario

TITLE:	Modulation of respiratory mucosal immunity against pulmonary tuberculosis
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NUMBER OF PAGES:	ix, 171

ABSTRACT:

Pulmonary tuberculosis (TB) remains one of the most infectious causes of death worldwide. *Mycobacterium tuberculosis (M.tb)*, the causative agent of TB is transmitted via infectious aerosols, and in the majority of cases the bacteria is effectively controlled, by the host, resulting in a chronic latent infection. Currently, the only available vaccine is the Bacillus Calmette-Guérin (BCG), which despite being successful in preventing childhood disseminated forms of TB, has failed to control the adult pulmonary TB epidemic. One of the major contributing factors in the failure of the BCG is that although antigen-specific T cells are present at the time of *M.tb* infection, the recruitment of such T cells into the site of infection is significantly delayed. This delay, while reduced compared to non-vaccinated hosts, allows the bacteria to replicate unchecked within the lung and establish a "foothold" prior to the arrival of protective T cells and subsequent immune control. Thus, novel initiatives seek to close this "immunological gap" through increasing the level of protective T cell responses within the airway mucosa immediately following *M.tb* infection. We therefore investigated the impact of deliberate modulation of T cell geography following BCG vaccination on the outcome of pulmonary *M.tb* infection. In addition, a number of environmental factors are also thought to affect the site of *M.tb* infection: the respiratory mucosa. However, little is currently known about the effects of environmental exposure to allergens and other substances such as cigarette smoke on the outcome of pulmonary TB. Throughout this thesis we have investigated the mechanisms of immune protection and failure of protection against pulmonary *M.tb* infection within the respiratory mucosa.

ACKNOWLEDGEMENTS:

The work performed throughout my Ph.D program would not have been possible without the continued support and direction of my supervisor Dr. Zhou Xing and my supervisory committee members Dr. Brian Lichty, Dr. Manel Jordana, and Dr. Fiona Smaill.

I would also like to thank the past and present members of my lab that provided continuous support and encouragement throughout the years. Special thanks to Ryan Shaler who provided both invaluable intellect and his hands, and without whom most of this work would not have been possible. The countless hours spent over cups of coffee made me a better scientist and person.

And finally, thank you to Mom, Dad, and Jennine who provided essential emotional support and encouragement throughout this whole process. You put up with my irregular hours and missing family functions for the sake of experiments, complained with me when they didn't work, and celebrated when they did. Luke, you are a source of constant inspiration and motivation throughout the writing and defense process.

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LIST OF ABBREVIATIONS:

APC- antigen presenting cell
TB – tuberculosis
M.tb – Mycobacterium tuberculosis
TNF – tumor necrosis factor
IL – interleukin
DC – dendritic cell
MHC – major histocompatibility complex
BCG – Bacillus Calmette-Guerin
HIV – human immunodeficiency virus
AIDS – acquired immunodeficiency syndrome
Ad – adenoviral
Ag – antigen
ALTs- airway luminal T cells
IP-10 - Interferon gamma-induced protein 10
MIP – macrophage inflammatory protein
MIG - Monokine induced by gamma interferon
MCP - monocyte chemotactic protein
DNA – Deoxyribonucleic acid
RNA - Ribonucleic acid
AM - Alveolar macrophage
PRRs – pattern recognition receptors
TLRs- toll-like receptors
LAM – lipoarabinomannan
CC – CC type chemokine
CXC – CXC type chemokine
CCR - CC type chemokine receptor
CXCR - CXC type chemokine receptor
CD - Cluster of Differentiation protein
MLN - mediastinal lymph node
HLA – Human Leukocyte Antigen
VCAM - vascular cell adhesion molecule
Th- T helper cell
NOS – nitric oxide synthase
iNOS – inducible nitric oxide synthase
IFN – interferon
NK – natural killer cell
NKT – natural killer T cell
NO Nitric Oxide
HDM House Dust Mite

DECLARATION OF ACCADEMIC ACHIEVMENT

Carly N. Horvath, Christopher R. Shaler, Mangalakumari Jeyanathan, Anna Zganiacz and Zhou Xing Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: A critical role of airway luminal T cells. ***Mucosal Immunol. 2012***; 5(4).

> **CNH -** primary author, contributed to the execution and completion of all experiments, data analysis and interpretation, manuscript preparation and revision.
> **CRS -** assisted in the design and execution of experiments and writing the manuscript
> **MJ and AZ -** assisted with the execution of experiments and animal care
> **ZX-** reviewed experimental design and data interpretation, edited and revised manuscript

Carly N. Horvath+, Christopher R. Shaler+, Anna Zganiacz, Amandeep Khera, Manel Jordana, and Zhou Xing. Allergic Immunity to pulmonary tuberculosis via the TLR4 – IL-17 – Nitric oxide pathway. **- To be submitted 2014**

> **CNH –** co-primary author, contributed to the execution and completion of all experiments, data analysis and interpretation, manuscript preparation and revision.
> **CRS-** co-primary author, assisted in the design and execution of experiments and writing the manuscript
> **AZ, AK -** assisted with the execution of experiments and animal care
> **MJ –** provided guidance for HDM model and experimental design, supplied reagents, aided in data review and interpretation
> **ZX-** reviewed experimental design and data interpretation, edited and revised manuscript

Christopher R. Shaler, **Carly N. Horvath**, Sarah McCormick, Mangalakumari Jeyanathan, Amandeep Khera, Anna Zganiacz, Joanna Kasinsj, Martin Stampfli and Zhou Xing. Continuous and discontinuous cigarette smoke exposure differentially affects protective immunity against pulmonary tuberculosis. ***PLOS One*. 2013** 2013;8(3):e59185

> **CNH –** assisted in the design and execution of experiments and writing the manuscript
> **CRS -** primary author, contributed to the execution and completion of all experiments, data analysis and interpretation, manuscript preparation and revision.
> **SM, MJ, AK -** assisted with the execution of experiments
> **AZ, JK –** assisted with animal care and smoke exposure
> **MS, ZX -** reviewed experimental design and data interpretation, edited and revised manuscript

CHAPTER 1: Introduction

[1.0] TB as a global health concern

[1.1] TB epidemiology

Tuberculosis (TB) remains a serious global health concern due to the current burden of disease throughout the world despite ongoing control efforts. The recent report from the World Health Organization in 2013 estimated that there are approximately 8.6 million new cases, and 1.3 million deaths per year from TB (1). While the global incidence rate is 122 cases per 100,000 individuals, 58% of all cases are located within Southeast Asia and the West Pacific (1). In particular, India and China have the highest burden of TB cases, and areas of Africa have the highest case rate per capita; reaching 1 case for every 100 individuals in South Africa and Swaziland (1).

Although 90% of cases are curable with first-line anti-tuberculosis drugs, lack of compliance combined with sub-standard health care in many of the regions with the highest incidence has further confounded the control of the epidemic, promoting the emergence of multi-drug resistant cases of TB (1). The danger of the increase in drug-resistant TB is most apparent in the increased likelihood of death to active TB, which is approximately double that of non-drug resistant cases (1). Further exacerbating the problem with TB control efforts, is the on-going HIV epidemic in regions of the world where TB incidence rates are also the highest. HIV is a major risk factor for TB that is evidenced by the fact that approximately 1.1 million of the total global cases and 300,000 deaths caused by TB are in HIV positive individuals (1).

[1.2] Vaccination and treatment of TB

Currently, the only licensed vaccine against tuberculosis is the BCG (Bacille Calmette Guerin), an attenuated strain of *Mycobacterium bovis,* which has been used throughout TB-endemic regions since the 1920's (2). Typically administered intradermally to infants shortly after birth, the BCG provides protection against childhood disseminated forms of TB; in particular milary and meningeal tuberculosis, which is a significant cause of morbidity and mortality (3,4). The ultimate goal of a vaccine against TB is to provide protection against infection with *Mycobacterium tuberculosis (M.tb);* the causative agent of TB. However, despite obtaining robust peripheral protection, the BCG does not protect against pulmonary *M.tb* infection and thus has not been sufficient in controlling the tuberculosis epidemic (2,5). Current efforts are therefore focused on boosting prior BCG immunity, and creating novel stand-alone vaccines that can provide better protective efficacy against TB than the BCG.

Despite ongoing routine BCG vaccination in TB-endemic areas, one third of the world's population is currently latently infected with *M.tb*, and must undergo treatment with anti-TB therapeutics if available. Further complicating the TB epidemic, diagnosis is typically ascertained via staining sputum spears obtained from patients for acid-fast bacilli, and conducting mycobacterium cultures from such samples with variable efficacy when it comes to diagnosing TB (6). Other more specific diagnostic methods do exist, one particularly effective measure is the use of the quantiFERON-TB gold test, and interferon-gamma release assay that detects the level of IFN-γ released from isolated cells in response to TB antigens (7). While this test is highly sensitive and specific, this assay

is more expensive, and therefore the older sputum tests are still most often employed (6,7).

Upon a positive diagnosis for TB, the first line drugs administered are a combination therapy of isoniazid, rifampin, pyrazinamide, and ethambutol for a typical duration of 6 to 9 months (8). While each of these drugs can be effective at treating TB, there has been an increase in the number of cases of multi-drug resistant TB (MDR-TB) which are caused by circulating strains of *M.tb* that are resistant to at least isoniazid and rifampin (8). Indeed, approximately 35% of all new TB cases in endemic areas are MDR-TB (9). In these cases, second-line treatments are required. These include the injectables (kanamycin, amikacin, capreomycin), fluoroquinolones, *p*-Aminosalicylic acid, cycloserine, ethionamide (9). These second line treatments are not only more expensive, but have increased toxicity, and the duration of therapy is extended to approximately 24 months (9). However, there are an increasing number of cases that are extensively drug resistant (XDR-TB), indicating that they are MDR as well as resistant to the second-line injectables and the fluoroquinolones (9). The situation becomes increasingly despairing with the recent emergence of totally-drug resistant strains of *M.tb;* those of which do not respond to any of the current therapeutic options (8,9). Clearly the demand for effective vaccination against *M.tb* infection, as well as novel therapeutics for infected individuals is urgently required.

[2.0] *M.tb* infection

[2.1] Mechanisms of infection

Tuberculosis is caused by infection with *Mycobacterium tuberculosis (M.tb)*, an intracellular pathogen that primarily infects macrophages and dendritic cells (10,11,12). Infection is established when an individual inhales an infectious aerosol depositing the bacilli in the lower respiratory tract (6,13). Upon entering the host, the bacilli gain entry to target cells via binding to a number of receptors. These include DC-SIGN, surfactant protein A, mannose receptors, complement receptors, and the class A scavenger receptors (10,12). Although some receptors such as the scavenger receptor MARCO have been found to be required for *M.tb* entry (14), others have shown that there is a redundancy in the requirement for all receptors (15). For example, Court and colleagues recently found that while the cytokines IFN-γ, TNF-α, and IL-1β are absolutely required for initiation of an immune response to *M.tb*, class A scavenger receptors as well as the C-type lectin receptors when absent have no impact on disease outcome or pro-inflammatory responses (15). The redundancy of various receptors is likely a result of the extensive co-evolutionary period between the pathogen and the host's immune system, of which multiple receptors can be used for entry by the bacilli.

Once macrophages are infected via receptor mediated phagocytosis, progression into the phagolysosome is arrested by bacterial evasion mechanisms (16). Such mechanisms include the *M.tb* lipid, mannose-capped lipoarabinomannan (ManLAM) along with the bacterial enzyme SapM that synergize to effectively inhibit phagosomal maturation. The target of both ManLAM and SapM is the host type 3 phosphaticylnositol-3-kinase (PI3P), which is required for proper membrane trafficking within the endosomal system and orchestrating late endosomal events (17,18). ManLAM

inhibits critical Ca^{2+} up-regulation required for P13P synthesis, and SapM is a P13P phosphatase; together ensuring a termination in phagosome maturation within the macrophage (17,18). This enables the bacilli to make use of the early endosomal system in order to gain access to critical nutrients such as iron and cholesterol from the host for survival and replication (19). In addition, mycobacteria are also able to regulate fusion and fission events, allowing for the deposition of lipids and proteins into the cytoplasm of the host cell (19). It is through these well-adapted methods of persisting within the host macrophages that are critical to the replication and establishment of a chronic infection.

In addition to alveolar macrophages, *M.tb* can also infect dendritic cells (DCs) (12). Although *M.tb* infection of DCs is possible, the bacteria are not able to replicate as efficiently as they do within alveolar macrophages, primarily due to bacterial location within this cell type. Although the bacteria are maintained within immature vacuoles upon entry, the DC does not make use of the endocytic recycling system, therefore the bacteria cannot take advantage of this pathway for nutrient exchange (20). Furthermore, it has been found that infected mature DCs do not participate in phagocytosis, and thus have no use for shuttling various extracellular components into internal vacuoles (20). In this manner, the bacteria are deprived of many essential nutrients and growth is arrested.

DCs also differ from macrophages in the context of mycobacterial infections in another important area. DC-SIGN is the primary method by which DCs recognize and internalize mycobacteria as opposed to scavenger receptors, mannose receptors, and complement receptors on macrophages (21). Binding through DC-SIGN activates a unique response by DCs as it can result in either the enhanced production of IL-12 or IL-

10 (21). It was originally thought that IL-10 production was advantageous to the pathogen as it allowed for suppression of the immune response, but it is now believed to be a method of regulating immunopathology (21). It is thought that during the chronic phase of the infection, the constant stimulation of DC-SIGN by mycobacterial lipid antigens results in IL-10 production in order to limit the amount of immune activation, preventing lethal immunopathology within the lung (21).

[2.2] Activation of the innate immune response

In addition to providing a niche for the bacteria to replicate, alveolar macrophages also serve as the frontline in host defense through the activation of the innate immune system through TLR signaling (22). In particular, TLR 2,4 and 9 have been shown to be the most critical in *M.tb* recognition. Indeed, the requirement for these particular TLRs in the recognition and initiation of the inflammatory response following *M.tb* infection is demonstrated by the rapid disease progression and enhanced pathology exhibited in knock-out mice (22).

However, although the innate immune system can induce a pro-inflammatory environment through TLR signaling following *M.tb* infection, primarily through the production of TNF-α, IL-1β, RANTES, IP-10 as well as other cytokines and chemokines, this response is not initiated for several days following infection (16,23). This delay is thought to allow *M.tb* critical time to replicate and establish a foothold in the lungs of the host. While there are many theories as to the reason behind this delay, the popular explanations include; direct suppression by *M.tb,* or infection with too few bacilli thus requiring sufficient bacterial replication to reach a threshold required for immune

activation (16). *Mycobacterium tuberculosis* can directly suppress the host immune responses in a number of ways. Virulence factors actively suppress macrophage activation through the down regulation of TLR expression, co-stimulatory molecules, MHC II expression, and active inhibition of phagosome-lysosome fusion (16). It is through this active suppression, that *M.tb* is able to replicate unchecked within the host, delaying innate activation of the pro-inflammatory response for several days (16). Furthermore, while TNF-α, IL-1β, and chemokine production is initially delayed, IL-10 is secreted almost immediately following *M.tb* infection (16).

Eventually, infected and *M.tb* antigen-harboring DCs are able to mature and migrate to the local draining lymph nodes where they process and present *M.tb* antigen to naïve antigen-specific T cells. However, due to the initial delay in the innate response, there is a delay of approximately 8 or 9 days before DCs bearing antigen are seen within the mediastinal lymph node following *M.tb* infection (24). Arrival of antigen in the local draining lymph node is correlated with activation and proliferation of effector cells, and effector T cell activation is not seen prior to this event (24). Indeed, this trafficking step is critical for adaptive immune activation, as CCR7 deficient mice; in which DCs are unable to traffic to secondary lymphoid organs are even further delayed in T cell activation kinetics (25).

[2.3] Adaptive immune response

Upon arrival to the lymph node, DCs are able to drive the critical activation of IFN-γ secreting effector T cells via IL-12 production during priming (26,27). CD4+ T cells are preferentially activated over CD8+ T cells due to the location of the

mycobacteria in endocytic vesicles, however CD8+ T cell priming does occur via cross presentation (28). It has been well established that Th1 polarized CD4+ T cells are critically required for control of an *M.tb* infection as they provide the essential production of IFN-γ (29). IFN-γ is required for the activation of infected macrophages to initiate phago-lysosome fusion and the production of nitric oxide in order to control bacterial growth (30). The absolute requirement of CD4+ T cells and IFN-γ for protection against *M.tb* is evidenced by the fact that MHC class II, CD4, and IFN-γ deficient mice all rapidly succumb to infection (31).

In addition to CD4+ T cells, CD8+ T cells also play an important role in anti-TB immunity. While they have proven to be dispensable in the acute phase of the infection, it has been shown by several groups that CD8+ T cells are required for control during the chronic or latent stage of the infection (32). It is thought that CD8+ T cells mediate critical effector responses through the production of IFN-γ, but more importantly, cytotoxic capabilities (33). These cytotoxic capabilities are evident in MHC class I deficient mice which display higher bacterial loads despite similar levels of IFN-γ as control animals, indicating that CD4+ T cells alone are unable to control bacterial replication (33). CD8+ T cell mediated cytotoxicity is thought to be required for inducing apoptosis in *M.tb* infected cells so that the bacilli can be ingested by cells that are more effectively able to limit bacterial growth.

The initiation of a dominant Th1 effector T cell response is required to limit bacterial replication within the lung, as the substantial delay in T cell priming permits *M.tb* to grow unchecked within the lung for approximately 3 weeks (24). Upon their

arrival to the lung, effector T-cells mediate protection by two primary mechanisms: (1) the activation of infected macrophages to produce antimicrobial substances, and (2) the physical segregation of infected cells to granuloma structures. While in 90% of infected humans these methods allow for the host to control *M.tb* dissemination and achieve latency, rarely is the bacteria ever cleared without therapeutic intervention (Figure 1) (34).

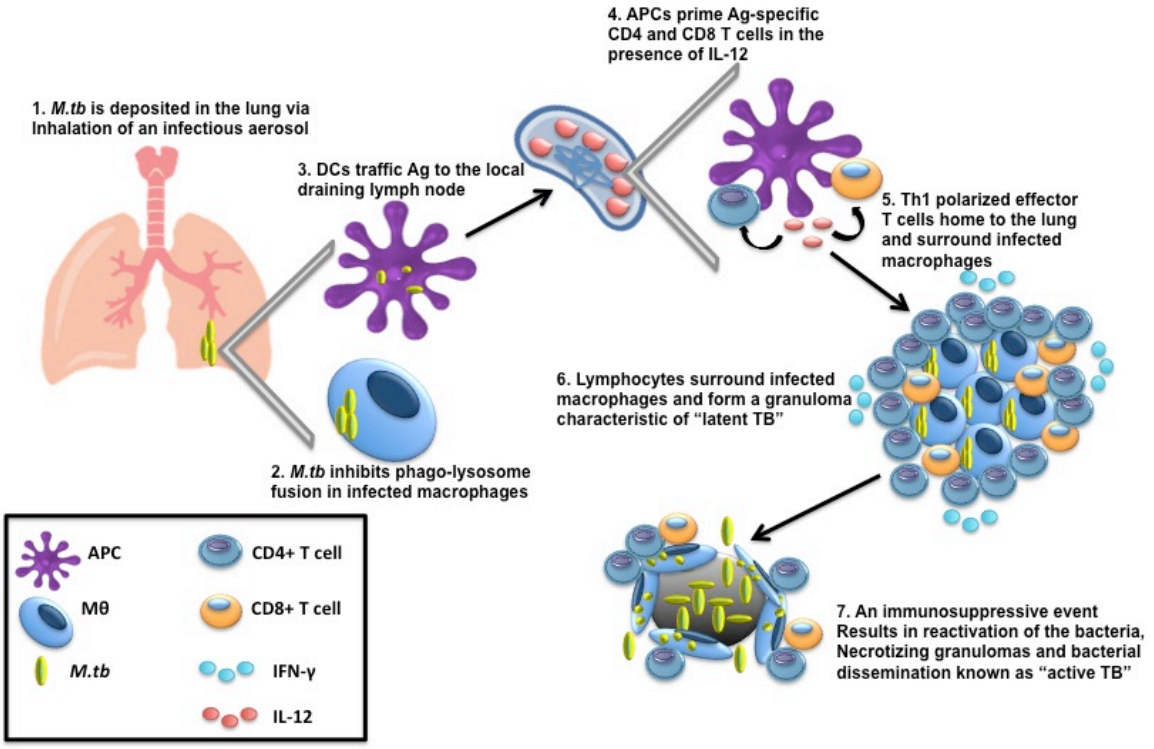


Figure 1: The immune response to pulmonary *M.tb* infection. Following the inhalation of an infectious aerosol, *M.tb* is deposited in the lungs, where it is taken up by receptor-mediated phagocytosis by alveolar macrophages. Virulence factors effectively prevent phago-lysosome fusion and the bacteria replicates within infected macrophages unchecked. Dendritic cells recruited to the site of infection are able to traffic *M.tb* antigen to the local draining lymph nodes for presentation to naïve antigen-specific T cells. T cell priming takes place under the Th1-polarizing cytokine IL-12, and antigen-specific CD4+ and CD8+ T cells are activated. Th1 polarized effector T cells then home to the site of infection and surround the *M.tb* infected macrophages. T cells secrete IFN-γ and activate infected macrophages to produce the bactericidal nitric oxide and mediate phago-lysosome fusion. The lymphocytes that surround the infected macrophages form a

barrier known as the granuloma, effectively walling-off the bacteria and preventing any further dissemination. The cessation of bacterial growth and granuloma formation is achieved in approximately 90% of *M.tb* infections and is referred to as "latent TB". However, in 5-10% of cases of latent TB, and 10% of primary infections, due to an immunosuppressive condition, granuloma structures disintegrate or fail to form and bacterial control cannot be achieved or maintained. This results in enhanced bacterial replication and dissemination resulting in enhanced transmission and morbidity known as "active TB".

The mucosal barrier within the lung, airway lumen is largely considered the primary site of infection. When effector T cells are recruited into the airway lumen, T-cell-derived IFN-γ activates infected AMs to mediate enhanced phago-lysosome maturation, up-regulation of MHC class II loading, and the production of highly toxic antimicrobial substances (24). The increase in MHC class II expression allows infected macrophages to be targeted by Th1 CD4 T-cells, and either activated to kill internalized bacteria, or removed by Fas/FasL or TNF-directed apoptosis (24,31). Following IFN-γ mediated activation, the infected macrophage produces both reactive oxygen substances (ROSs) and reactive nitrogen intermediates (RNIs) (35). Although the generation of ROS, such as H_2O_2 has been demonstrated following *M.tb* infection, it is believed that the major mediator of anti-mycobacterial action is through the generation of RNI, specifically nitric oxide (NO) by the inducible nitric oxide system (iNOS) (35,36). Despite the essential function of IFN-γ in activating iNOS and subsequently control bacterial growth and dissemination (30,37), IFN-γ cannot function alone. iNOS induction requires the presence of TNF-α signaling, and thus models of TNF-α deficiency display similar kinetics of bacterial growth and host survival as those lacking IFN-γ (38). Unfortunately, resistance to RNIs is a common feature of mycobacteria, with the most virulent strains of

M.tb and *M.bovis* being almost completely resistant (39). Although sterile clearance is rarely achieved, the activation of an infected macrophage is thought to be strongly bacteriostatic, facilitating the persistence of *M.tb* within the host (34).

[2.4] Establishment of the granuloma and latency

The segregation of the bacteria within the lung following the arrival of effector T cells and subsequent activation of infected macrophages initiates the cascade of events that form the hallmark of tuberculosis: the granuloma. Granuloma formation following *M.tb* infection represents the primary mechanism of long-term protection via bacterial segregation; an event that occurs in 90% of infections (34). The process of granuloma formation is induced only following the arrival of effector T cells into the site of infection and is denoted by a number of well-defined histopathological changes (34,40). Firstly, the infected macrophages are surrounded by effector T cells, which form a barrier against bacterial movement known as the lymphocytic cuff (34). The combination of the formation of the lymphocytic cuff and the production of inflammatory mediators by the activated T cell and macrophage populations, results in two major morphological changes to the partitioned infected macrophage population. The first of these changes is the transformation into an epithelial-like appearance resulting in epithelioid macrophages, and the second is the fusion of the macrophage populations to form what is known as multinucleated giant cell populations (41). While the granuloma functions to control bacterial replication, it is not a quiescent, but rather a dynamic interplay between the immune system and the bacteria. Indeed, virulent strains of *M.tb* have been shown to induce a large amount of cell death to infected macrophages, creating granulomas with

large necrotic foci, allowing bacteria to leach out and facilitate the recruitment of new host cells (34). In the later stages of granuloma evolution, fibrotic encapsulation and calcification occur, most likely to prevent trafficking of cells in and out of the granuloma to prevent dissemination of the bacteria (34). Although granulomas are the hallmark of a tuberculosis infection, they are extremely heterogeneous even within the same individual. Analysis of granulomas isolated from both humans and primates have shown a large variability in the granuloma size, centralized necrosis, bacterial levels, as well as fibrotic encapsulation and calcification (34). Despite the role of the granuloma in bacterial segregation and limiting bacterial growth during latent infection, the granuloma is also thought to be the mechanism by which transmission is facilitated should the infection progress to active disease.

[3.0] Vaccination against TB

[3.1] Successes and failures of BCG as a TB vaccine

As previously mentioned, the only vaccine currently available against TB is the BCG; an attenuated strain of *Mycobacterium bovis* typically administered intradermally shortly after birth in TB endemic areas. Although the vaccine is extremely effective at preventing childhood disseminated forms of tuberculosis in children, it is largely ineffective at preventing adult pulmonary TB, which constitutes the greatest burden of disease (5). As such, the majority of individuals with cases of active TB have been previously BCG immunized, resulting in a highly variable efficacy of the vaccine, ranging from 0-80% (5). The immunologic mechanisms underlying the failure of the lung protection provided by the BCG remain poorly understood however it is suggested

that it is due a variety of factors ranging from host genetics, environmental mycobacteria, and the type of memory T cells generated from the immunization (2,42).

The immune response towards BCG immunization begins when an individual is intradermally injected with a dose of BCG. Upon injection, the bacilli infect either skin resident macrophages, or are picked up by Langerhans cells residing within the skin (43). Langerhans cells carrying BCG or BCG antigen migrate to the local draining lymph nodes where they prime naïve antigen-specific T cells in the presence of IL-12 (44). Antigen-specific T cells are activated, differentiate into IFN-γ+ effector cells, undergo proliferation, and finally migrate to the site of injection (45). Upon arrival in the periphery, Th1 polarized CD4+ effector cells exert effector responses on infected macrophages consisting primarily of IFN-γ secretion (46). IFN-γ production allows infected macrophages to become activated and bacterial control is obtained (30,37). In addition to CD4+ T cells, BCG immunization also primes a small subset of CD8+ T cells that mediate effector function primarily through (cytotoxic lymphocyte) CTL functionality resulting in the apoptosis of infected cells (47). Finally, a memory T cell pool is generated which persists within the periphery, including the spleen and lung interstitium (48). Although these sites are the most relevant when it comes to a subsequent mycobacterial infection, it is known that memory cells will also reside within the skin, gut, and lymph nodes (48).

Peripheral memory populations are maintained through homeostatic proliferation and stimulation by IL-7, and thus memory cells express the receptor CD127 (49). However, memory populations within the lung are known to have a limited lifespan and require

continuous recruitment from systemic sites for maintenance (49). As memory populations at peripheral sites begin to dwindle, so does protection against *M.tb* (49)**.** Of note, investigation into the phenotype of memory populations has determined that BCG vaccination yields primarily effector memory T cells, with a small subset of central memory T cells (49). It has therefore been suggested that the dominating effector memory T cells may be the cause of variable vaccine efficacy as there is a lack of "backup" responses from central memory cells upon *M.tb* infection (49). In addition, these populations may also wane due to the lack of systemic recruitment for maintenance of lung resident memory T cells.

Despite its shortcomings, BCG is the ''Gold Standard'' for vaccine-mediated protection against *M.tb* infection, and since the vaccine has been widely administered for almost a century, BCG or an improved BCG vaccine will continue to be used in the foreseeable future. Thus, the continuing effort is required to better understand the immune mechanisms behind poor lung protection by parenteral BCG immunization and develop effective boost immunization strategies to fill such an ''immunologic gap'' in order to enhance the protective efficacy in the lung. Due to the variable efficacy discussed above, there is also a movement to replace BCG strains currently used, with recombinant organism-based vaccines that elicit greater protective immunity. Candidates for heterologous prime boosting of pre-existing immunity provided by BCG vaccination include genetic-based vaccines consisting of viral and DNA vectors expressing various *M.tb* antigens, as well as subunit vaccines comprised of *M.tb* proteins combined with adjuvants.

[3.2] Current vaccine initiatives

[3.2.1] Live-organism

One method of improving the current vaccination platform is the replacement of the BCG with a more immunogenic organism. With this goal in mind, several groups have generated recombinant strains of BCG that express *M.tb* proteins such as ESAT-6 (50). These strains have demonstrated greater immunogenicity and protective efficacy compared to the current BCG (50). Similarly, recombinant *M.tb* strains that have had virulence factors removed have shown particularly promising results, with protective efficacy greater than that of recombinant BCG (51). Indeed, great strides have been made in the development of novel vaccine candidates, and the future of TB vaccination is promising.

In addition to recombinant mycobacterial strains, the potential to enhance the protective efficacy of the current BCG vaccine by altering the route of immunization has demonstrated promise. In theory, BCG can be delivered parenterally, intranasally, or by aerosol. Indeed, intranasal immunization in mice was shown to provide better protection against pulmonary *M.tb* challenge than the subcutaneous route (52). Such improved protection was associated with more rapid T cell responses in response to mycobacterial challenge (52). While it is unknown whether mucosal administration of BCG resulted in a population of T cells located within the airway lumen at the site of infection, there was an increase in the lung interstitial T cell population over that of parenteral administration (52). The observation that respiratory mucosal delivery of BCG results in enhanced protection is not only limited to murine models, as aerosol-delivered BCG was also found

to provide better protection than standard subcutaneous or intradermal BCG administration in guinea pigs (53,54). While the use of BCG as an intranasal vaccine has shown great promise in experimental animal models, its safety and efficacy in humans remains to be validated.

[3.2.2] Viral vector

In the search for novel heterologous prime boost methods of TB vaccination, viral vectors expressing *M.tb* antigen are one of the primary candidate methods currently considered to be used in conjunction with a parenteral BCG immunization. Indeed, such vaccines achieve enhanced protection against *M.tb* compared to BCG alone in many experimental models. Given the experimental promise, many viral vector based vaccines have resulted in several candidates progressing into clinical trials. Although several candidates exist, one of the most promising viral vector candidates is the human type 5 adenovirus (Ad5) due to its thorough pre-clinical efficacy upon intranasal delivery and as such, is pioneering the way for novel methods of protection against *M.tb*. Ad5-based vectors make good vaccine candidates due to a high level and sustained transgene expression, tropism for the human respiratory tract, high immunogenicity and can be administered both intranasally as well as parenterally (55). Engineered to express the *M.tb* antigen 85A (AdAg85A), this vaccine was the first replication-defective TB vaccine to be manufactured and evaluated as both a parenteral and intranasal vaccine candidate (55).

It was found that while parenteral administration of the vaccine yielded robust antigen-specific T cell responses in the periphery, it failed to provide significant

pulmonary protection in murine models against *M.tb* (55). However, upon administration of the vaccine intranasally, there were reduced numbers of antigen-specific cells located within the periphery, but elevated levels within the lung interstitium, and upon closer examination, the airway lumen as well (56). The enhanced levels of T cells within the lung and airway lumen correlated with superior protection to both parenteral immunization with AdAg85A and even that of BCG (56).

Insight into the mechanism by which this enhanced protection was achieved indicated that a significant population of antigen-specific CD8+ T cells within the airway lumen that was not present following intramuscular immunization (56). Clearly, the geographical distribution of antigen-specific T cells is highly associated with the route of immunization and the protective efficacy against pulmonary *M.tb* infection. In addition to Ad5-based TB vaccines, other viral-vectored TB vaccines have also been developed of which include Ad35-based and vesicular stomatitis virus-based vaccines. However, following single intranasal immunization in murine models, these candidates do not display the same degree of protective efficacy as the Ad5-based counterpart (57).

[3.2.3] Protein-conjugate

Unlike live organism and viral vector-based vaccine candidates, the lack of immunogenicity of purified and recombinant *M.tb* proteins, there is a requirement for the addition of immune adjuvants and repeated deliveries in order to produce protective T cell responses (58). Despite this challenge, there are several protein-based vaccines against TB that have been tested in both animal models and human clinical trials. While head to head comparisons of mucosal and parenteral administrations are not yet possible

due to differential use of adjuvants with different routes, one study (59) has compared the effect of subcutaneous with intranasal inoculation of a protein-based TB vaccine consisting of *M.tb* culture filtrate proteins and the adjuvant lipophilic quaternary ammonium salt, dimethyldioctadecylammonium bromide (DDA). In this study, researchers found that when the vaccine was delivered intranasally but not via a subcutaneous route, there was a greater level of antigen-specific T cell activation and resulted in a significantly greater protection against pulmonary *M.tb* challenge (59). While this study did not fully characterize the T cell geography following the two vaccination routes, the results clearly support the advantage in vaccinating mucosally against pulmonary TB.

Although most of the protein-based TB vaccine formulations are delivered parenterally, several studies have successfully used such vaccines intranasally to boost parenteral BCG immunization. Repeated intranasal deliveries of the fusion protein vaccine consisting of Ag85B-ESAT6 in LTK63 (59) or CTA1-DD/ISCOMs (60) induced potent T cell responses which then boosted the protection provided by parenteral BCG immunization. However, intranasal administration of an arabinomannantetanus toxoid conjugate (AM-TT) combined with a Eurocine adjuvant only enhanced protection in the spleen and not in the lungs of parenteral BCG-immunized mice (60). Therefore, it appears that the efficacy of intranasal protein-based boost immunization in parenteral BCG hosts is dependent on the formulation of such boost vaccines.

[3.3] Importance of T cell geography in TB vaccination

It is now widely accepted that one of the critical defects in the immune response

following pulmonary *M.tb* infection is the delay in the initiation of T cell priming in the local draining lymph nodes (24). This delay in T cell priming results in delayed recruitment of effector T cells to the airway lumen and lung interstitium, the principal site of infection. Thus, *M.tb* is able to replicate at a logarithmic rate within the lungs of the infected host for approximately 3 weeks, creating a robust ‘‘foothold’’ before the arrival of antigen-specific effector T cells to the site of infection (24). Concurrent with the influx of abundant effector T cells into the lungs, a plateau in bacterial growth is reached (Figure 2).

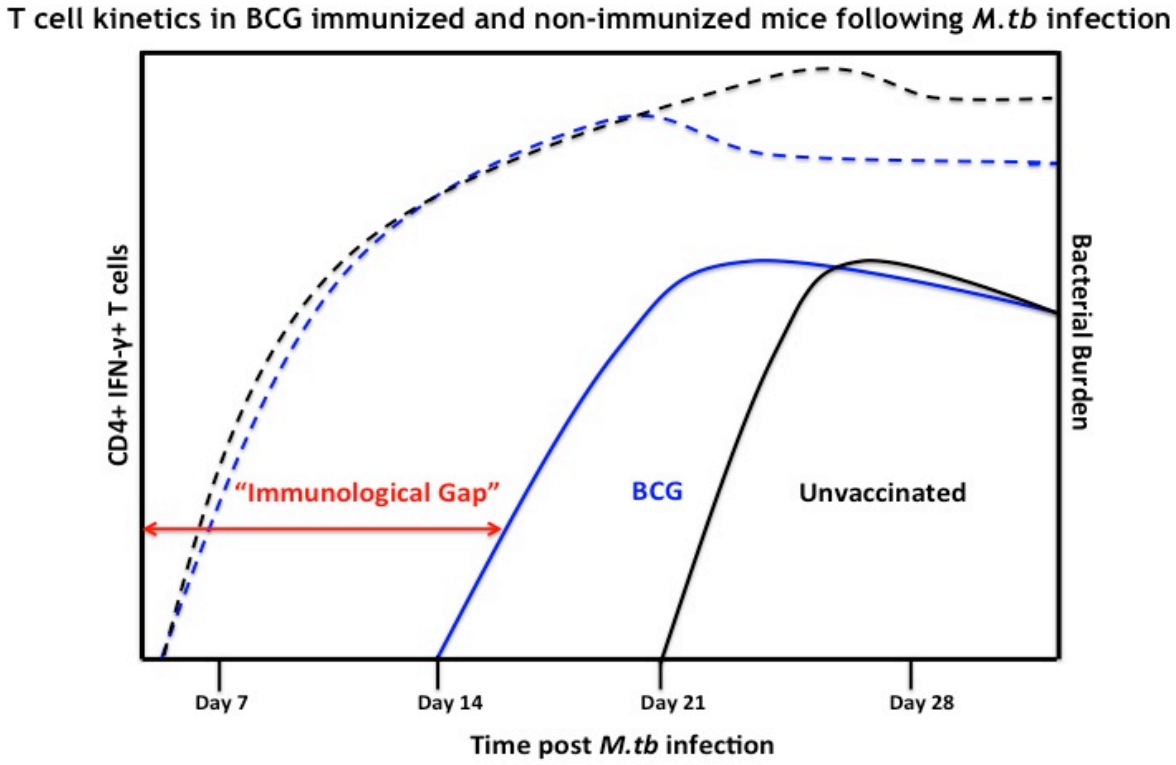


Figure 2: T cell kinetics in BCG immunized and non-immunized mice following pulmonary *M.tb* infection. In non-immunized hosts, the bacteria replicates unchecked within the lungs until the arrival of effector T cells approximately 3-4 weeks post infection. While the delay in trafficking of *M.tb* antigen to the local draining lymph nodes permits bacterial growth initially in the lungs of BCG vaccinated hosts, BCG-primed T cells are rapidly mobilized back to the site of infection resulting in an earlier

plateau of bacterial growth. However, the delay in the arrival of antigen-specific effector T cells in the lungs of BCG vaccinated hosts still allows for an initial "immunological gap" whereby the bacteria replicates unchecked.

Immune control of *M.tb* in the lung following primary infection does not begin until the arrival of Ag specific T cells in the lung following T cell priming in the local draining lymph nodes (61). Although they bypass the initial priming stage in parenterally TB vaccinated hosts, peripherally located T cells must still enter the lung, particularly the airway lumen, in order to mediate effector function. While early vaccine studies primarily focused on the T cell distribution in the spleen and lymph nodes, more recent studies have begun to examine T cell responses in both the lung interstitium and the spleen following parenteral TB immunization (62). It is evident from such studies that parenteral immunization elicits robust T cell responses in the lung interstitium and peripheral lymphoid tissues such as the spleen, although the lung population varies with each vaccine candidate (62). However, when head-to-head comparisons are made between parenteral and mucosal administration of the same vaccine, it becomes apparent that robust T cell responses in the lung interstitium and spleen are not always correlated with protection against *M.tb* infection locally in the lung to the same extent as the presence of an airway luminal T cell population (62).

Therefore, mucosal immunization fills the immunological void that is coupled with parenteral immunization through the installation of antigen-specific T cells at the respiratory mucosal surface within the lung prior to pulmonary *M.tb* infection. It is this mounting evidence that confirms the importance of viewing the lung as two separate compartments when considering T cell geography following TB vaccination. The first is

the lung interstitium which resides between the alveoli and the vasculature, and the second is the mucosal surface of the lung which consists of the conducting airways and the alveoli (62). It is apparent from recent comparisons of parenteral and mucosal vaccination strategies that the majority of past studies in pulmonary *M.tb* infection and vaccination models solely focused on evaluating T cell responses in the whole lung, without separately analyzing both entities (62).

The importance of installation of such an airway luminal T cell population is represented in the case of respiratory or parenteral boosting of a BCG prime immunization by AdAg85A. Although the vaccine when delivered alone provides substantial protection against *M.tb* infection, when delivered mucosally as a booster to a BCG prime immunization in animals, immune protection was enhanced even further. In contrast, boosting intramuscularly with AdAg85A had only marginal effects on the protective outcome of *M.tb* challenge (63). Investigation into the mechanism of how such a marked enhancement in protection was achieved, determined that while parenteral boosting of BCG with AdAg85A enhanced the number of Ag-specific T cells in the lung interstitium and the spleen, only mucosal boosting yielded a population of Ag-specific T cells within the airway lumen (Figure 3) (63).

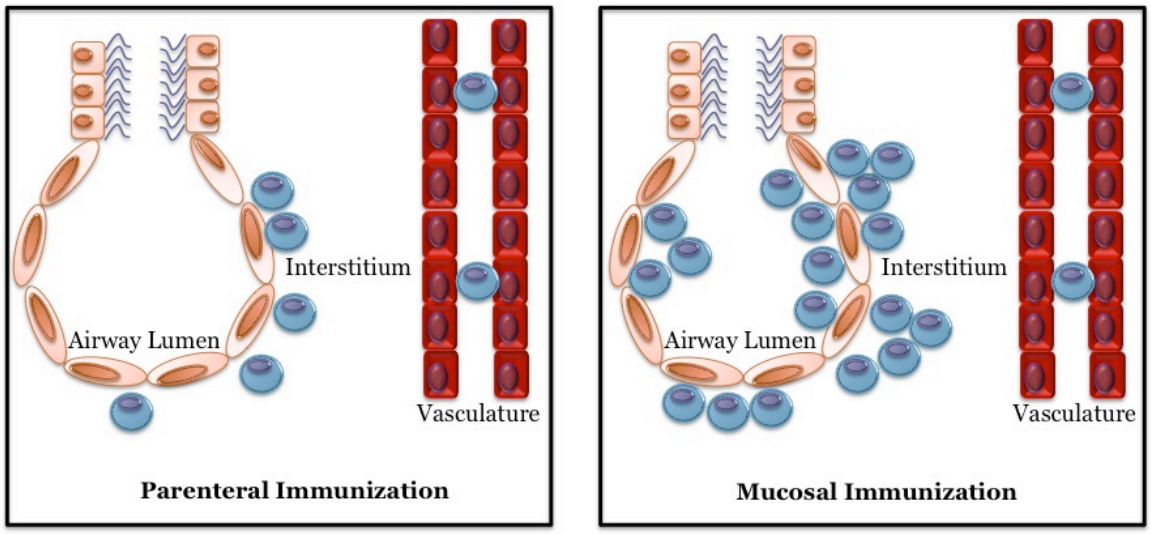


Figure 3: T cell geography in the lung interstitium and airway luminal compartments following differential routes of immunization. Following parenteral immunization, antigen-specific T cells are peripherally located. While a population of antigen-specific T cells resides within the lung interstitium, they do not penetrate the mucosal surface of the lung to populate the airway lumen. In contrast, following intranasal immunization, antigen-specific T cells can be found locally within the lung, and reduced in peripheral locations. Thus following intranasal immunization, antigen-specific T cells populate both the airway lumen and the lung interstitium.

Taken together, these results from both murine and guinea pig models provide justification for evaluation of mucosal vaccination against TB in humans in order to correct the observed deficiency in BCG immunized individuals.

[4.0] T helper cell paradigm

Traditionally, according to the T helper cell paradigm, depending on the immune response required for the type of infection encountered, activated T cells secrete varying profile of cytokines. Typically, Th1 polarized T cells secrete IFN-γ as well as TNF-α and Th2 polarized T cells secrete IL-4, 5, and 13 (64). The reason for the discrepancy between the specific T cell phenotypes is attributed to the type of infection; with a Th1

dominant population required for control of microbial infections, and Th2 dominated responses for control of those primarily parasitic in nature (64). Indeed, in the event of the generation of an improper immune response, increased pathology and often death ensues (64).

In addition to differential cytokine profiles, Th1 and Th2 phenotypes also differ with respect to adhesion receptors and transcription factors. T cells displaying a Th1 phenotype express both the IL-12Rβ chain and the IFN-γRβ chain required for continuous Th1 signaling following activation, and the Th1 homing receptors CXCR3 (IP-10) and CCR5 (RANTES) (64). The transcription factors that determine a Th1 polarization and the production of IFN-γ are T-bet and ERM, both of which are activated by IL-12 activation of STAT4 (64). Once activated, T-bet and downstream IFN-γ secretion are maintained via autocrine signaling through the IFN-γR activation of STAT1, and IL-12 production by activated macrophages (64). Due to the nature of infections that require a dominate Th1 response, the primary function of IFN-γ and TNF-α secretion by such effector cells is to classically activate macrophages to kill ingested pathogens, and to reinforce cytotoxic T cells to target virally infected cells (65). Th1 polarization and effector response are illustrated in Figure 4.

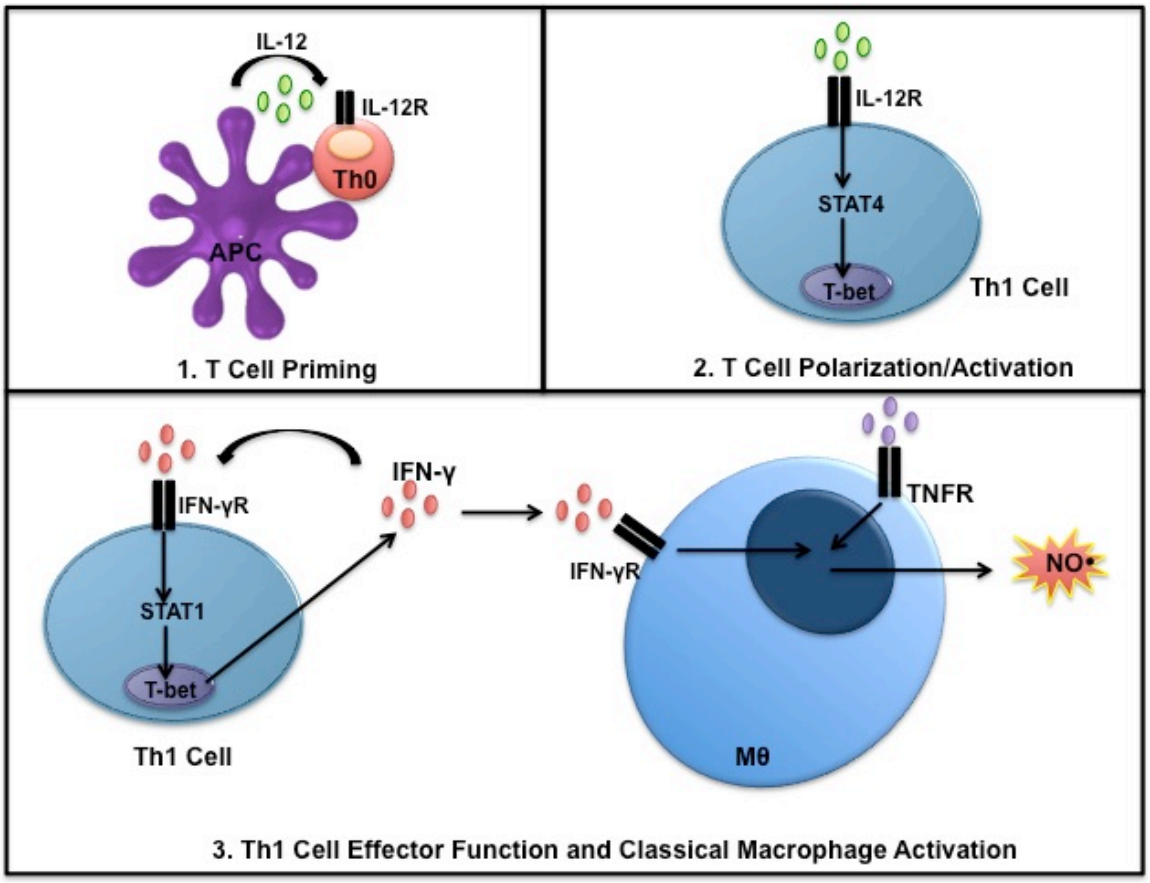


Figure 4: Th1 polarization and effector response. Th0 cells are primed in the presence of IL-12 which signals via the IL-12 receptor to phosphorylate STAT4 and activate T-bet to polarize the T cell to a Th1 phenotype. The predominant effector response of Th1 polarized cells is the secretion of IFN-γ. T-bet facilitates the production of IFN-γ which results in a positive feedback mechanism on the Th1 effector cell via signaling through the IFN-γR. IFN-γR activation results in the phosphorylation of STAT1 which continues to activate T-bet and subsequent production of IFN-γ by the T cell. This IFN-γ production also binds to the IFN-γR on infected macrophage populations, which in conjunction with TNF secretion, results in classical macrophage activation denoted by the secretion of nitric oxide.

On the other hand, T cells of a Th2 phenotype express the eotaxin receptor CCR3, the Th2 homing chemokines CCR4 and CCR8, and the IL-33 receptor T1/ST2 (64). Th2 polarization occurs in the presence of IL-4, and independent of IL-4 in the presence of TSLP (64,66). IL-4 phosphorylates STAT6, which goes on to activate the prototypical

Th2 transcription factors GATA3 and cMaf which then signal the secretion of IL-4, IL-5, and IL-13 by these activated T cells (64). IL-4 is required for B cell class switching to IgE as well as alternative activation of macrophages (64). IL-5 stimulates eosinophil maturation in the bone marrow, and is involved in the activation and recruitment of eosinophils (64). IL-13 is responsible for increased mucous production, collagen deposition, eosinophil recruitment, and along with IL-4 can alternatively activate macrophages (67,68). The mast cell activation via IgE production, eosinophilic granule release, as well as alternative activation of macrophages to release arginase, is well adapted to defend against parasitic infections (67,68). Th2 polarization and effector response are illustrated in Figure 5.

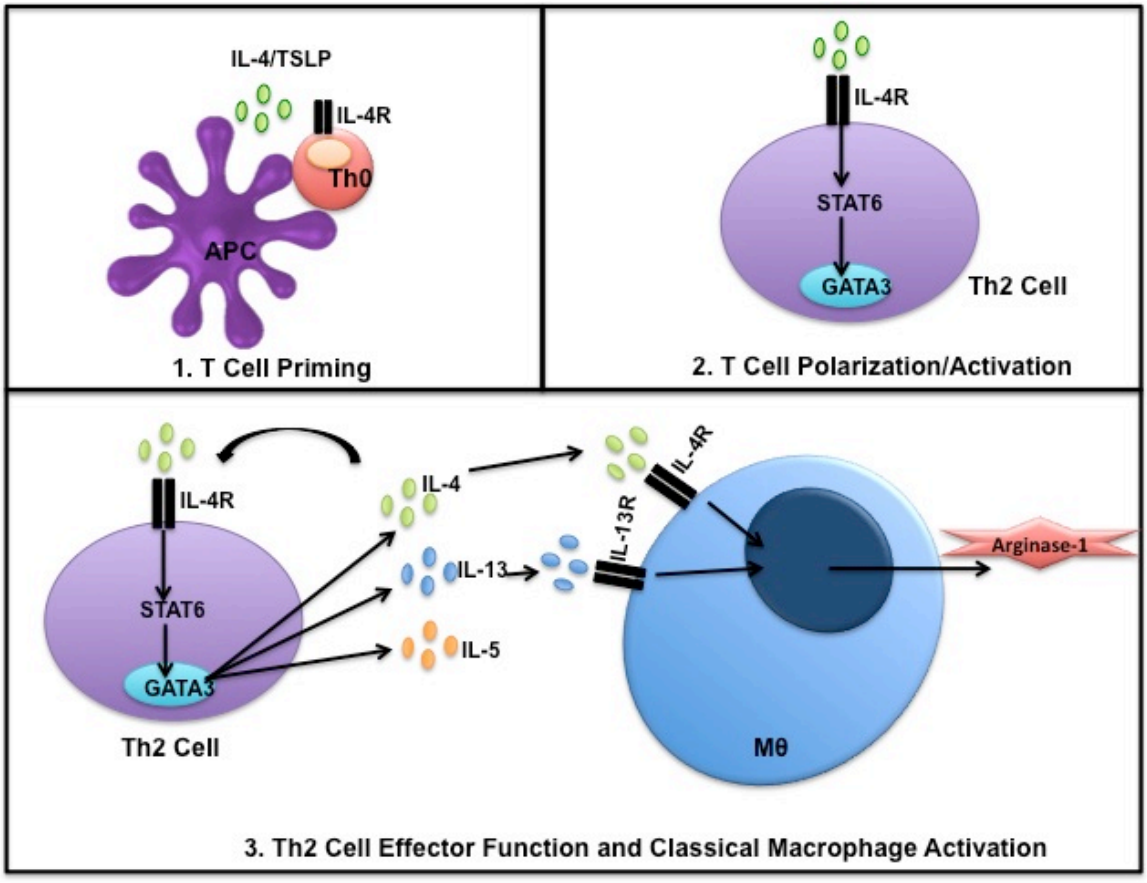


Figure 5: Th2 polarization and effector response. Th0 cells are primed in the presence of IL-4 which signals via the IL-4 receptor to phosphorylate STAT6 and activate GATA3 to polarize the T cell to a Th2 phenotype. The predominant effector response of Th2 polarized cells is the secretion of IL-4, 5 and 13. GATA3 facilitates the production of IL-4 which results in a positive feedback mechanism on the Th2 effector cell via signaling through the IL-4R. IL-4R activation results in the continued phosphorylation of STAT6 which continues to activate GATA3 and subsequent production of Th2 cytokines by the T cell. This IL-4 and IL-13 production also binds to the IL-4R and IL-13R on nearby macrophage populations, which results in alternative macrophage activation denoted by the secretion of arginase-1.

[4.1] Th17 polarization and effector response

While the Th1/Th2 paradigm was characterized over 25 years ago, recently, another distinct T helper cell subtype has emerged that is separate in both phenotype and effector function from both Th1 and Th2 cells known as Th17 cells. It is thought that Th17 cells are required for protective responses to pathogens that cannot be controlled by Th1 or Th2 cell responses alone (69). Polarization of Th17 cells occurs in the presence of both IL-6 and TGF-β production by APCs during T cell priming (69,70,71). These newly activated Th17 cells are characterized by the up-regulation of the IL-23 receptor, and activation of the RAR-related orphan receptor γt (RORγ); the transcription factor required for IL-17 production (71).

However, while IL-6 and TGF-β are both required for Th17 polarization, the presence of IL-23 dictates the effector function of this cell type. In the absence of IL-23 production, these Th17 polarized T cells become non-inflammatory Th17 cells and down-regulate the IL-23 receptor and while still able to produce IL-17, additionally produce IL-10 (71). On the other hand, if IL-23 is present, Th17 cells are induced to become inflammatory Th17 cells and up-regulate the IL-23 receptor and RORγt and secrete IL-17,

Il-22, GM-CSF, and often also IFN-γ (70). The IL-23 receptor promotes the secretion of IL-17 via signaling via JAK2 and TYK2 to activate STAT3 and subsequently IRF-4 which in turn activates RORγt (71). IL-23 is secreted primarily by DC and macrophage populations, while the receptor complex is expressed by γδ T cells, iNK T cells, NK cells in addition to Th17 cells.

Phenotypically, Th17 cells can be characterized by the surface expression of RANKL, CCR6, IL-23R and RORγt (69). RANKL has been associated with Th17-mediated pathology in the joints as it stimulates osteoclastogenesis resulting in the breakdown of cartilage and bone (69). CCR6 expression has been characterized on both murine and human Th17 cells as a chemotactic signal to CCL20 (69,70). Th17 cells themselves secrete CCL20, and mice deficient in either CCR6 or CCL20 have ablated trafficking of Th17 cells(71). While the IL-17 family of cytokines consists of IL-17A through E, Th17 cells pre-dominantly secrete IL-17A and F (69). While 40% homology exists between IL-17A and F, IL-17A has been the most extensively characterized. IL-17A interacts primarily on epithelial cells of mucosal surfaces and induces a number of effects. IL-17A signaling on epithelial cells induces the production of CXCL1/2, IL-6, IL-8, KC, CCL28 and GM-CSF (70). This results in APC maturation, the recruitment of neutrophils, regulation of tight junctions and adhesion molecule expression as well as facilitating the influx of IgE secreting B cell populations (70). Th17 polarization and effector response are illustrated in Figure 6.

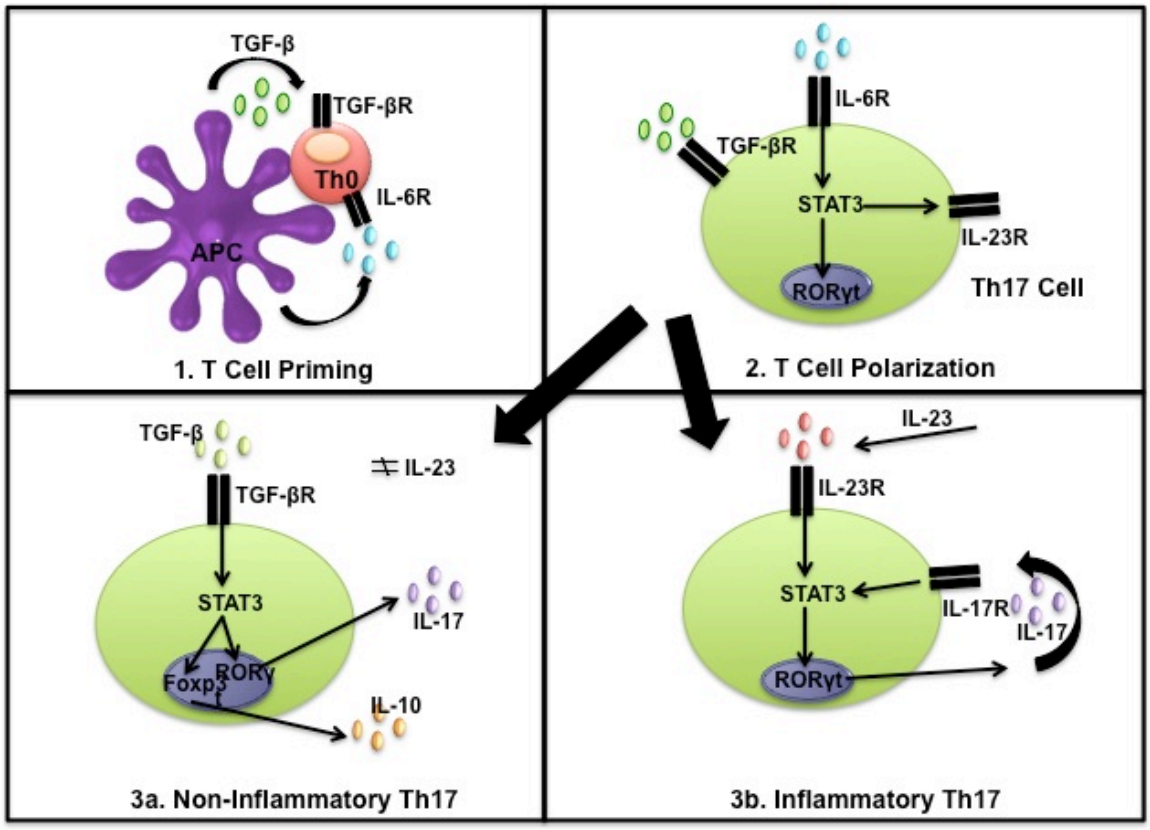


Figure 6: Th17 polarization and effector response. Th0 cells are primed in the presence of TGF-β and IL-6 which signals via the TGF-β and IL-6 receptors to phosphorylate STAT3 and activate RORγt to polarize the T cell to a Th17 phenotype. This involves not only the activation of RORγt, but also the up-regulation of the IL-23R. Unlike Th1 and Th2 cells, Th17 cells effector response is dictated by the presence or absence of IL-23 production. In the absence of IL-23, Th17 polarized T cells become non-inflammatory Th17, characterized by signaling via the TGF-βR to phosphorylate STAT3 to activate both RORγt and Foxp3. The activation of these transcription factors results in the production of both IL-17 and IL-10 from the Th17 effector cell. However, in the presence of IL-23, Th17 polarized T cells become inflammatory effector Th17 cells. Signaling through the IL-23R results in the phosphorylation of STAT3 and the activation of RORγt which results in a positive feedback mechanism on the Th17 effector cell via signaling through the IL-17R. IL-17R activation results in the continued phosphorylation of STAT3 which continues to activate RORγt and subsequent production of IL-17 by the T cell.

Interestingly, memory Th17 cells remain controversial. Unique to this cell type is the extreme plasticity that they exhibit compared to Th1 and Th2 counterparts. This is

because Th17 cells will readily alter the cytokine profile that they secrete as effector cells based on environmental cues (71). The most common change in cytokine profile, is the propensity of Th17 cells to become IFN-γ producing cells (71). This is largely thought to be due to the fact that IL-23 signaling is also highly capable of signaling via STAT1 and the subsequent activation of T-bet (71). Indeed, there are several studies that have demonstrated the requirement of Th17 cells or IL-23 production in order for Th1 generation (69,72,73,74). While memory responses remain enigmatic, there is a clear involvement of Th17 cells in mucosal immunity; particularly in pulmonary bacterial infections and allergic inflammation (70).

[5.0] Risk factors for TB

As a risk factor for TB, HIV infection is a complicating factor for a variety of reasons. Firstly, HIV results in immunosuppression, an effect detrimental to the control of a latent TB infection, and thus results in the reactivation and dissemination of the bacteria leading to severe disease progression. This reactivation of a latent TB infection is a direct result of the disintegration of the granuloma structure due to the decline of adaptive immunity responsible for bacterial segregation and growth inhibition (75,76). HIV-TB co-infected individuals represent a unique challenge as anti-tuberculosis treatments have been shown to interfere with several anti-retroviral drugs resulting in increased HIV viremia due to enhanced viral resistance to protease and reverse-transcriptase inhibitors (77,78). Similarly, HIV anti-retrovirals have been shown to interact with the metabolism of rifampin, one of the current first line drugs used against TB as well as affect the absorption of anti-TB therapeutics in general (78,79,80,81,82).

Not only does this interfere with the control of bacterial growth in TB infected individuals, the resulting ineffectiveness promotes the development of drug-resistant strains of TB in HIV-TB co-infected individuals. Far from ideal, there has been a consensus among health care professionals that these interactions warrant separate treatment regimes, with anti-TB treatment commencing first, and subsequent treatment with anti-retrovirals for HIV (83,84,85,86).

While HIV is the most significant risk factor for TB, other risks include socio-economic status, genetic factors, anti-TNF therapy, as well as alcohol and tobacco use. It is well accepted that tuberculosis is a disease predominately among those of low socio-economic status throughout the world. This is largely attributed to the association between poverty and increased crowding, poor nutrition, lack of appropriate ventilation, drug and alcohol abuse, and cigarette smoke consumption (87,88,89). Over-crowded living conditions act to facilitate the spread of the infection by increasing the number of household contacts, and transmission is further assisted by the lack of appropriate ventilation found among many individuals living in poverty (79,87).

Moreover, malnutrition is of particular concern with 2 major factors contributing to TB disease. The first is that a malnourished individual is more likely to progress to active disease as cell-based immunity required for control of tuberculosis and the establishment of a latent infection is a highly energetic process and is severely hindered (90). Indeed, malnourished individuals have been found to be up to 10 times more likely to progress to active TB than their nourished counterparts (87,88). Secondly, tuberculosis is a disease that often promotes malnutrition, creating a vicious cycle of anorexia, nutrient

malabsorption, and muscle and fat wasting due to alterations in metabolism (87,89). Thus, individuals who are malnourished at the time of infection face a worse prognosis than their healthy counterparts.

Alcohol and tobacco smoking are both significant risk factors for TB for several key reasons. While in general, high levels of alcohol consumption are associated with impoverished conditions and go hand in hand with malnutrition, the major effect alcohol abuse places on individuals at risk for TB primarily is due to its impact on the immune system. Alcoholism has been shown to decrease neutrophil and monocyte chemotaxis, decrease the phagocytic capability of macrophages as well as their capacity to produce oxygen and nitrogen radicals, and globally suppresses signaling molecules and the production of pro-inflammatory cytokines all key processes required for anti-TB immunity (87,89,91,92).

Of particular concern, tobacco smoking rates are the highest in China and India, where the number of TB cases are also the highest. Similar to alcohol consumption, cigarette smoke exposure has a detrimental effect on the host immune response to TB. Cigarette smoke exposure has been shown to decrease mucosal secretions, the phagocytic ability of macrophages, and reduce CD4 T cell counts resulting in lymphopenia (87,89,93). Therefore, tobacco smoke exposure is associated with increased rates of infection, active TB cases, and death from TB (87,89,93).

In addition to poverty-associated risks, there are also several genetic factors that have been correlated with an increased susceptibility to TB. These include polymorphisms that affect the functionality of key genes such as mannose binding lectin

protein (entry of mycobacteria into a target cell), vitamin D (macrophage activation), HLA-D (determines which mycobacterial antigens are presented), natural resistance-associated macrophage protein (NRAMP-1; regulation of phagocytic cations), haptoglobin (regulation of lymphocyte function), and cytokine/cytokine receptors; in particular those that impact T cell functionality and polarization (IL-12/TNF-α/IFN-γ) (87,89,94). While genetic pre-disposition does play a role in TB susceptibility, it is often difficult to uncouple these risks from those associated with poverty.

[6.0] TB risk factors: cigarette smoke exposure

[6.1] Epidemiology: smoking and TB

Globally, rates of cases of TB among smokers are increasing, as cigarette sales continue to rise, particularly among females and within developing countries (95). Indeed, developing countries account for approximately 71% of the global tobacco consumption, and also have the highest burdens of active TB cases and TB related deaths (95). Several studies have shown that not only does cigarette smoking increase the likelihood of *M.tb* infection, but a reduced prognosis as smokers progress to active TB more frequently, and are associated with TB-related death than ex-smokers and never-smokers (96). Indeed, it has been shown that smokers are twice as likely to become infected with *M.tb*, and there are approximately 50% more deaths from TB among smokers than non-smokers (95). Due to the unequivocal evidence of the risk of cigarette smoke exposure on infection and disease outcome for cases of TB, it is highly recommended that tobacco control programs as well as smoking cessation be implemented in affected countries (95,96).

[6.2] Impact of cigarette smoke exposure on anti-TB immunity

Given that the epidemiological evidence that smoke exposure is undoubtedly a risk factor for subsequent infection with *M.tb,* cases of TB, and TB associated mortality; there has been a recent effort into specifically investigating the mechanisms underlying this predisposition to TB and other respiratory infections.

It is astounding that the rates of tobacco sales continue to increase to a point where there are approximately 1.3 billion smokers worldwide, and a global average of 6 million deaths due to smoking-related illness (95). In particular, it is well known that smoking results in an enhanced predisposition to respiratory infections, including the frequency, severity, duration, and efficacy of treatment (95). Of note, second hand smoke exposure can actually further enhance this risk, as it has been found that those exposed to second hand smoke have double the risk of acquiring respiratory tract infections compared to non-smokers (95). This enhanced susceptibility to both upper and lower respiratory tract infections is due to the interference of airway host defenses; in particular the mucocilliary escalator, and impairment of alveolar macrophage populations (95).

The mucocilliary escalator lines the airway luminal surface and has the primary function of entrapping and expelling pathogens from the lower airways by coating microbes with mucous and propelling them upward and out of the lungs (95). In this respect, the escalator combines a combination of goblet cell secreted mucous with the rhythmic beating of the ciliated respiratory epithelium (95). Cigarette smoke exposure impedes this process by decreasing the cilliary beating of the respiratory epithelium, promotes mucous hypersecretion and squamous cell metaplasia, and increases the

epithelial cell permeability (95). This increased permeability is of particular importance as it compromises the structural integrity of tight junctions by decreasing gene expression of junctional proteins (95). Epithelial cells are also compromised in the ability to secrete β-defensins, IFN-β, CCL5, IP-10 and IFN-γ, rendering the lung more susceptible to infection, and impairing clearance by restricting recruitment of protective T cell populations (95).

In addition to the mucocilliary escalator, alveolar macrophages are also impaired by cigarette smoke exposure. It has been well documented that smoke exposure specifically impairs alveolar macrophage phagocytosis, pro-inflammatory cytokine production, TLR and other PRR surface expression, activation of NF-κB and AP-1, and scavenger receptor functionality (95). This combined with a decreased concentration of opsinins within the airway results in impaired overall functionality of the alveolar macrophage (95). In addition to the alveolar macrophage, dendritic cells display decreased antigen presentation capacity, IL-12 and IFN-γ secretion, and CCR7 expression leading to decreased trafficking to local draining lymph nodes in response to cigarette smoke exposure (95).

In addition to impairing the innate immune response, adaptive responses are also impaired by smoke exposure. Smoke exposure models demonstrate a decreased amount of total IgG and an increase in IgE combined with an overall impairment in antibody synthesis, as well as an inhibition of T cell proliferation and subsequent IFN-γ production (95). The result is a skewing of the immune response from a predominant Th1 protective response, leading to impaired mycobacterial control. Indeed, it was found that when

monocyte-derived macrophages and alveolar macrophages from humans were exposed to cigarette smoke and infected with BCG, there was a decrease in the capacity to produce IFN-γ, TNF-α, and IL-10 in addition to impaired mycobacterial containment (97).

It was found that one mechanism behind this impairment in cytokine production by mycobacterially-infected macrophages is that cigarette smoke exposure results in increased oxidative and nitrosative stress and subsequently an increase in carbonyl groups in a dose and time-dependent manner (98). These carbonyl groups then inhibit the functionality or cause the degradation of transcription factors such as NF-κB and AP-1, and an increase in suppressor of cytokine signaling 1 (SOCS1) and interleukin-1 receptor-associated kinase M (IRAK-M) of which regulate cytokine production and TLR activation respectively (98).

As a result of impaired host immunity, pathogens are able to gain a foothold over the nasal passages, oral cavity, and upper respiratory tract in particular (95). This leads to increased pathogen biofilm formation leading to enhanced transfer of genetic material resulting in increased pathogen virulence and decreased susceptibility to antibiotics (95). This combination of immune impairment and pathogen virulence in the smoke exposed individual, results in a dire prognosis upon infection, and accounts for the increased mortality rates from infectious disease; infection with *M.tb* as one, among smokers (88,96).

[6.3] Model of cigarette smoke exposure

The protocol established to achieve a comparable smoke exposure phenotype in murine models to that found in humans is a whole body exposure system. This involves

smoking mice twice daily for 50 minutes, 5 days a week using 12 reference cigarettes with the filters removed for a total of 6 weeks (99). Control animals are exposed to room air for the same duration. This exposure model achieves blood levels of carboxyhaemoglobin and cotinine levels that are comparable to that found in the average human smoker (99,100,101,102).

[7.0] Impact of allergic asthma on anti-TB immunity

While the influence of many environmental factors such as cigarette smoke exposure and pollutants are well characterized as risk factors to subsequent *M.tb* infection, the impact of allergic asthma remains largely unknown. However, given the increasing prevalence of this disease of the respiratory mucosa throughout the world, investigation into the relationship between allergic asthma and pulmonary *M.tb* warrants future investigation.

[7.1] Allergic asthma and pulmonary *M.tb* infection

It is historically well documented that there is an inverse relationship between asthma and pulmonary *M.tb* infection. However, throughout the literature, the observation that pulmonary tuberculosis is a protective factor against the development and progression of asthma dominates the relationship between the two diseases (103,104,105). In regards to the role of preexisting asthma and Th2 dominated immune responses on subsequent host immunity to *M.tb* is absent in animal models, and is controversial in human anecdotal evidence. Some reports from patients with TB and asthma describe TB to be slow and unprogressive, and that pre-existing asthma is a

protective factor for developing active TB, where as others describe asthma as a risk factor to TB, and an association with a more severe prognosis (106,107,108,109).

[7.2] Allergic asthma and BCG vaccination

It has been recognized for several years that there is an inverse correlation between the presence of allergic responses and exposure to mycobacteria (107). Furthermore, BCG has been found to reverse established allergic airway disease, and prevent allergic responses in several models (110,111,112,113,114). The mechanism by which this is thought to occur is through the suppression of Th2 responses via Th1 polarization by enhanced levels of IL-12 or by the induction of regulatory T cells which suppress Th2 functionality following exposure to BCG (114,115).

In humans, the role of BCG vaccination in atopic disease has proved to be highly controversial. Several studies have shown an inverse correlation between BCG immunization and allergic asthma, whereby TST positivity and scar formation are negatively associated with asthma (107,116,117,118). In contrast, other studies have found positive correlations and no-correlation at all between BCG immunization status and allergic symptoms (119,120,121). Furthermore, in TB-endemic areas, BCG immunization coverage is approximately 84% in rural areas, and over 90% in urban areas (3), many countries of which are experiencing increased prevalence of asthma. Therefore it remains unknown if BCG vaccination status has any impact on the ability of an individual to develop allergic asthma.

[8.0] Allergic asthma: prevalence, disease, and treatment

[8.1] Epidemiology of allergic asthma

Approximately 300 million individuals worldwide suffer from asthma making this condition the most common chronic disease in the world, attributed to 1 in every 250 deaths (122,123). In addition, the prevalence of asthma has been increasing over the last several years due to the introduction and expansion of industrialization and urbanization throughout the world, but in particular, the developing world (122,123,124). Although largely considered to be a disease of the developed world, with over 10% of North America and Western Europe's population diagnosed with asthma, there has been an increasing incidence of asthma in the urban centers of the developing world (122,123,124). Importantly, due to the lack of available treatment in the developing world, the highest rates of hospitalization and death due to asthma occur in these regions (122,123,124). In particular, several African countries as well as China and India are experiencing an increasing prevalence of asthma (122,123,124). The reasons for the augmented incidence of asthma are believed to be a result of primarily increased urbanization, sanitation, and westernized lifestyle within these areas of the developing world (122,123,124). These assertions are in agreement with the current "hygiene hypothesis" which states that increased levels of sanitation, personal hygiene, decreased family size, and consequential decreased exposure to microorganisms has resulted in enhanced Th2 polarized responses, which has facilitated the increased prevalence of atopic diseases (122,123,124). Clearly, worldwide trends of asthma are changing as the prevalence of asthma is becoming widespread, and can now be considered a global concern, not just that of westernized populations.

[8.2] Immune profile of allergic asthma

Allergic asthma in humans is characterized by numerous heterogeneous symptoms which include obstruction of airflow, airway inflammation, and airway hyper-responsiveness (125). These symptoms are reversible through either the administration of treatment such as bronchodilators and glucocorticoids, or spontaneous resolution (126,127). Factors known to cause asthma include viral infections, exercise, irritants, and exposure to allergens, and the disease typically manifests during infancy or early childhood (122,125). The disease is separated into two distinct phases, the acute and chronic stages. Acute symptoms include the presence of allergen-specific IgE, mast cell infiltration and activation, Th2 lymphocytes, and eosinophil influx as visualized in lung histological sections from afflicted individuals (125,128,129). The cytokine profile in the lung of asthmatic individuals is dominated by the Th2 cytokines IL-13, IL-4, IL-5 as well as mast cell production of type 2 pro-inflammatory chemokines resulting in increased cellular infiltration (122,126,128,129). There are several additional cytokines that have recently been implicated allergic asthma in addition to the characteristic IL-4, 5, and 13. IL-33 is a newly characterized cytokine produced during allergic asthma (130,131). It has been shown that IL-33 secretion is responsible for the enhanced viability and survival of eosinophils (130,131). Interleukin-17 and 23 are two other cytokines recently recognized in the involvement of allergic airway disease. It has been shown that IL-23 and IL-17 work synergistically to promote the recruitment of neutrophils into the lung, but additionally increase levels of eotaxins, thereby enhancing the recruitment of eosinophils in response to allergic stimuli (132). In particular, the recruitment of eosinophils into the

lung results in airway inflammation, hyper-responsiveness, and obstruction of airflow through granule secretion and tissue injury (133). Lastly, TSLP (thymic stromal lymphopoietin) also facilitates the induction of allergic airway disease by acting on DCs to drive CD4+ T cells towards a pro-inflammatory Th2 phenotype (66). Over time, this repeated and prolonged Th2 response within the lung results in the chronic effects of asthma.

The chronic phase of asthma although variable, is characterized by myofibroblast activation and collagen deposition, which cause airway remodeling which leads to irreversible loss of lung function (134,135). This is the result of increased vascularity, airway muscle thickness as well as the abundance and size of smooth muscle cells that are able to achieve increasing levels of broncho-constriction (134,135). In addition, the chronic inflammatory conditions leads to extensive damage of the airway epithelial cells and stimulates goblet cells to secrete increasing amounts of mucous (129,134). The disruption to the mucosal epithelium of the airways also poses an additional problem of impairing the natural barrier of this surface against subsequent pathogen exposure (129,136).

[8.3] Prevention and treatment

While efforts to understand the mechanisms behind the development of allergy and asthma have led to the development of novel therapeutic options, the most effective treatment remain inhaled glucocorticoids and bronchodilators. Inhaled glucocorticoids combat the inflammation within the lung via globally suppressing immune activation (137). Specifically, glucocorticoids act on a wide range of cell types, primarily by

suppressing the transcription factors nuclear factor-κB (NF-κB) as well as activator protein-1 (AP-1) (137). While suppressing immune activation, glucocorticoids are also able to simultaneously control inflammation via increasing the transcription of anti-inflammatory proteins such as IL-10 and IL-1 receptor agonists (137). The second major method of treatment for allergic asthma is utilization of β2 agonists which act to relieve bronchoconstriction by binding to the β2-adrenoreceptor on the bronchial airway smooth muscle cells, causing them to dilate (138). Depending on the severity and frequency of asthma symptoms, the use of these therapeutics vary from heavy daily use, to occasional doses (139,140,141). The most recent treatment administered to patients with severe allergic asthma is Omalizumab; a human monoclonal anti-IgE antibody which acts to suppress allergic asthma by binding to serum IgE and preventing IgE from binding to its receptor and propagating the inflammatory response (142,143,144). This is especially helpful to patients who do not respond well to the glucocorticoids or the bronchodilators in controlling their allergic asthma.

Efforts to develop novel treatments for allergic asthma, with the exception of Omalizumab have had disappointing results. Therapeutic targets for the majority of the inflammatory pathways of allergic asthma have been tested in a wide range of clinical trials. These novel approaches include the cysteinyl leukotriene receptor antagonists (145,146,147), phosphodiesterases (148,149,150), anti-IL-4, anti-IL-5, and anti-IL-13 (127,133,151,152,153), anti-TNF (154), CCR3 agonists which block the function of eotaxin (155), and adhesion molecule antagonists (156) all of which aim to target the various aspects of the pathology of allergic asthma. While clinically most of these drugs

have been disappointing, it has become apparent that such a complex disease such as asthma may have to be approached with multiple therapeutics, and combination therapy is most likely the strategy of the future

[9.0] House dust mite

[9.1] Models of allergic asthma

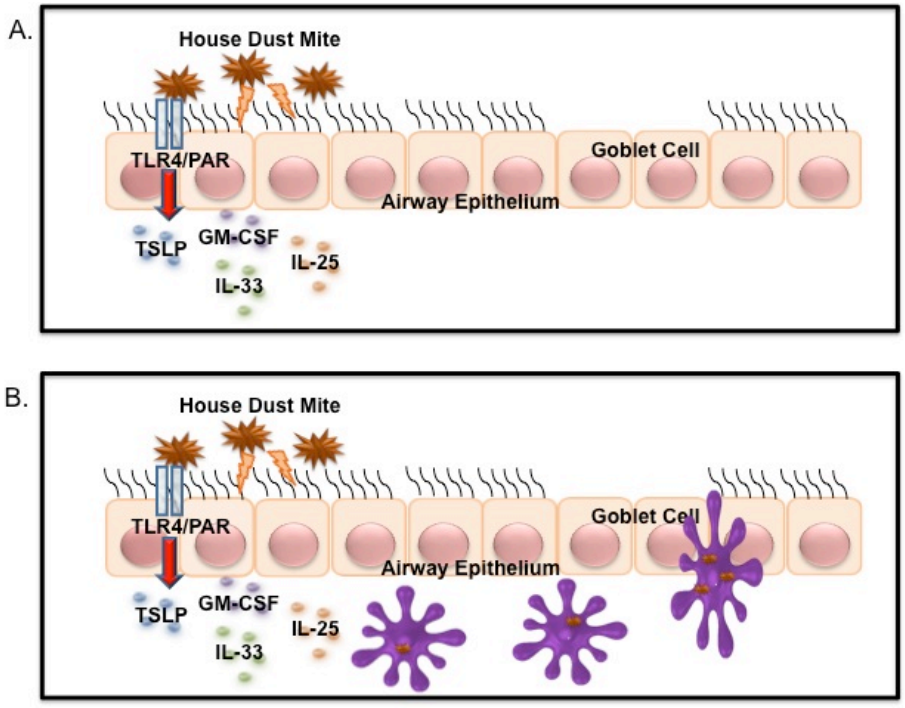
There have been several models developed in attempt to recapitulate the hallmarks of asthma seen in humans. However, several of these models use innocuous antigens such as OVA that lead to immunological tolerance if administered without the presence of adjuvants (157). In addition, the current standardized models of allergic asthma using OVA combined with adjuvants must utilize a sensitization and challenge model unrealistic of human exposure, and as such do not manifest the chronic stages of allergic asthma (128,134,158,159). Recently, an effective model of chronic allergic asthma has been developed through repeated exposure to a common human allergen: house dust mite (HDM). A chronic allergic model using house dust mite is a more relevant model as it comprises a significant portion of indoor allergens that causes atopic symptoms in approximately 10% of exposed individuals (122,160,161,162,163). Furthermore, approximately 80% of all cases of allergic asthma are caused by HDM (122,161,162,163). The reason for this high prevalence of allergic asthma caused by HDM, is due to the ubiquitous occurrence of this organism throughout the globe (160,162,163). Although there are several species of house dust mite, *Dermatophagoides pteronyssinus* has been implicated as the most frequently encountered source of allergic asthma (160,164,165,166).

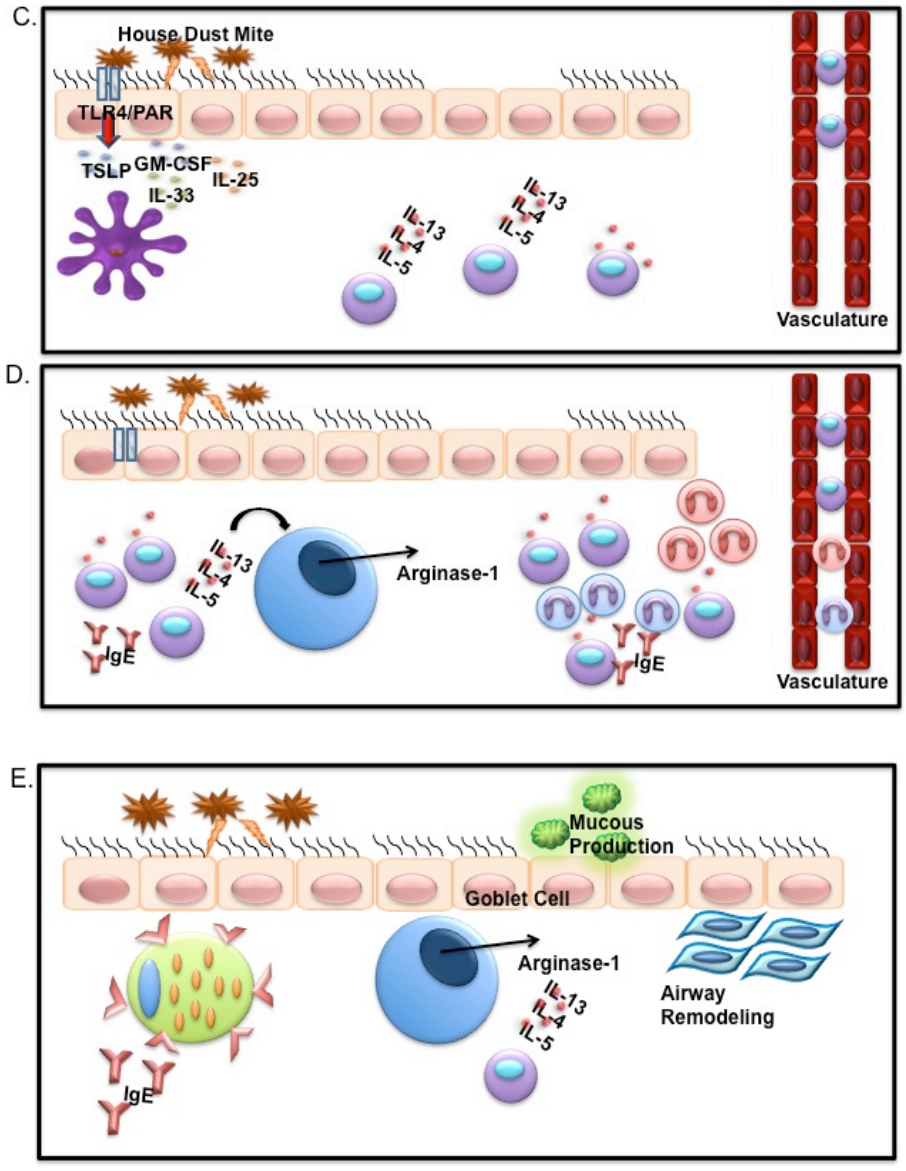
[9.2] Immune response to house dust mite

It has been shown in murine models that intranasal delivery of HDM as a whole mite extract results in a potent inflammatory response (167). The extract contains LPS in addition to the HDM antigens Der p 1,3,5, and 9 of which are cysteine and serine proteases (160,164,165,168). These proteases function via stimulation of airway epithelial cells, signaling via TLR4 (168,169) and protease activated receptors (PAR) that then secrete a number of cytokines, including GM-CSF, TSLP, IL-33, and IL-25 (130,151,160,170). Repeated administration and cytokine secretion results in the recruitment and maturation of dendritic cells, which polarize HDM-specific T cells to a Th2 phenotype characterized by the secretion of IL-4, 5, 13 (130,170,171,172).

These T cells then promote the production of HDM-specific IgE and IgG1 as well as home back to the site of allergen exposure in the lungs (167,170,173,174). IL-5 production results primarily in the recruitment of eosinophils into the airway, where as IL-4, and 13 result in the activation of macrophages to produce arginase (67,68). The production of arginase leads to the downstream vascularization, production of collagen, and airway remodeling by myofiberblasts (67,68). In addition, the chronic exposure of the airway epithelium to the damaging proteases found in the HDM extract results in increased goblet cell hyperplasia characterized by enhanced mucus production (167). The enhanced inflammation and contractibility of the airway smooth muscle results in increased airway hypersensitivity, and ultimately reduced lung function (167). Although upon cessation of HDM, inflammation is eventually resolved, the airway remodeling caused by the HDM exposure is irreversible (167). Therefore, this model is a useful tool

for the study of allergic asthma in a variety of different contexts. The immune response to house dust mite resulting in the hallmarks of allergic asthma is illustrated in Figure 7.




C.
House Dust Mite
TLR4/PAR
TSLP
GM-CSF
IL-25
IL-33
IL-13
IL-4
IL-5
Vasculature
D.
IL-13
IL-4
IL-5
Arginase-1
IgE
IgE
Vasculature
E.
Mucous Production
Goblet Cell
Arginase-1
IL-13
IL-4
IL-5
Airway Remodeling
IgE

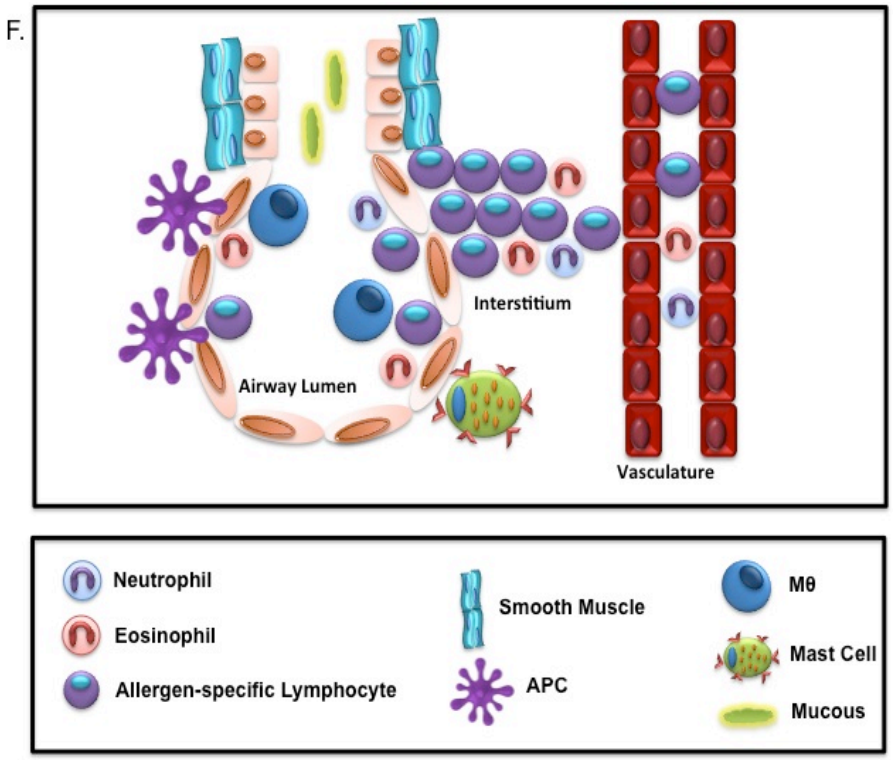


Figure 7: The immune response to chronic aeroallergen exposure. Initiation of the immune response to chronic house dust mite (HDM) exposure begins with the interaction of house dust mite with the respiratory epithelium. The airway epithelium detects various components of HDM primarily via TLR4 and PARs (protease activated receptors). This results in the secretion of the cytokines TSLP, GM-CSF, IL-33, and IL-25 **(A)**. The secretion of these cytokines results in the recruitment and maturation of dendritic cells which uptake HDM components and traffic to the local draining lymph nodes **(B)**. In the local draining lymph nodes, antigen-specific T cells are primed and polarized towards a predominant Th2 phenotype. These Th2 cells then migrate to the respiratory epithelium where they mediate their effector response through the secretion of IL-4, 5, and 13 **(C)**. The production of these cytokines results in the recruitment of IgE secreting B cells, alternative activation of macrophage populations to produce arginase-1, and the maturation and recruitment of both eosinophils and neutrophils **(D)**. Chronic exposure of HDM, and the resulting continued inflammation results in the hallmarks of chronic allergic asthma. IgE production results in mast cell activation, and the continuous production of Th2 cytokines, arginase-1 production, and epithelial cell damage results in increased mucous production, and smooth muscle activation and airway remodeling **(E)**. Collectively, the continuous exposure to HDM results in both the Th2 dominated allergic inflammation combined with the chronic effects of such inflammation denoted by goblet cell hyperplasia and airway remodeling leading to the clinical manifestation of allergic asthma **(F)**.

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[10.0] Central hypothesis and objectives:

[10.1] Central hypothesis: T cell geography is critical to host defense against mycobacterial infection. Therefore, the longer the delay in T cell arrival to the airway lumen following infection, the greater the delay in bacterial control. Environmental exposure of the respiratory mucosa to immune modulatory agents will result in altered host immune response, and subsequent bacterial control following pulmonary mycobacterial challenge.

[10.2] Objectives:

[1] To determine the T cell distribution following BCG parenteral immunization, and whether exposure to the respiratory mucosa to immune modulatory agents can manipulate T cell distribution to enhance the protective efficacy of BCG.

[2] To investigate the impact of respiratory mucosal exposure to allergens on host anti-mycobacterial control.

[3] To explore the mechanisms by which exposing the respiratory mucosa to cigarette smoke alters the host immune response to mycobacterial infection, and impairs pulmonary bacterial control.

CHAPTER 2: Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: A critical role of airway luminal T cells

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Published in *Mucosal Immunology*. 2012, 5(4): 420-431

Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: A critical role of airway luminal T cells

In this publication, we sought to address the mechanisms by which parenterally BCG immunized hosts have delayed T cell responses and bacterial control following pulmonary *M.tb* infection. In addition, we aimed to improve the efficacy of the BCG through the manipulation of T cell geography. For the first time, our study demonstrated that the lack of early immune protection following *M.*tb infection in parenterally BCG immunized hosts, is the result of delayed Ag-specific T cell recruitment into the airway lumen. We then redistributed BCG-primed T cells into the airway lumen via the intranasal administration of soluble *M.tb* culture filtrate proteins which resulted in robust early T cell responses in addition to improved protection against pulmonary *M.tb* infection.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

ARTICLES

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Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: A critical role of airway luminal T cells

CN Horvath[1,2], CR Shaler[1,2], M Jeyanathan[1,2], A Zganiacz[1,2] and Z Xing[1,2]

The immune mechanisms underlying unsatisfactory pulmonary mucosal protection by parenteral Bacillus Calmette–Guérin (BCG) immunization remain poorly understood. We found that parenteral BCG immunization failed to elicit airway luminal T cells (ALT) whereas it induced significant T cells in the lung interstitium. After *Mycobacterium tuberculosis* (*M.tb*) challenge, ALT remained missing for 10 days. The lack of ALT correlated with lack of lung protection for 14 days post-*M.tb* challenge. To further investigate the role of ALT, ALT were elicited in BCG-immunized animals by intranasal inoculation of *M.tb* culture-filtrate (CF) proteins. Installment of ALT by CF restored protection in the early phases of *M.tb* infection, which was linked to rapid increases in ALT, but not in lung interstitial T cells. Also, adoptive transfer of T cells to the airway lumen of BCG-immunized animals also accelerated protection. This study thus provides novel evidence that unsatisfactory lung protection by parenteral BCG immunization is due to delayed ALT recruitment after pulmonary *M.tb* exposure.

INTRODUCTION

An estimated two billion people worldwide are currently latently infected with *Mycobacterium tuberculosis (M.tb)* and there are approximately 9.3 million new cases of active tuberculosis (TB) and 1.7 million deaths each year.[1] The Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is the only approved vaccine against TB and has been administered parenterally via the skin shortly after birth throughout TB endemic areas for nearly a century.[2] However, while such parenteral BCG vaccination strategy is effective in preventing severe disseminated forms of childhood TB, it has failed to control pulmonary TB, the major cause of the current global TB burden.[3–6] Despite some progress made in the understanding of the variable efficacy of the BCG,[7] the precise immune mechanisms underlying the limited capacity of parenteral BCG immunization to protect against TB locally in the lung still remain poorly understood. As parenteral BCG vaccination will continue to be implemented in the human immunization program, enhanced knowledge in this regard will be critical to developing the most effective boosting strategies to enhance the protective immunity in the lung by parenteral BCG priming vaccination.

T-cell-mediated immunity is known to be critical to anti-TB host defense.[8] As primary TB infection occurs at the respiratory mucosa and it is increasingly recognized that the immunity present at the mucosal site of pathogen entry determines the level of protection,[9,10] an increased effort is directed towards understanding the kinetics of T-cell recruitment to the lung following pulmonary mycobacterial challenge in naïve or parenterally BCG-vaccinated animals and its relationship with lung protection.[8,11,12] It has been found that significant T-cell recruitment into the lung of non-vaccinated hosts does not occur until approximately 20 days post-pulmonary myco bacterial challenge.[13–16] By comparison, a limited number of studies have examined lung T-cell recruitment in parenterally BCG-vaccinated animals and found that vaccination accelerated T-cell recruitment to the lung only by 4–5 days after pulmonary mycobacterial challenge.[15,17] The general consensus is that although increased lung recruitment of T cells precedes enhanced lung protection, the lung, whether or not with parenteral BCG

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Received 29 August 2011; accepted 26 February 2012; published online 28 March 2012. doi:10.1038/mi.2012.19

vaccination, is still left unprotected in the first 14–20 days after pulmonary mycobacterial challenge.[12,18] However, of note, all of the studies to date have examined anti-mycobacterial T-cell responses only in the context of the whole lung and it is unclear whether such T cells are limited to a specific lung compartment. Recent studies on genetic TB vaccines have begun to divide the lung into the two critical tissue compartments—the lung interstitium and airway lumen, when considering T-cell distribution and lung protection.[9,12,19] A close examination of published studies has revealed that the presence of T cells in the lung interstitium is not a reliable indicator of lung protection against pulmonary *M.tb* challenge, whereas those present in the airway luminal space are a more accurate determinant of lung protection.[12,20–22] Thus, airway luminal T cells (ALT) are believed to play a critical role in lung mucosal immunity against *M.tb* exposure[12,23,24] and viral infection.[25] However, it still remains largely unclear whether antigen (Ag)-specific T cells populate the airway lumen following parenteral BCG vaccination, how quickly Ag-specific T cells are recruited into the airway lumen after pulmonary *M.tb* challenge, and what is the relationship between ALT and lung protection in parenteral BCG-vaccinated hosts. Such knowledge will be critical not only to understanding the immune mechanisms of unsatisfactory lung protection by parenteral BCG vaccination but also to developing the effective boosting strategies to enhance protective immunity in the lung by BCG in humans.

In the present study, we have used a murine model of parenteral BCG vaccination to investigate whether parenteral BCG-primed CD4 T cells populate the airway lumen and how quickly these T cells can be mobilized into the airway lumen upon pulmonary *M.tb* challenge. Also, we have investigated the role of the ALT in lung protection against pulmonary *M.tb* infection in these hosts.

RESULTS

Lack of ALT in parenteral BCG-immunized hosts

The geographical distribution of T cells plays an important role in mucosal protection from intracellular infections, including TB.[9,12,26] To examine the tissue distribution of antigen-specific T cells, particularly CD4 T cells, following parenteral BCG immunization, we immunized mice subcutaneously with BCG and examined Ag-specific $CD4^+IFN\text{-}\gamma^+$ (interferon-γ) T-cell populations residing in the airway lumen (bronchoalveolar lavage (BAL)), lung interstitium, and spleen 9 weeks post vaccination. There were hardly any detectable Ag-specific CD4 T cells in the airway lumen (BAL; **Figure 1a**). By contrast, substantial levels of such T cells were seen in the lung interstitium (lung) and spleen (**Figure 1a**). These data suggest that parenteral BCG immunization elicits T-cell responses localized to the peripheral tissue sites but not to the airway luminal compartment.

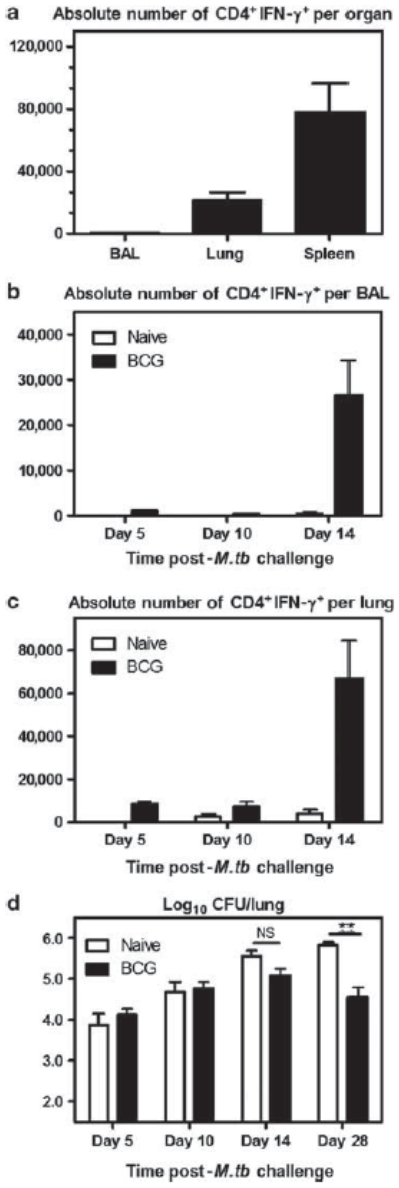
Delayed lung protection and its association with failure of pulmonary *M.tb* challenge to elicit ALT for up to 10 days in parenteral BCG-immunized hosts

As the airway lumen was virtually devoid of antigen-specific T cells following parenteral BCG immunization, we next examined whether and when pulmonary *M.tb* challenge would be able to elicit Ag-specific airway luminal CD4 T cells (ALT) in parenteral BCG-immunized animals and compared these with the responses in *M.tb* $H_{37}Ra$ challenged non-immunized hosts. We found that up to 14 days post challenge there was a lack of ALT in non-immunized animals (naïve group) (**Figure 1b**). In comparison, there was still a lack of ALT for at least 10 days post challenge in BCG-immunized animals (BCG group) although the number of ALT was markedly increased by 14 days (**Figure 1b**). In the lung interstitium of non-immunized animals, the levels of Ag-specific T cells were very small for at least 14 days post challenge (**Figure 1c**). In comparison, the levels of such T cells did not markedly rise above BCG-elicited levels until 14 days post challenge (**Figure 1c**). Thus, after pulmonary *M.tb* challenge, the overall kinetics of T-cell responses in the lung interstitium of naive or parenteral BCG-immunized animals (**Figure 1c**) mirror those in the airway lumen (**Figure 1b**). To examine the relationship between lung T-cell responses and protection, we examined the level of lung immune protection at various time points post-mycobacterial challenge in naive and BCG-immunized animals. We observed that in spite of the presence of Ag-specific CD4 T cells in the lung interstitium at early times (**Figure 1c**), the bacterial burden remained comparable in the lungs of naive and BCG-immunized mice for at least 14 days post infection and a marked reduction was not seen until day 28 in BCG-immunized mice (**Figure 1d**). These results together suggest that (1) parenteral BCG immunization fails to confer any protection in the lung for at least 14 days post challenge; (2), this delayed lung protection is correlated with a complete lack of ALT for up to 10 days of time post challenge in BCG-immunized hosts; and (3) the significant airway luminal T-cell responses (day 14) need to occur before *M.tb* infection can be markedly reduced in the lung.

Selection of crude *M.tb* antigenic preparations for recruiting parenteral BCG-primed T cells into the airway lumen

As parenteral BCG immunization fails to elicit ALT and there is yet still a much delayed appearance of ALT after pulmonary mycobacterial exposure, which is closely linked to the delayed lung protection (**Figure 1a–d**), we next explored the ways to elicit the ALT in parenteral BCG-immunized mice and compared the efficacies of intranasal (i.n.) inoculation of soluble *M.tb* antigenic preparations, including recombinant Ag85A (rAg85a), Ag85 complex proteins, and *M.tb* culture filtrate (CF). To this end, parenteral BCG-immunized mice were exposed to two doses (1 week apart) of selected soluble *M.tb* Ag and the ability of each Ag to recruit $CD4^+IFN\text{-}\gamma^+$ T cells into the airway lumen (BAL) and lung interstitium was assessed 4 days later (**Figure 2a**). Although administration of rAg85a (BCG-rAg85a) or Ag85 complex protein (BCG-Ag85 complex) had only a modest effect on eliciting ALT (**Figure 2b**), exposure to *M.tb* CF (BCG-immunized/CF-treated (BCG/CF)) exerted a significant increase of ALT ($P \leq 0.005$; **Figure 2b**). Similarly, rAg85a or Ag85 complex protein hardly increased the level of lung interstitial T cells above that by parenteral BCG immunization alone (**Figure 2c**). By contrast, the delivery of CF proteins

significantly increased the number of T cells in the lung interstitium ($P \leq 0.05$; **Figure 2c**). CF administrations to naive non-immunized animals did not induce any detectable Ag-specific T-cell responses in any tissue compartment, indicating that CF on its own is incapable of T-cell priming (data not shown). As CF delivery was the most effective in eliciting ALT of several tested *M.tb* antigenic preparations, we carried out histological examination of lung sections and found that compared with BCG, BCG-rAg85a, and BCG-Ag85 complex lungs, BCG/CF lungs had only a mildly increased cellular infiltration around the airway, in keeping with significantly increased ALT (**Supplementary Figure S1A–D** online). In addition, the levels of the pro-inflammatory cytokine tumor necrosis factor-α measured in the BAL fluids were also comparable among all the four treatment groups (**Supplementary Figure S1E** online). Taken together, these data suggest that respiratory mucosal inoculation of soluble CF proteins, but not other mycobacterial Ag preparations, represents an effective and safe way to elicit ALT, which is otherwise missing in parenteral BCG-immunized hosts.



Recruitment of parenteral BCG-primed T cells into the airway lumen by respiratory mucosal inoculation of *M.tb* CF proteins

Having identified the i.n. delivery of CF to be the most effective way to elicit ALT in parenteral BCG-immunized animals, we further evaluated its effects in various regimens. First, mice were immunized subcutaneously. with BCG and left for 4 weeks before they were inoculated i.n. with CF, and the Ag-specific CD4 T-cell responses in the airway lumen (BAL) and lung interstitium were examined 2 weeks after the second CF delivery (**Figure 3a**). Consistent with the data from the previous experiments (**Figures 1** and **2**), there was a lack of ALT in the lung of parenteral BCG-immunized mice without receiving CF (BCG group; **Figure 3b**). However, there were a great number of ALT induced by CF treatment ($P \leq 0.005$; BCG/CF group) (**Figure 3b**). On the other hand, as shown in the previous experiments (**Figures 1** and **2**), parenteral BCG immunization alone elicited a significant level of T cells in the lung interstitium and this level was further increased by i.n. CF delivery ($P \leq 0.0005$; **Figure 3c**). Based on these findings, we next examined the effect of CF delivery on ALT in a varied immunization regimen whereby the interval between BCG immunization and CF treatment was prolonged from 4 weeks (**Figure 3a**) to 8 weeks (**Figure 3d**). We observed that even with an interval of 8 weeks, i.n. CF treatment elicited a significant level of ALT in contrast to a complete lack of ALT in the lung of mice with BCG immunization alone ($P \leq 0.005$; **Figure 3e**). Similarly, the initially BCG-elevated levels of lung interstitial T cells were further enhanced by CF treatment ($P \leq 0.0005$; **Figure 3f**).

Figure 1 Lack of airway luminal antigen (Ag)-specific T cells in parenteral Bacillus Calmette–Guérin (BCG)-immunized animals before and after pulmonary mycobacterial challenge. (**a**) Mice were killed 9 weeks following BCG immunization and Ag-specific T cells in the bronchoalveolar lavage (BAL), lung interstitium, and spleen were examined. (**b–d**) Naïve non-immunized and BCG-immunized mice were challenged with *Mycobacterium tuberculosis* (*M.tb*) 9 weeks after BCG immunization, and killed at 5, 10, and 14 days post infection for T-cell analysis or at 5, 10, 14, and 28 days post infection for assessing the level of protection (infection) in the lung. Ag-specific T-cell responses were analyzed in the BAL (**b**) and lung interstitium (**c**). Lungs from infected mice were assessed for the level of protection (infection) at various time points post challenge (**d**). Data are expressed as mean values±s.e.m. of 4–5 mice/time/group, representative of 2–3 independent experiments. $^{**}P \leq 0.005$ compared with the indicated groups. IFN, interferon; CFU, colony-forming units.

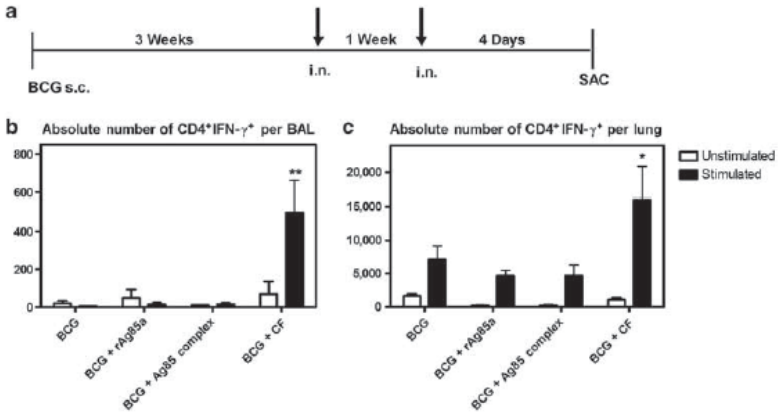


Figure 2 Selection of crude *Mycobacterium tuberculosis* (*M.tb*) antigenic preparations for recruiting parenteral Bacillus Calmette–Guérin (BCG)-primed T cells into the airway lumen. (**a**) Experimental schema. (**b**, **c**) Four days after the last intranasal (i.n.) delivery of a selected *M.tb* antigenic preparation (recombinant antigen (rAg)85A protein, Ag85 complex proteins, or *M.tb* culture-filtrate (CF) proteins), Ag-specific T cells were analyzed in the bronchoalveolar lavage (BAL) (**b**) and lung interstitium (**c**). Data are expressed as mean values±s.e.m. of 3–4 mice per group. $^{*}P \leq 0.05$, $^{**}P \leq 0.005$ compared with all the other groups. IFN, interferon; SAC, sacrifice; s.c., subcutaneously.

We next addressed whether the ALT elicited by CF delivery to parenteral BCG-immunized hosts according to the regimen depicted in **Figure 3a** could persist beyond 2 weeks of time. Thus, mice were set up as in **Figure 3a** except that the Ag-specific CD4 T-cell responses in the airway lumen (BAL) and lung interstitium were examined 4 weeks after the second CF delivery (**Figure 4a**). Indeed, compared with the BCG immunization alone group, we detected sharply raised levels of ALT in CF-treated BCG-immunized animals ($P \leq 0.05$; **Figure 4b**). By comparison, the number of lung interstitial T cells in the BCG/CF group did not statistically significantly rise above that in the BCG immunization alone group (**Figure 4c**). The above data together suggest that as long as parenteral BCG-primed T cells are present within the periphery, they can be effectively mobilized into the airway lumen by airway CF exposure.

Activated effector memory phenotype of ALT elicited by CF in parenteral BCG-immunized hosts

We next evaluated the immune phenotype and property of ALT elicited by CF in parenteral BCG-immunized hosts. To characterize the activation–memory status of these ALT, we gated on CD4+ and CD44+ T cells and then analyzed the expression of surface markers CD62 L and CD127 on these cells. These surface markers are commonly used for analysis of T-cell phenotypes in the lung.[25,26] Most of the ALT were found to be CD62 L-CD127 – or CD127 + T cells (**Figure 5a**), suggesting an effector memory and recently activated effector memory phenotype. The T cells in the lung interstitium assumed predominantly an effector memory phenotype, being mostly CD62 L-CD127 + (**Figure 5b**). These data indicate that the ALT elicited by CF treatment in parenteral BCG-immunized hosts are primarily of an activated effector memory phenotype.

The installment of ALT by CF delivery is associated with accelerated and enhanced protection against pulmonary *M.tb* challenge in parenteral BCG-immunized hosts

To examine whether the reinstated ALT in CF-treated parenteral BCG-immunized animals would confer lung protection that was otherwise missing for up to 14 days post-pulmonary mycobacterial challenge in BCG-immunized hosts (**Figure 1d**), naïve non-immunized (naive), BCG-immunized (BCG), and BCG/CF mice were challenged via the airway with virulent *M.tb* $H_{37}Rv$ 4 weeks after the last CF administration and the level of lung protection was assessed at 2 weeks post challenge (**Figure 6a**). Despite the significant presence of Ag-specific T cells within the lung interstitium of BCG-immunized animals at the time of challenge and consistent with the data in **Figure 1d**, the lungs of BCG mice were not any better protected than the naive controls (**Figure 6b**). However, a significantly enhanced level of protection was seen in the lung of BCG/CF mice ($P \leq 0.005$; **Figure 6b**). This suggests that the installment of ALT at the time of pulmonary *M.tb* exposure is associated with accelerated lung protection that is completely lacking in the early phases of pulmonary *M.tb* infection in parenteral BCG-immunized hosts.

We next went on to address whether this accelerated lung protection in the early phases of *M.tb* infection seen in BCG/CF animals would continue into the later point of infection. To this end, mice were set up as in **Figure 6b** except that the level of lung protection was assessed at 4 weeks post-*M.tb* challenge (**Figure 6a**). As expected and also in keeping with the data in **Figure 1d**, parenteral BCG immunization alone by this time conferred a significantly enhanced level of lung protection compared to naive controls ($P \leq 0.005$; **Figure 6c**). However, the level of lung protection in BCG/CF animals was significantly further enhanced ($P \leq 0.05$) over the BCG group (**Figure 6c**).

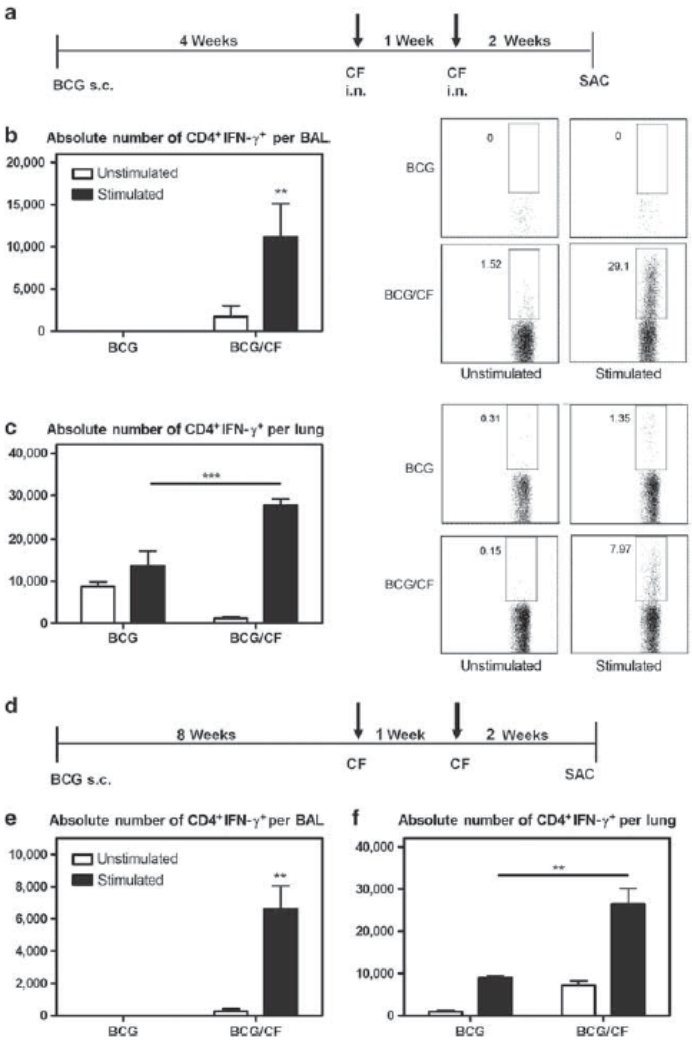


Figure 3 Recruitment of parenteral Bacillus Calmette–Guérin (BCG)-primed T cells into the airway lumen by respiratory mucosal inoculation of crude non-formulated *Mycobacterium tuberculosis* culture-filtrate (CF) proteins. (**a**, **d**) Experimental schema. (**b**, **c**) The absolute numbers and representative dot plots of antigen (Ag)-specific T cells in the bronchoalveolar lavage (BAL; **b**) and lung interstitium (**c**) analyzed 2 weeks after the second intranasal (i.n.) CF in the mice that received the first CF from 4 weeks post-BCG immunization. (**e**, **f**) The absolute numbers and representative dot plots of Ag-specific T cells in the BAL (**e**) and lung interstitium (**f**) analyzed 2 weeks after the second i.n. CF in the mice that received the first CF from 8 weeks post-BCG immunization. Data are expressed as mean values±s.e.m. of 3–4 mice per group, representative of two independent experiments. $^{**}P \leq 0.005$, $^{***}P \leq 0.0005$ compared with the indicated groups. IFN, interferon; SAC, sacrifice; s.c., subcutaneously.

There were also correspondingly decreased levels of bacterial burden in the spleen (data not shown). With regard to improved lung protection, the BCG/CF group had much improved lung histopathology compared with the non-immunized naive or BCG immunization groups (**Figure 6e** and **f**). The above findings together suggest that airway CF delivery and ALT installment in parenteral BCG-immunized animals accelerate and enhance lung protection against pulmonary *M.tb* infection.

Highly reactive secondary airway luminal T-cell responses to pulmonary *M.tb.* challenge in parenteral BCG/CF animals

To further understand the mechanisms by which airway CF treatment enhances lung protection in parenteral BCG-immunized hosts, we examined the kinetics of secondary Ag-specific CD4 T-cell responses in the airway lumen following pulmonary mycobacterial challenge. To this end, the mice were set up similarly as depicted in **Figure 6a** except that T-cell

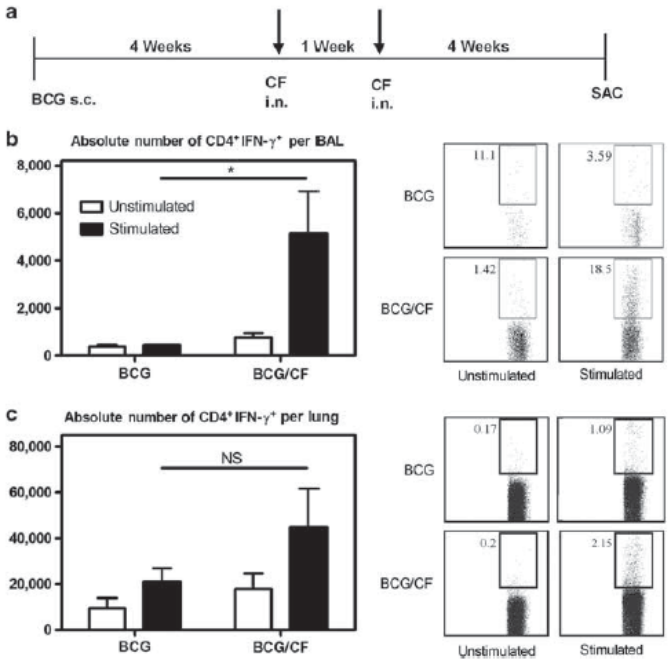


Figure 4 Retained airway luminal T cells by respiratory mucosal inoculation of crude non-formulated *Mycobacterium tuberculosis* culture-filtrate (CF) proteins in parenteral Bacillus Calmette–Guérin (BCG)-immunized animals. (**a**) Experimental schema. (**b**, **c**) The absolute numbers and representative dot plots of antigen (Ag)-specific T cells in the bronchoalveolar lavage (BAL; **b**) and lung interstitium (**c**) analyzed 4 weeks after the second intranasal (i.n.) CF in the mice that received the first CF from 4 weeks post-BCG immunization. Data are expressed as mean values±s.e.m. of 4–5 mice per group, representative of four independent experiments. *$P \leq 0.05$ compared with the indicated group. IFN, interferon; NS, not statistically significant; SAC, sacrifice; s.c., subcutaneously.

responses were assessed at 0, 5, 10, and 14 days post-*M.tb* $H_{37}Ra$ challenge (**Figure 7a**). In keeping with the data presented in **Figure 1b**, there was hardly any detectable ALT in BCG-immunized animals until 14 days post-*M.tb* challenge (**Figure 7b**). In sharp contrast, a raised level of ALT (CD4 T cells) was already present before *M.tb* exposure in BCG-immunized–CF-treated animals (day 0; **Figure 7b**) as seen in previous experiments (**Figure 4b**), and subsequent pulmonary *M.tb* exposure quickly further increased the levels of ALT several times above the base level at days 5, 10, and 14 (**Figure 7b**). On the other hand, in keeping with the data in **Figure 1a–c**, parenteral BCG immunization alone elicited a significant T-cell population in the lung interstitium before pulmonary mycobacterial challenge (day 0), and this population did not increase in size until day 14 (**Figure 7c**; BCG group). In comparison, although BCG/CF animals had a larger T-cell population in the lung interstitium before pulmonary mycobacterial challenge (day 0), it remains unchanged between day 5 and day 14 post-mycobacterial challenge (**Figure 7c**; BCG/CF group), contrasting the highly responsive ALT in these mice (**Figure 7b**). Similarly, increased Ag-specific CD8 T cells, albeit in smaller numbers than CD4 T cells, were also seen at various time points in the BCG/CF group following *M.tb* challenge (**Table 1**). Thus, the markedly increased secondary T-cell responses soon after pulmonary *M.tb* exposure in the airway lumen of the BCG immunized/CF-treated animals group were associated with a significantly increased level of lung protection at day 14 (**Figure 6b** and data not shown). These findings suggest that airway CF treatment accelerates and enhances lung protection in parenteral BCG-immunized hosts by installing highly mycobacterium-reactive T cells in the airway lumen in the early stages of pulmonary *M.tb* infection, which are otherwise absent in parenteral BCG animals if untreated with CF.

Adoptive transfer of Ag-specific T cells into the airway lumen of parenteral BCG-immunized mice restores lung protection in the early phases of pulmonary *M.tb* infection

Thus far, we have found that prior respiratory mucosal CF delivery to parenteral BCG-immunized animals accelerates lung protection in the early phases of pulmonary *M.tb* infection (**Figure 6a**) and this was associated closely with dramatically increased responses of ALT (**Figure 7b**), but not with lung interstitial T cells (**Figure 7c**). To further investigate the role of ALT in lung protection in the early phase of pulmonary *M.tb*

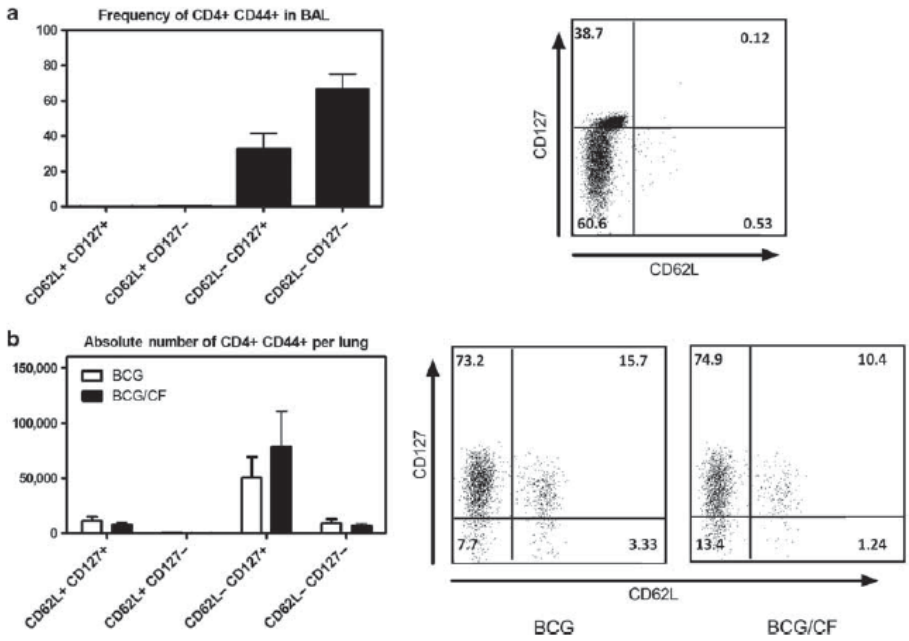


Figure 5 Activated effector memory phenotype of airway luminal T cells elicited by *Mycobacterium tuberculosis* culture filtrate (CF) in parenteral Bacillus Calmette–Guérin (BCG)-immunized animals. The mice were immunized with BCG for 4 weeks, intranasally inoculated with CF and killed 2 weeks after the second CF as depicted in **Figure 3a**. The immune phenotype of antigen-specific T cells in the bronchoalveolar lavage (BAL; **a**) and lung interstitium (**b**) were analyzed. Data are expressed as mean values±s.e.m. or representative dot plots of 4–5 mice per group, representative of three independent experiments.

infection, we addressed whether adoptive transfer of Ag-specific CD4 T cells to the airway lumen of parenteral BCG-immunized animals (without CF treatment) would accelerate lung protection in the early phase of pulmonary *M.tb* infection, similarly as did the airway CF delivery. This approach would allow us to specifically examine the role of ALT in early lung protection without altering or increasing the levels of lung interstitial T cells as we have previously shown that the T cells adoptively transferred to the airway lumen do not transmigrate to the other sites, including the lung interstitium.[26] To this end, mice were immunized subcutaneously with BCG for 9 weeks and adoptively transferred intratracheally with purified T cells isolated from the lungs of BCG/CF mice (BCG-AT (BCG/CF) group) or from the lungs of naive mice as a control (BCG-AT (naïve) group). Additional control groups were naive non-immunized (naive), parenteral BCG alone (BCG), and parenteral BCG/CF–treated (BCG/CF) mice. The mice were then challenged via the airway with virulent *M.tb* H_{37}Rv on the following day (16 h) after T-cell transfer and killed 2 weeks post challenge for assessment of early lung protection (**Figure 8a**). As shown in **Figures 1d** and **6b**, there was a lack of lung protection by day 14 post-*M.tb* challenge in parenteral BCG-immunized mice (naïve vs. BCG groups) (**Figure 8b**). However, the BCG mice receiving adoptively transferred Ag-primed T cells isolated from BCG/CF mice (BCG-AT (BCG/CF) group) demonstrated a significantly enhanced level of lung protection ($P \leq 0.0005$; **Figure 8b**), which was comparable with that accomplished in BCG/CF animals (**Figure 8b**). By contrast, the BCG mice receiving adoptively transferred unprimed T cells isolated from naive mice (BCG-AT (naïve) group) did not have enhanced lung protection (**Figure 8b**). Collectively, these data further indicate that the Ag-specific ALT is a critical determinant of lung protection in the early phases of pulmonary *M.tb* infection.

DISCUSSION

To date, the precise immune mechanisms underlying the unsatisfactory protection against pulmonary TB in BCG-vaccinated individuals remains poorly understood. Our current study reveals that parenteral BCG immunization fails to elicit the ALT although a substantial population of Ag-specific T cells can be detected within the lung interstitium. The absence of ALT in BCG-immunized animals persists for a significant period of time (at least 10 days) even after pulmonary *M.tb* challenge and this is well correlated with the complete lack of immune protection in the early phase of pulmonary mycobacterial infection in spite of the presence of lung interstitial T cells. Thus, by recruiting a population of parenterally BCG-primed T cells into the airway lumen via respiratory mucosal delivery of crude unformulated or unadjuvanted mycobacterial proteins (CF), the lung is granted a significantly enhanced level

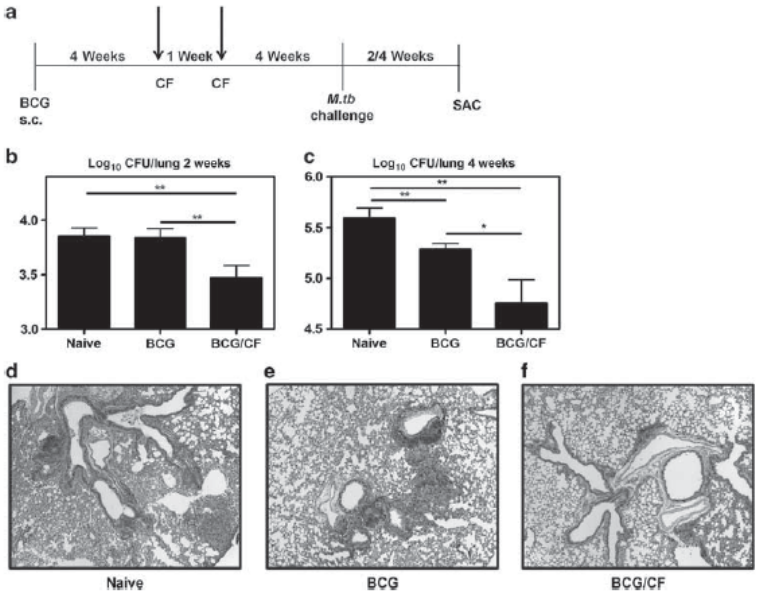


Figure 6 Accelerated and enhanced immune protection from pulmonary *Mycobacterium tuberculosis* (*M.tb*) challenge by culture filtrate (CF)–mediated installation of airway luminal T cells in parenteral Bacillus Calmette–Guérin (BCG)-immunized hosts. (**a**) Experimental schema. (**b**, **c**) The levels of immune protection in the lung (bacterial colony-forming units (CFU) burden) were assessed either 2 weeks (**b**) or 4 weeks (**c**) post-*M.tb* challenge. Data are expressed as mean values±s.e.m. of 5–6 mice per group, representative of two independent experiments. (**d–f**) The extent of lung immunohistopathology was assessed at 4 weeks post-*M.tb* challenge in naïve non-immunized (**d**), parenteral BCG immunization alone (**e**) and parenteral BCG-immunized/CF–treated (**f**) mice. Each hematoxylin and eosin –stained histomicrograph is representative of 5–6 mice per group (×4 magnification). $^{*}P \leq 0.05$, $^{**}P \leq 0.005$ compared with the indicated groups. SAC, sacrifice; s.c., subcutaneously.

of immune protection in the early phases of pulmonary *M.tb* infection. Different from lung interstitial T cells, these initially implanted ALT by mucosal CF delivery are able to undergo a rapid and robust expansion upon mycobacterial exposure. We also demonstrated that the mere equipment of ALT by adoptive airway T-cell transfer, without a change to the size of lung interstitial T-cell population, in parenteral BCG-immunized animals, is able to restore early lung immune protection as effectively as airway CF delivery. These findings together provide new insights into the immune mechanisms behind the delayed and unsatisfactory protection locally in the lung by parenteral BCG immunization, and point to the importance of Ag-specific T cells present on the surface of respiratory mucosa in immune protection in the early phases of pulmonary *M.tb* infection.

It has been long believed that the magnitude of immune protection against a mucosal infection is closely associated with the level of immune effectors present at that mucosal site.[9,10,25] This concept has increasingly been applied to understanding anti-TB immunity and vaccination.[8,11,12,18] However, although a limited number of studies have begun to address the relationship between the level of immune protection and Ag-specific CD4 T cells in the lung of naïve [13–16] or parenteral BCG-immunized animals,[15,17,27] these studies were carried out only by taking the lung as a single entity. In other words, it remains unclear whether the T-cell responses examined in these studies were localized to the lung interstitium, airway lumen, or both. Recent studies on parenteral and mucosal genetic TB vaccination have suggested the importance of dissecting anti-TB T-cell responses separately on the surface of respiratory mucosa (ALT) and in the lung interstitium. This is because different from the ALT, the mere presence of such T cells within the lung interstitium may not well inform about the level of anti-TB protection in the lung.[12,20–22] In addition to genetic vaccines, it is believed that the ALT also hold the key to superior lung protection by respiratory mucosal immunization with other forms of TB vaccines.[23,24,28] Notably, different from parenteral genetic TB vaccination, parenteral BCG immunization does provide a significant level of lung protection in the later stage of pulmonary *M.tb* infection as shown in the current study in addition to many other studies. However, we have found that parenteral BCG immunization is unable to protect the lung in the early phases of pulmonary *M.tb* infection (up to at least 14 days) in spite of a significant presence of lung interstitial Ag-specific CD4 T cells. The delayed immune protection in the lung of these animals coincides with the lack of ALT, whereas the increased protection in later

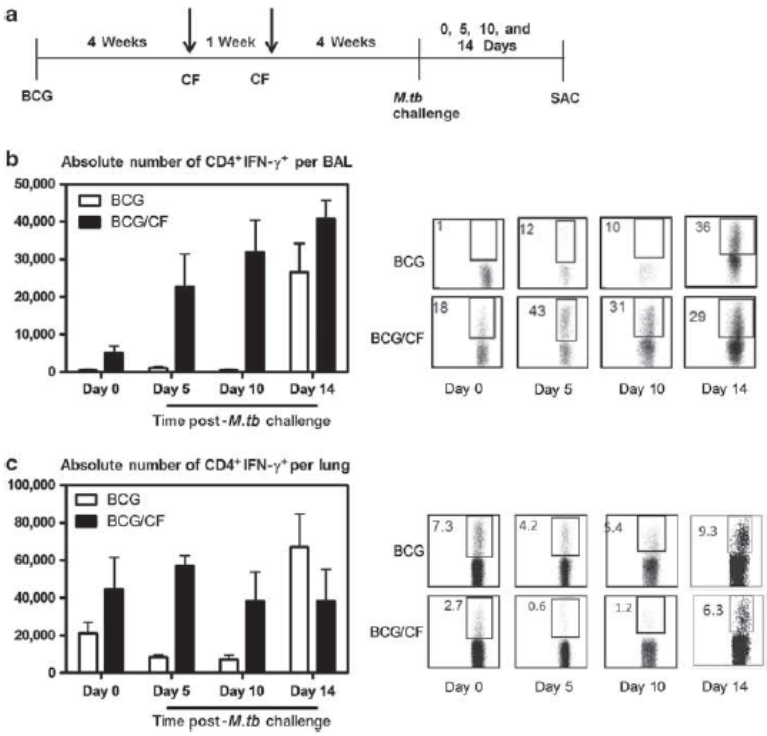


Figure 7 Highly reactive secondary airway luminal T-cell responses to pulmonary *Mycobacterium tuberculosis* (*M.tb*) challenge in parenteral Bacillus Calmette–Guérin (BCG)-immunized/CF (culture filtrate)–treated hosts. (**a**) Experimental schema. (**b**, **c**) The absolute numbers and representative dot plots of antigen-specific T cells in the bronchoalveolar lavage (BAL; **b**) and lung interstitium (**c**) analyzed at various time points post-*M.tb* challenge in the mice immunized with BCG and not treated with CF (BCG) or immunized with BCG and treated with CF (BCG/CF). Data are expressed as mean values±s.e.m. of 4–5 mice per group, representative of two independent experiments. IFN, interferon; SAC, sacrifice.

Table 1 CD8⁺IFN-γ⁺ T cells in the BAL and lung following pulmonary *M.tb* infection

	BAL		Lung	
	BCG	**BCG/CF**	**BCG**	**BCG/CF**
Day 0	77.4±30.8	223.3±77.8*	4,834.5±1437.7	2,815.3±773.1
Day 5	140.9±26.3	1,792.6±499.3**	774±298	1,180±396
Day 10	42.1±19.3	1,590.1±442.4***	3,123.3±110.9	8,370±207.2
Day 14	1,512±615.7	2045±357.5	6,443.3±1438	9,316.6±4330.5

Ag, antigen; BAL, bronchoalveolar lavage; BCG, Bacillus Calmette–Guérin; CF, culture filtrate; IFN, interferon; *M.tb*, *Mycobacterium tuberculosis*.
Accelerated secondary airway luminal CD8 T cell responses to pulmonary *M.tb* $H_{37}Ra$ challenge in parenteral BCG-immunized/CF-treated hosts. The absolute numbers of Ag-specific CD8 T cells in the BAL and lung interstitium analyzed at various time points post-*M.tb* challenge in the mice immunized with BCG alone (BCG) or immunized with BCG and treated with CF (BCG/CF). Data are expressed as mean values±s.e.m. of 4–5 mice per group, representative of two independent experiments.
*$P<0.05$, **$P<0.005$, ***$P<0.0005$ compared with BCG alone counterparts.

phases of *M.tb* infection (from 4 weeks onward) in BCG-immunized hosts is preceded by a marked response of ALT (day 14) (**Figures 1b, d** and **6b, c**). The fact that significantly increased protection lags behind markedly raised ALT suggests that ALT-mediated protection takes some time to ramp up. In comparison, naïve non-immunized animals still lack ALT by day 14 after *M.tb* challenge and, thus, do not have increased protection in the lung by 4 weeks. Also, we have observed a similarly delayed lung protection from *M.tb*H37Ra or *M.tb*H37Rv challenge in BCG-immunized hosts, suggesting the delayed ALT responses to be a trait of infection by both attenuated and virulent strains of mycobacteria. These findings together suggest that although

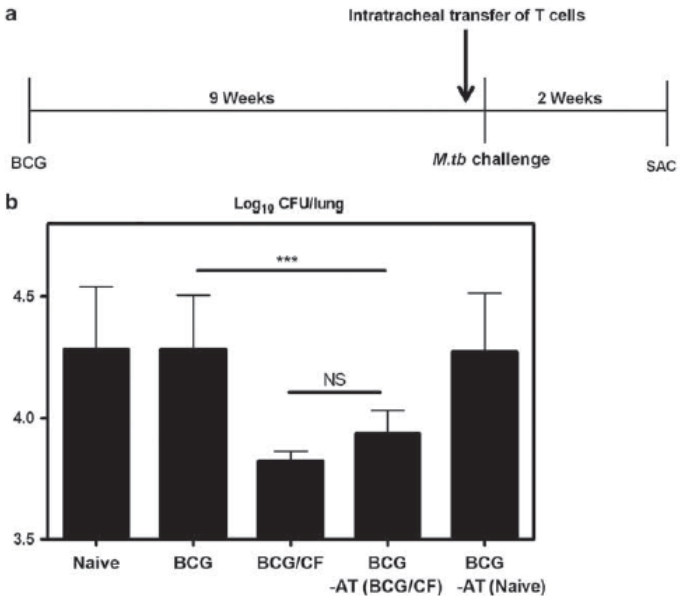


Figure 8 Adoptive transfer of antigen-specific T cells into the airway lumen of parenteral Bacillus Calmette–Guérin (BCG)-immunized mice restores lung protection in the early phase of pulmonary *Mycobacterium tuberculosis* (*M.tb*) infection. (**a**) Experimental schema. (**b**) The levels of immune protection in the lung (bacterial colony-forming units (CFU) burden) were assessed 2 weeks post-*M.tb* challenge in naïve mice, BCG-immunized mice, BCG-immunized/CF-treated mice (BCG/CF), BCG-immunized mice receiving the *in vivo* primed T cell transfer (BCG-AT (BCG/CF)), and BCG-immunized mice receiving the naive T-cell transfer (BCG-AT (naïve)) as control. Data are expressed as mean values±s.e.m. of five mice per group, representative of two independent experiments. ***$P \leq 0.0005$ compared with the indicated groups. CF, *M.tb* culture–filtrate; NS, not statistically significant; SAC, sacrifice.

compared with naive non-immunized hosts, parenteral BCG immunization allows the primed CD4 T cells to populate the lung interstitium, and upon pulmonary mycobacterial exposure, it accelerates T-cell recruitment to the airway lumen and lung interstitium by a few days, it still leaves a significant "immunological gap" where the lung is devoid of the ALT and left completely unprotected. We believe that this significant delay in engendering the ALT and lung protection in the early phases of pulmonary *M.tb* infection to be an important mechanism underlying the relative inefficacy of parenteral BCG immunization to protect from pulmonary TB in humans.

The molecular mechanisms responsible for the sluggish generation of ALT in response to pulmonary *M.tb* exposure in parenteral BCG-immunized hosts still remain to be fully understood. It, however, has been speculated that the slow-growing nature of mycobacteria and the immune-evasive property of mycobacterial infection may have a role in delayed T-cell recruitment and lung protection.[18] Regardless of which mechanisms, our current findings suggest that the future boosting strategies developed for enhancing protective efficacy of parenteral BCG immunization in humans should aim to fill up the "immunological gap" in the lung or to increase the number of Ag-specific T cells not only in the lung interstitium but also, more importantly, on the respiratory mucosal surface (airway lumen). As we have investigated in the current study, a potent way to accomplish this is to i.n. inoculate simple doses of crude non-formulated soluble mycobacterial proteins (CF), which elicits a population of ALT that is highly expandable upon pulmonary *M.tb* exposure and helps accelerate lung protection in the early phases of pulmonary *M.tb* infection. Compared with the approach of respiratory mucosal boost vaccination,[12,29] the airway delivery of CF differs in that it represents a "vaccine-less" and perhaps even safer approach, as we have shown that the CF itself is incapable of T-cell priming and that it causes little undesired lung immunopathology. The potency of CF in eliciting and retaining the ALT in parenteral BCG-immunized animals is likely attributable to its capability to engage the TLRs and that it contains the mycobacterial antigens shared by BCG vaccine. Our previous studies suggest that both pro-inflammatory signals and mycobacterial Ags are required for eliciting and retaining the ALT.[22,26]

In summary, our current study has identified an immune mechanism, which we call an "immunological gap", underlying the delayed and unsatisfactory protection in the lung of parenteral BCG-immunized hosts. We believe that such knowledge will help develop the effective mucosal boosting strategies for enhancing the protective efficacy within the lung provided by parenteral BCG immunization in humans.

METHODS

Mice. Female BALB/c mice aged 6–8 weeks were purchased from Harlan Laboratory (Indianapolis, IN). Mice were housed in a specific pathogen-free (level B) facility. All experiments were conducted in accordance with the animal research ethics board at McMaster University.

Mycobacteria. *M. bovis* BCG (Connaught), virulent *M.tb.* ($H_{37}R_V$; ATCC 27,294) and attenuated *M.tb* ($H_{37}Ra$; ATCC 25,177) were grown in Middlebrook 7H9 broth supplemented with Middlebrook oleic acid–albumin–dextrose–catalase enrichment (Invitrogen Life Technologies, Carlsbad, CA), 0.002% glycerol, and 0.05% Tween 80 for <10–15 days, then aliquoted and stored in −70 °C until needed.[20,30,31] Before each use, *M.tb* bacilli were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 80 twice and passed through a 27-gauge needle 10 times to disperse clumps.

BCG immunization and delivery of soluble *M.tb* antigenic proteins. Mice were immunized subcutaneously with 5×10^4 colony forming units (CFU) of BCG in 100 µl of PBS. For experiments involving the administration of soluble *M.tb* CF proteins, recombinant Ag85A (rAg85A) or purified *M.tb* Ag85 complex proteins (Ag85 Complex containing purified Ag85A, B, and C proteins), 2 µg of each in 20 µl of PBS was delivered i.n. to BCG-immunized mice. All of these *M.tb* reagents were provided by the BEI Resources (Manassas, VA).

Pulmonary M.tb challenge. Mice were challenged i.n. with a dose of 0.5×10^6 CFU *M.tb*H_{37}Ra in 40 µl of PBS or with a dose of 1,000 CFU (depositing 100–200 CFU to the lung) *M.tb*H_{37}Rv in 25 µl of PBS. The mice were killed at 2 or 4 weeks following *M.tb* challenge. The use of attenuated H37Ra strain allowed us to perform detailed analyses of airway luminal T-cell responses under the P2 conditions (we cannot perform the BAL in H37Rv-infected mice in the P3 facility due to technical constraint). The level of bacterial burden in the lung and spleen was assessed at each time point by plating serial dilutions of lung homogenates in triplicate onto plates containing Middlebrook 7H10 agar plates containing Middlebrook oleic acid–albumin–dextrose–catalase enrichment. Plates were placed into semisealed plastic bags and incubated at 37 °C for 21 days. Colonies were then enumerated, calculated, and expressed as $\log_{10}$ CFU per organ.

Lung histology. Isolated lungs were fixed for 72 h in 10% formalin, processed, paraffin embedded, sectioned, and stained with hematoxylin and eosin.

Cell isolation from the airway lumen, lung interstitium, and spleen. The intra-airway luminal cells were removed from the lung by exhaustive lavage as previously described.[20,22] Following lavage, the lungs were perfused through the left ventricle with Hanks' buffer to remove leukocytes and red blood cells from the pulmonary vasculature. The lungs were then cut into small pieces and incubated with collagenase type 1 (Sigma-Aldrich, Oakville, Ontario, Canada) for 1 h at 37 °C. The digested lungs were then crushed through a 100-µm filter. Splenocytes were isolated as previously described.[20,22] All isolated cells were resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and 1% penicillin and streptomycin.

Immunological analyses by intracellular cytokine staining and fluorescence-activated cell sorting. Isolated mononuclear cells were cultured in a U-bottom 96-well plate at a concentration of 20 million cells ml^{-1} for spleen and lung cells, and 0.5–1 million cells ml^{-1} for cells from the BAL. For intracellular cytokine staining, cells were cultured for 24 h in the presence or absence of mycobacterial antigens (*M.tb* CF and crude BCG). Golgi plug (5 µg/brefeldin A; BD Pharmingen, Mississauga, Ontario, Canada) was added after 18 h of stimulation. Cells were then washed and blocked with CD16/CD32 in 0.5% bovine serum albumin/PBS for 15 min on ice and stained with cell surface antibodies. Cells were washed, permeabilized, and stained with in accordance with the manufacturer's instructions for intracellular cytokine staining (BD Pharmingen). The following fluorochrome-labeled antibodies were used: CD3-Per chlorophyll protein Cy5.5, CD4-PE-Cy7, CD8-APC-Cy7, IFN-γ-APC, TNF-α-FITC, IL-2-PE, CD44-PE-Cy5, CD62 L-PE-Texas Red, CD127-FITC (BD Pharmingen). Following staining, cells were run on a LSR II (BD Pharmingen) and 100,000–200,000 events were collected for each sample. The fluorescence-activated cell sorting data were analyzed using FlowJo Software (Tree Star, Ashland, OR). The CD4+ T cells that were positive for interferon-γ intracellular cytokine staining were defined as Ag-specific CD4+ T cells.

T-cell purification and adoptive airway luminal transfer. T cells were purified from the lung mononuclear cells isolated from naïve and BCG/CF-treated mice by using Pan T Cell Isolation Kit II (Miltenyi biotec, Auburn, CA). Briefly, single-cell suspensions were then pooled and first incubated with a cocktail of biotin-conjugated monoclonal antibodies against CD11b, CD11c, CD19, CD45R, CD49b, CD105, Ter-119, and anti-major histocompatibility complex class II. Cells were then incubated with anti-biotin Microbeads (Miltenyi biotec) and passed through an MS column using the OctoMACS separator (Miltenyi biotec). The negative (unlabeled) fraction was collected and run through a second MS column to achieve highly purified T cells. T-cell purity was determined by fluorescence-activated cell sortingto be consistently >90%. Purified T cells were then adoptively transferred intratracheally (2×10^6 cells/mouse in 40 µl PBS) into the airway lumen of recipient mice. Recipient mice were then challenged via the airway with *M.tb*H_{37}Rv 16 h after adoptive transfer and killed 2 weeks post challenge for assessment of bacterial burden.

Statistical analysis. Statistical analysis was conducted to evaluate the significance between two differences. For two-sample comparison, Student's *t*-test was used. For comparison between two or more groups, analysis of variance was used; wherever applicable, a *post hoc* Fisher's least significant difference test was used for further comparison. The difference was considered to be statistically significant when $P<0.05$.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGMENTS

We thank BEI Resources for providing *M.tb* antigens. This work was supported by funds from the Canadian Institutes for Health Research.

DISCLOSURE

The authors declared no conflict of interest.

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CHAPTER 3: Allergic immunity to pulmonary tuberculosis via the TLR4 – IL-17 – Nitric oxide pathway

Carly N. Horvath, Christopher R. Shaler, Anna Zganiacz, Amandeep Khera, Manel Jordana, and Zhou Xing

Allergic Immunity to pulmonary tuberculosis via the TLR4 – IL-17 – Nitric oxide pathway

In this study we investigated the impact of continuous allergen exposure on the outcome of subsequent pulmonary *M.tb* infection. We utilized an established method of chronic exposure to the common aeroallergen, house dust mite to induce allergic inflammation in the lungs of mice. We have documented for the first time, that continuous exposure to house dust mite results in enhanced and sustained protection against pulmonary tuberculosis. Furthermore, we found that the mechanism by which this enhanced protection is achieved is via a TLR4-depandant phenotypic switch of allergen-specific T cells from that of a Th2/Th17 dominated to a Th1/Th17 dominated phenotype. Increased Th17 cells resulted in enhanced production of IL-17 following *M.tb* infection, which was able to mediated enhanced protection independently of IFN-γ. The mechanism by which IL-17 was able to achieve such enhanced protection against *M.tb* was found to be due to an IFN-γ independent activation of infected macrophages to produce bactericidal nitric oxide.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

Allergic Immunity provides enhanced protection against pulmonary tuberculosis via the TLR4-IL-17-nitric oxide pathway

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Keywords: tuberculosis, lung, T cell immunity, allergic immunity, interleukin 17, mycobacteria, nitric oxide, TLR4

Abstract

Over 300 million individuals worldwide have asthma, 80% of which are characterized as allergic asthma. Allergic immunity can be induced by a variety of allergens however, a significant proportion of allergic asthma cases are caused by house dust mite (HDM). While the majority of global cases of allergic asthma occur in the developed world, there has been an increase in the number of cases in areas of the developing world, in areas where there is an overlap with infections with *Mycobacterium tuberculosis*. Historically, Th1 and Th2 immune responses are reciprocally antagonizing and while it has been firmly established that strong Th1 polarizing immune responses can reverse or prevent the establishment of Th2 responses, it remains unknown what effect prior established allergic immunity in the lung will have on *M.tb* infection which requires robust Th1 immunity for bacterial control. In the present study, we set out to address the impact of HDM-driven allergic immunity in the lung on virulent *M.tb* infection. To our surprise, we found that HDM exposure provided early and sustained enhanced protection against *M.tb* infection over saline controls. In addition, we demonstrated that this enhanced protection was dependent on the TLR4 triggering of IL-17 production required for nitric oxide production. Furthermore, we found that this mechanism of HDM-mediated protection was independent of IFN-γ production. Thus, we have established a novel mechanism by which protection against pulmonary *M.tb* infection can be paradoxically achieved by allergic immunity.

Introduction

Over 300 million individuals worldwide have asthma, a disease characterized by chronic inflammation of the conducting airways induced by a variety of factors and results in periodical and reversible airway obstruction and bronchial hyper-responsiveness [1,2]. The greatest prevalence of asthma is found in the developed world where 5-10% of the population is affected [3]. Allergic asthma, comprising up to 80% of all asthma endotypes is characterized by allergen-driven airway hypersensitivity induced by Th2 driven chronic inflammation and cytokines, the presence of eosinophils in the airway, and allergen-specific IgE [2,4,5]. Allergic immunity can be driven by a variety of allergens, however between 50 and 85% of all cases of allergic asthma are caused by house dust mite (HDM) [5]. Indeed experimentally, chronic exposure to HDM induces all of the distinctive features of allergic asthma including allergen driven allergic inflammation, HDM-specific IgE production, eosinophilia, goblet cell hyperplasia, and the chronic features of airway remodeling such as collagen deposition and increased airway smooth muscle which result in airway hypersensitivity [6]. While the majority of the global cases of allergic asthma reside within the developed world, there has been a growing increase in the number of cases in the developing world within the last twenty years as these countries become increasingly more industrialized [3].

Interestingly, it is within the developing world where there is a growing overlap within the geographical distribution of allergic asthma and infection with *Mycobacterium tuberculosis* (*M.tb*) the causative agent of tuberculosis. Tuberculosis remains a global health concern, with 8.7 million new cases and 1.4 million deaths per year worldwide [7].

Immune control of *M.tb* infection requires a strong Th1 polarized immune response characterized by IFN-γ, IL-12 and TNF-α production [8-12]. While controlled but never cleared, granuloma formation is required to restrict bacterial growth and dissemination a process, which requires both CD4 and CD8 T cells to maintain [13-16].

Classically, Th1 and Th2 T cells are considered to be reciprocally antagonizing responses. Indeed, in the context of allergic asthma, a highly polarized Th2 environment consisting of eosinophils and the Th2 cytokines IL-4, 5, and 13 can be reversed by subsequent exposure to strong Th1-inducing agents such as bacteria and bacterial antigens [17-22]. These observations are in accordance with the renowned 'hygiene hypothesis' where the increase in allergy in the developed world has been thought to be a result of improved hygiene and lack of infectious disease[23-26]. However, while such protection afforded by *M.tb* on allergic asthma has been thoroughly characterized, it remains unknown if the prior induction of allergic immunity in the lung alters the ability of the host to control subsequent *M.tb* infection.

Unexpectedly we found that HDM induced allergic immunity was capable of enhancing protection against subsequent *M.tb* infection. Interestingly, this HDM-dependent protection was attributed to enhanced IL-17 responses and was independent of established Th2 immunity. Furthermore, following *M.tb* infection TLR4 signaling was required for the early limitation of bacterial growth by IFN-γ independent IL-17 dependent production of nitric oxide.

Materials and Methods:

Ethic statement

All animal experiments including animal care and procedures were conducted in accordance with the guidelines from 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 10-04-23.

Mice

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories (Charles River, St Constant, Quebec, Canada). IL-4-/- (BALB/c-IL-4^{tm2Nnt}/J #002496), TLR4-/- (C.C3-$Tlr4^{Lps\text{-}d/}$J #002930), IFNγ-/- (C.129S7(B6)-$Ifng^{tm1Ts}$/J #002286), and iNOS-/- (CByJ.129P2(B6)-*Nos2*^{tm1Lau}/J #007072) mice were purchased from Jackson laboratories (Bar Harbor, Maine, USA). All mice were housed under specific pathogen-free conditions in a level B facility at McMaster University. Mice were either infected with *M.tb* H_{37}Ra and housed at level II bio-hazardous conditions, or *M.tb* H_{37}Rv and housed at level III bio-hazardous conditions. All mice were maintained in specific pathogen-free environments regardless of containment level. All animals were maintained on a constant light:dark 12:12 cycle and given free access to food and water. For all experiments, mice were euthanized by exsanguination of the abdominal artery under anesthesia.

HDM Administration

In experiments evaluating allergic asthma or inducing allergic asthma prior to *M.tb* infection, mice were exposed to purified HDM extract (Greer Laboratories, Lenoir, NC) delivered intranasally (25μg of protein in 10μl of saline) for 5 days/week for up to five consecutive weeks as described previously[6]. Following *M.tb* infection, mice received

HDM (25μg of protein in 10μl of saline) intranasally every other day until time of sacrifice. Control mice received 10μl of saline intranasally at the time of HDM delivery.

Mycobacterial preparation and Challenge

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 enrichment (Invitrogen Life Technologies, Carlsbad, CA), 0.002% glycerol, and 0.05% Tween 80 for < 10– 15 days, then aliquoted and stored in −70°C until needed. Before each use, *M.tb* bacilli were washed with phosphate-buffered saline (PBS) containing 0.05 % Tween 80 twice and passed through a 27-gauge needle 10 times to disperse clumps.

Mice were challenged i.n. with a dose of 0.5x10 6 CFU/mouse *M.tb* H_{37}Ra in 40 μl of PBS or with a dose of 10,000 CFU/mouse *M.tb* H_{37}Rv in 25 μl of PBS. A higher inoculum of *M.tb* H_{37}Ra was used to compensate for its avirulent nature, allowing generation of Th1 immune responses highly similar to those seen following *M.tb* challenge.

Bacterial Enumeration

The bacterial load in the lung and spleen were enumerated as previously described. Briefly, half lungs and whole spleens were sterilely collected at the time of sacrifice and homogenized in PBS. Lung and spleen homogenates were subjected to serial dilution and plated on Middlebrook 7H10 agar plates, supplemented with Middlebrook OADC enrichment (Invitrogen). Bacterial plates were incubated at 37°C for 15-17 days

until colonies were visible, at which time colonies were enumerated and the bacterial burden at time of sacrifice calculated.

Lung Cell isolation and purification

Following extraction, the lungs were perfused through the left ventricle with Hanks' buffer to remove leukocytes and red blood cells from the pulmonary vasculature. The lungs were then cut into small pieces and incubated with collagenase type 1 (Sigma-Aldrich, Oakville, Ontario, Canada) for 1 h at 37°C. The digested lungs were then crushed through a 100-µm filter. All isolated cells were resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and 1% penicillin and streptomycin. In experiments where cells were isolated and subsequently purified, CD11c+ APCs, and T cells were isolated from single cell suspensions of total lung mononuclear using CD11c microbeads (Miltenyi biotec, Auburn, California, USA) for purification of CD11c+ antigen presenting cells (APCs) and T cells by using Pan T Cell Isolation Kit II (Miltenyi biotec, Auburn, CA). Labelled cells were passed through an MS column on the OctoMACS separator (Miltenyi biotec). Samples were run through MACS separation columns twice to achieve high purity. Cells were counted and their viability was verified by Trypan blue exclusion. Purity of recovered CD11c+ APC and T cell populations was determined by FACS and was consistently >90%.

Cell stimulation and culture

Total isolated cells ($0.25x10^6$/well) were seeded into a 96-well flat bottom plate and cultured at 37°C and 5% CO_2 with or without mycobacterial antigen stimulation for various time points depending on experimental readout. For stimulations observing

responses to *M.tb,* the antigens used for *ex vivo* stimulation were a mixture of *M. tuberculosis* culture filtrate proteins (*M.tb*-CF) (2μg/well) and crude BCG preparation (50,000 CFU/well). For stimulations carried out for the purpose of observing responses to HDM, cells were stimulated with 5μg/well of HDM. In some experiments, cells were cultured in the presence of the nitric oxide inhibitor molecule, L-NAME at a concentration of 10μg/well. Cells were cultured in a total volume of 250μl of cRPMI. Culture supernatants were collected at various time points and stored at -20°C until cytokine/chemokine measurement, and cell pellets were collected for further analysis.

Immunological analyses by intracellular cytokine staining and fluorescence-activated cell sorting.

Isolated mononuclear cells were cultured in a U-bottom 96-well plate at a concentration of 20 million cells ml^{-1} for spleen and lung cells, and 0.5 – 1 million cells ml^{-1} for cells from the BAL. For intracellular cytokine staining, cells were cultured for 24 h in the presence or absence of mycobacterial antigens (*M.tb* CF and crude BCG). Golgi plug (5 μg/ brefeldin A; BD Pharmingen, Mississauga, Ontario, Canada) was added after 18 h of stimulation. Cells were then washed and blocked with CD16 / CD32 in 0.5 % bovine serum albumin / PBS for 15 min on ice and stained with cell surface antibodies. Cells were washed, permeabilized, and stained with in accordance with the manufacturer' s instructions for intracellular cytokine staining (BD Pharmingen). The following fluorochrome-labeled antibodies were used: CD3-Per chlorophyll protein Cy5.5, CD4-PE-Cy7, CD8-APC-Cy7, IFNγ-APC, IL-17A-V450, and IL-4-PE (BD Pharmingen). Further, in some experiments, cells stained for transcription factors T-bet-APC, GATA3-

V450, and RORγT-PE (BD Pharmingen) using ebioscience's transcription factor staining buffer according to the manufacturer's protocol. Following staining, cells were run on a LSR II (BD Pharmingen) and depending upon the sample, 100,000 – 200,000 events were collected per sample. The fluorescence-activated cell sorting data were analyzed using FlowJo Software (Tree Star, Ashland, OR).

Cytokine quantification

Cytokine concentrations in cell cultures, lung homogenates, and BAL fluids for IL-4, IL-5, IL-17A, IFN-γ IL-12p40, IL-12p70, and IL-23 were measured by using duoset ELISA kits (R&D systems).

Nitric oxide production quantification and inhibition

The release of nitric oxide (NO) by airway lumen and granuloma derived cells was determined by measuring the end product of NO, nitrite, as previously described. Briefly, diluted supernatants were added at a 1:1 ratio with Griess reagent buffer (Sigma-Aldrich). The absorbance was measured at 540 nm by a spectrophotometer. The final concentration of nitrite was calculated by referring to a standard curve prepared from 0 to 100μM of sodium nitrite concentrations. In experiments where NO was inhibited, the inhibitor molecule L-NAME (Sigma Aldrich, Oakville, Ontario, Canada) was used to inhibit nitric oxide production.

Neutralizing Abs for in vivo depletion

In certain experiments, mice received anti-CD4/CD8, anti-IFNγ (made in house) or anti-IL-17A (BioXcell) blocking antibodies. In experiments where T cells were depleted, anti-CD4/CD8 antibodies were administered flowing two weeks of HDM

administration prior to *M.tb* challenge with a single dose of 200μg/mouse. Antibodies against INF-γ and IL-17A were administered one day prior to *M.tb* challenge and delivered at a dose of 150μg/mouse twice weekly until time of sacrifice. All antibodies were delivered via the intraperitoneal route in 200μL. Appropriate isotype matched controls were delivered to control groups.

In vitro killing assay

In experiments where *M.tb* infected APCs were evaluated for bactericidal capacities, both T cells and APCs were purified from total isolated lung mononuclear cells and resuspended in cRPMI. In a U-bottom 96 well plate, purified APCs were plated at 400,000 cells/well and incubated overnight with *M.tb* H_{37}Ra at a ratio of 5 CFU/cell in a total volume of 250μL. The next day, cells were centrifuged to remove any remaining extracellular bacteria, and additional cells or stimulations were added. For wells receiving purified T cells, the T cells purified the previous day were incubated overnight in cRPMI at 37°C 5% CO_2, enumerated, and added to wells at a ratio of 1:5 APC to T cells. Recombinant IFN-γ and IL-17A (R&D systems) were added at a concentration of 1000pg/well, and the NO inhibitor molecule L-NAME was used at a concentration of 10μg/well. Cells were then incubated at 37°C 5% CO_2 for various time points as indicated, at which time plates were centrifuged. Supernatents were collected and stored at -20°C for future analysis of cytokines and nitric oxide production. The cell pellets were resuspended in 300μL of ddH_2O, diluted, and plated on Middlebrook 7H10 agar plates, supplemented with Middlebrook OADC enrichment (Invitrogen). Bacterial plates

were incubated at 37°C for 15-17 days until colonies were visible, at which time colonies were enumerated and the bacterial reduction over time could be calculated.

Histology

For the evaluation of histological changes, isolated lungs were fixed for 72 h in 10 % formalin, processed, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Stained sections were mounted to slides and histological evaluation was performed by conventional light microscopy at various magnification (5x, 10x, 20x), looking for structural, cellular and morphological changes associated with allergic asthma, mycobacterial infection, and overall pathological changes within the lung.

Cytospins and cell enumeration

The intra-airway luminal cells were removed from the lung by exhaustive lavage as previously described [27]. Cell pellets were re-suspended in PBS and slides were prepared by cyto-centrifugation at 300 rpm for 2 min. The slides containing the cells were then stained with Diff-Quik (Baxter, McGraw Park, IL). Differential counts of BAL cells were determined from at least 500 leukocytes using standard hemocytological procedures to classify the cells as neutrophils, eosinophils, lymphocytes, or macrophages/monocytes.

Quantification of HDM-specific IgG1

For detection of HDM-specific IgG1, MaxiSorp plates (NUNC Brand Products) were coated with 5 μg HDM in carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Subsequently, coated wells were blocked with 1% w/v BSA in PBS for 2 h at RT. After washing, serum samples (four serial dilutions) were incubated overnight at 4°C, washed and developed with biotin-labeled IgG1 (Southern Biotechnology Associates) overnight

at 4°C. Plates were washed and incubated with alkaline-phosphatase streptavidin (Zymed, South San Francisco, California, USA) for 1 h at RT. The color reaction was developed with p-nitrophenyl phosphate (Sigma) in 10% v/v diethanolamine buffer (Sigma) and ODs were read at 450 nm. For HDM-specific IgG1, units correspond to the maximal dilution that results in an OD that is greater than the blank plus two standard deviations.

Statistical Analysis

Statistical analysis was conducted to evaluate the significance between two differences. For two-sample comparison, Student's t-test was used. For comparison between two or more groups, analysis of variance (ANOVA) was used; wherever applicable, a post hoc Tukey test was used for further comparison. All statistics were conducted using the statistical analysis component of GraphPad Prism software. Values of $p<0.05$ were considered statistically significant.

Results

Continuous exposure to HDM provides early and sustained protection against M.tb infection

We first confirmed the induction of allergic immunity in the lung by HDM by utilizing the chronic exposure model previously established [6]. Daily exposure to HDM for 2, 4, and 6 weeks (Supplemental Figure 1A) resulted in increased cellular recruitment into the airway (Supplemental Figure1B) with 30% of total infiltrating cells being eosinophils (Supplemental Figure 1C). In addition, there was an increase in HDM-specific IgG1 (Supplemental Figure 1D) and production of the Th2 cytokines IL-4, 13, and IL-5 (Supplemental Figure 1E and data not shown). Furthermore, histopathologically

there was a time dependent increase in cellular infiltration, including eosinophils surrounding the major airways with pronounced goblet cell hyperplasia and mucous production (Supplemental Figure 1F).

We next asked whether the establishment of allergic immunity by HDM exposure had an impact on subsequent *M.tb* infection. We found that unexpectedly, HDM exposure provided enhanced protection against virulent *M.tb* challenge and restrained bacterial growth within the lung as early as the first 7 days post infection, and was sustained for as long as 4 weeks post infection (Figure 1A). We found that although HDM exposure enhanced protection against *M.tb* infection, continuous exposure both prior to, and following *M.tb* challenge was required to achieve maximal levels of protection (Supplemental Figure 2A&B). The enhanced protection afforded by continuous HDM exposure following *M.tb* challenge was associated with decreased lung immunopathology as denoted by fewer and less diffuse granuloma structures and overall cellular infiltration compared to saline controls (Figure 1B). Additionally, it was also noted that in accordance with published literature [28-30], despite the continuation of HDM exposure *M.tb* infected mice did not display any sign of allergic immunity as indicated by the absence of eosinophils, goblet cell hyperplasia or Th2 cytokines (Figure 1B and data not shown).

We next sought to investigate the cellular and cytokine responses to *M.tb* in the context of HDM exposure that would be responsible for the enhanced protection that was seen in infected mice. We found that following *M.tb* infection in HDM exposed mice, there was no marked increase in IL-4 production (Figure 1C), or IFN-γ production (Figure 1D) in the lung homogenates compared to saline controls. However, from day 7

to 14 there was a highly significant ($p<0.001$) surge of IL-17 production in the lungs of HDM exposed mice compared to the saline controls which displayed only a minor increase in IL-17 during the first week post infection (Figure 1E).

Since control of *M.tb* infection is known to require T cells, we next profiled T cell responses in the lungs of HDM exposed animals following infection. We found that in accordance with the above cytokine data, the T cells residing within the lungs during the first two weeks post infection did not produce heightened levels of either IL-4 or IFN-γ in comparison to saline controls (Figure 1F and G). However, in line with the significant increase in IL-17 production that was noted within the lungs, we also found that CD4+IL-17+ T cells were heightened over saline controls for the first two weeks following *M.tb* challenge (Figure 1H). Thus taken together, continuous HDM exposure was found to provide enhanced protection against *M.tb* infection, characterized by predominant IL-17 production and increased Th17 response in the lungs of dually exposed mice.

HDM-primed T cells are an essential source of IL-17 following M.tb challenge required for enhanced protection

Given the pronounced production of IL-17 following *M.tb* challenge in HDM exposed mice, we next examined if IL-17 was critical for HDM-mediated enhanced protection against *M.tb*. To this end, mice were exposed to HDM daily for two weeks and challenged with *M.tb* while depleted of IL-17 with a depleting antibody (Figure 2A). We found that indeed, in the absence of IL-17, HDM-mediated protection against *M.tb* was completely lost (Figure 2B). We then asked if HDM-primed T cells were required for this protection as we saw a significant enhancement of Th17 cells following infection

(Figure 1H). To address this question, we utilized depleting antibodies against CD4 and CD8 4 days prior to *M.tb* infection following two weeks exposure to HDM. This would allow any T cells induced by HDM to be depleted, however would not impact the normal kinetics of T cell priming following *M.tb* as such responses take approximately 14 days [31], by which time the T cell repertoire would be replenished as depletion only remains in effect for 7 days (data not shown). We found that in HDM exposed mice depleted of T cells prior to infection, the protective effect of HDM against *M.tb* was lost, with the level of bacterial burden equal to that of the saline, and saline depleted controls (Figure 2C).

Given that HDM-primed T cells are required for protection, we hypothesized that such T cells were undergoing a phenotypic switch following *M.tb* infection from a predominant Th2 phenotype to that of predominantly Th17. We found that CD4+ T cells from HDM exposed mice *ex vivo* stimulated with *M.tb* and HDM significantly ($p<0.00001$) reduced GATA3 expression by 50% (Figure 2D). Conversely to GATA3, T-bet expression increased in response to *M.tb* and HDM by almost 6 fold (Figure 2E) and RORγT expression, elevated from HDM exposure underwent a further significant ($p<0.01$) increase following stimulation (Figure 2F). Indeed in accordance with the decrease in GATA3 expression on T cells, there was a reduction in the level of IL-4 production (Figure 2G), an increase in IFN-γ production (Figure 2H), and a highly significant ($p<0.0001$) increase in IL-17 production (Figure 2I). Together this data indicates that following *M.tb* infection in HDM exposed mice, T cells undergo a phenotypic switch from being primarily Th2 and Th17 polarized to a Th1 and Th17 phenotype.

TLR4 is required for HDM induced IL-17 mediated protection against M.tb

Following the observation that HDM-primed T cells undergo a phenotypic switch in response to *M.tb* and HDM, we wondered what was enhancing the Th17 responses following *M.tb* infection in the context of HDM exposure. Several groups have noted that Th17 responses can be induced by TLR4-mediated production of IL-23 by APCs [32-36]. We therefore asked whether TLR4 stimulation was required for HDM-mediated enhanced protection against *M.tb* infection. To this end, we induced an allergic phenotype in TLR4-/- and wild type mice by 2 weeks of chronic HDM exposure (Supplemental Figure 3A-D) and infected both groups of mice with *M.tb.* We found that in the absence of TLR4, HDM-mediated protection against *M.tb* challenge was completely lost as denoted by both the lack of reduction in bacterial burden within the lung, as well as the enhanced lung histopathology in HDM exposed TLR4-/- mice infected with *M.tb* (Figure 3A and B).

In addition, we found that in accordance with the lack of protection, in the absence of TLR4, we saw a significant reduction in the level of IL-17 production in dually exposed TLR4-/- mice at day 7 following *M.tb* infection (Figure 3C). Furthermore, while RORγT expression on wild type CD4 T cells increased upon *M.tb* and HDM stimulation, this did not occur in TLR4-/- mice (Figure 3D). Wild type CD4+ T cells also underwent approximately double the level of proliferation in response to dual stimulation when compared to TLR4-/- CD4+ T cells (Figure 3E). Moreover, of the CD4+ T cells that proliferated in wild type mice, the vast majority (over 40%) of cells were RORγT+ (Figure 3F). In line with the lack of up-regulation of RORγT expression on TLR4-/-

CD4+ T cells, these cells produced 78% less IL-17 following *ex vivo* stimulation (Figure 3G).

Given the poor production of IL-17 production in TLR4-/- mice we hypothesized that a deficiency in IL-23 production may contribute to the lack of IL-17 and reduced bacterial control seen following *M.tb* infection. Indeed, following *M.tb* infection TLR4 -/- mice produced very little IL-23 for the first 14 days post *M.tb* infection, whereby in contrast IL-23 levels exceeded 2000pg/mL in the lung homogenates of wild type mice exposed to HDM (Figure 3H). Taken together, this data implies that protection against *M.tb* in HDM exposed animals is via TLR4 induction of IL-17, likely through the production of IL-23.

IL-17 mediated bacterial control is achieved via enhanced production of nitric oxide

Having discovered that HDM induced allergic immunity was providing enhanced protection against *M.tb* infection via a TLR4-IL-17 pathway, we next sought to determine the means by which IL-17 was capable of mediating such enhanced protection. We first asked whether the production of nitric oxide, critical for mycobacterial killing after classical activation of *M.tb* infected macrophages was intact in the absence of IL-17. We found to our surprise that in the absence of IL-17, HDM exposed lung MNCs isolated from *M.tb* infected mice produced significantly ($p<0.0001$) less nitric oxide than the undepleted counterparts, and even less than saline controls (Figure 4A). In addition, we also observed almost 4 fold higher levels of nitric oxide from cultures derived from undepleted HDM mice, than the saline controls (Figure 4A). Since TLR4 was seen to be required for maximal IL-17 production in HDM exposed mice infected with *M.tb*, we

then wondered if TLR4-/- mice displayed a decreased ability to produce nitric oxide. As expected, in the absence of TLR4, mice exposed to HDM and infected with *M.tb* displayed a significantly ($p<0.01$) reduced capacity to produce nitric oxide (Figure 4B). This impairment in nitric oxide production further corresponded to a reduction in bacterial killing by 50% in APCs isolated from TLR4-/- mice exposed to HDM, when stimulated with both rIL-17 and HDM-primed T cells (Figure 4C).

Having noted the defect in nitric oxide production in both the absence of TLR4 and IL-17, we next wished to confirm the requirement of nitric oxide in HDM-mediated protection against *M.tb* infection *in vivo.* To this end, we delivered the selective iNOS inhibitor L-NAME to *M.tb* infected HDM and saline exposed mice. We found that in the absence of nitric oxide, HDM-mediated protection against from infection was completely lost despite slightly elevated levels of IL-17 in the lung homogenates (Figure 4D&E).

Given the role for HDM-primed T cells (Figure 2B) and the fact that they achieved the same degree of bacterial killing as rIL-17, we wondered if these T cells could still mediate enhanced protection in the absence of nitric oxide. To address this question, we paired APCs and T cells purified from HDM exposed mice in the presence of a selective nitric oxide inhibitor L-NAME. We found that in accordance with the *in vivo* data, in the presence of this inhibitor, there was approximately 90% reduction in the ability of HDM exposed APCs to kill *M.tb* despite the presence of T cells from HDM exposed mice (Figure 4F). Collectively, these data suggest that HDM provides enhanced protection against *M.tb* via TLR4 and IL-17 dependent production of nitric oxide.

IL-17 induced production of nitric oxide in HDM-exposed mice following M.tb infection is independent of IFN-γ

Having demonstrated the essential requirement for IL-17, we wondered if IFN-γ played a role in HDM-mediated enhanced protection against *M.tb* given the established requirement for this cytokine for macrophage activation in primary mycobacteria infection models [8,12]. To address this question, we depleted IFN-γ for the duration of *M.tb* infection in HDM-exposed animals (Figure 4G). In accordance with published literature, IFN-γ depletion in infected saline controls had a significant increase in bacterial burden within the lungs at 2 weeks post infection (data not shown). However, unexpectedly, HDM-exposed mice in the absence of IFN-γ displayed no loss of bacterial control and compared to the undepleted HDM exposed controls (Figure 4H).

We found that the enhanced protection of HDM exposed mice in the absence of IFN-γ, there was no impairment of nitric oxide production (Figure 4I). We then confirmed the dispensable role of IFN-γ for bacterial killing of *M.tb* in HDM-mediated protection by pairing APCs derived from HDM exposed wild type or iNOS-/- mice with wild type or IFN-γ-/- T cells. We found that maximal killing was dependent on the capability of *M.tb* infected APCs to produce nitric oxide, but not on the ability of T cells to produce IFN-γ (Figure 4J). Thus, while IL-17 production is essential for nitric oxide mediated bactericidal function in HDM exposed animals infected with *M.tb*, IFN-γ is dispensable, and our results indicate even a hindrance to HDM-mediated protection.

Collectively, our data suggests that HDM induced allergic immunity consists of a mixture of Th1, Th2, and Th17 cells that reside within the lung. Upon infection with

M.tb and under continuous HDM exposure, triggering of TLR2 by *M.tb* and TLR4 by *M.tb* and HDM induces the production of both TNF and IL-23. This production of IL-23 then stimulates the up-regulation of RORγT on CD4+ T cells as well as the proliferation of such T cells. The production of IL-17 by these T cells, combined with TNF induces the production of nitric oxide which ultimately results in the killing of *M.tb* before *M.tb*-specific T cells can be primed and recruited to the site of infection (Figure 4K).

Discussion

To date, the impact of prior allergic inflammation on subsequent *M.tb* infection remains unknown. Our current study thus paradoxically demonstrates for the first time that continuous respiratory exposure to a common allergen elicits early and sustained protection against *M.tb* infection. Following chronic HDM exposure, the dominant inflammatory response is characterized by Th2 cytokine production, tissue eosinophilia, allergen-specific IgE production, and the accumulation of lymphocytes. Within this lymphocyte population, we and others [37-40] demonstrate that in an allergic lung, the dominant population of T cells is of a Th2 phenotype, with a mixture of Th17, and Th1 populations forming the remainder. However, upon *M.tb* infection these resident T cells were shown to undergo a phenotypic switch from a dominant Th2 population, to that of a Th17 phenotype. The ability of HDM-primed T cells to obtain a Th17 phenotype characterized by the secretion of IL-17 was shown to be required for enhanced protection against *M.tb* and dependent on TLR4 signaling. The mechanism by which IL-17 was able to provide enhanced protection against *M.tb* was determined to be the production of high levels of nitric oxide independently of IFN-γ. Collectively, these findings provide

novel insight into plasticity of polarized T cell responses in response of competing antigens, increased understanding of protective immune mechanisms against *M.tb,* and further challenges the dogma that IFN-γ production is essential for both the production of nitric oxide, and protection in the context of an *M.tb* infection.

It has long been known that mycobacteria and mycobacterial antigens provide a protective role against atopic diseases including allergic asthma, acting to both resolve established allergic inflammation [5,29] in addition to preventing the development of pathological allergic responses [17,18,20,21,30,41]. It is currently understood that the mechanism by which this protection is achieved is via the stimulation of the Th1 cytokines IL-12 and IFN-γ that serve to suppress an allergic immune response by both down-regulating Th2 cytokine production and promoting classical macrophage activation [17,18,30]. However, while mycobacteria and their products are clearly protective against allergic asthma, the reciprocal effect of allergic inflammation on subsequent host mycobacterial control has not been addressed to date.

To our initial surprise, our data has shown that continuous HDM exposure in the context of an *M.tb* infection provides "allergic immunity" against pulmonary tuberculosis characterized by enhanced bacterial control in addition to a reduction in lung pathology. In accordance with the literature, we observed a resolution of allergic lung pathology as well as Th2 cytokine production following *M.tb* infection (data not shown). However, we present novel alternative means by which protection against *M.tb* can be achieved without the requirement of IFN-γ. IFN-γ has been long perceived as essential for protection against *M.tb* infection based upon several studies demonstrating the rapid fatality in

animal models deficient in this cytokine [8,16]. The source of IFN-γ is derived from *M.tb*-specific T cells, and cannot be compensated by other cell types as T cell deficiencies are also universally fatal due to overwhelming bacterial growth following *M.tb* infection [13,42-44]. IFN-γ is critical for mycobacterial control as it is required to synergize with TNF-α in order to produce iNOS and subsequently nitric oxide as well as mediate phago-lysosome fusion [8,12,13,45]. Nitric oxide as a radical, interacts with many downstream products and facilitates the destruction of the bacterial cell wall and creates a toxic environment for bacterial growth, effectively allowing the bacterial burden within the lungs of infected hosts to plateau and establish immune control known as the latent stage of the infection. Our results demonstrate that in the context of HDM exposure, while nitric oxide production is a requirement for enhanced protection against *M.tb,* we have shown for the first time, that the production of this toxic radical is independent of IFN-γ in the context of a *M.tb* infection.

While IFN-γ is dispensable for HDM-mediated enhanced protection, the cytokine can not compensate for the requirement of IL-17 for the production of nitric oxide. While never described in the context of a mycobacterial infection, IL-17 has been shown to both independently and synergistically with IFN-γ drive the transcription of iNOS in the presence of TNF-α following TLR stimulation [32,34,35]. Although IL-17 has been found to be dispensable for bacterial control in unvaccinated animals, this cytokine has recently gained attention its requirement in vaccine-mediated protection. In particular, it has been shown that early IL-17 in parenterally BCG-immunized mice is required for vaccine-mediated enhanced protection via the generation of Ag-specific Th1 responses [46,47].

Furthermore, several mucosally delivered TB vaccines have been shown to provide enhanced protection at the site of *M.tb* infection due to the generation of Th17 cells within the lung [46,47]. Thus, the present study provides insight into the mechanism by which IL-17 in the context of TB vaccination is a protective indicator in instances where IFN-γ is not.

Despite our findings that continuous exposure to HDM provides allergic immune protection against pulmonary TB, the clinical implications of this phenomenon have yet to be investigated. Current trends indicate that there is an increase in allergic asthma, especially due to HDM and cockroach allergens in the urban intercity populations of areas of the developing world such as India and South Africa [48]. It is within these populations that the prevalence of TB is decreasing, similar to the association between these two diseases in developed countries [49,50]. Despite these trends, in the absence of clinical data in humans, no conclusions in this regard can be made but warrant future exploration.

In summary, our study has identified a paradoxical role of pre-existing allergic immunity exerting a protective role on subsequent infection with *M.tb*; a prototypical Th1 infection. This highlights the degree of plasticity of polarized T cells when exposed to competing antigens. Furthermore, this study has identified a novel mechanism of protection against pulmonary tuberculosis, in which early bacterial control can be obtained in the absence of IFN-γ.

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Figures:

Figure 1: *Continuous intranasal allergen delivery provides early and sustained protection against pulmonary M.tb infection*

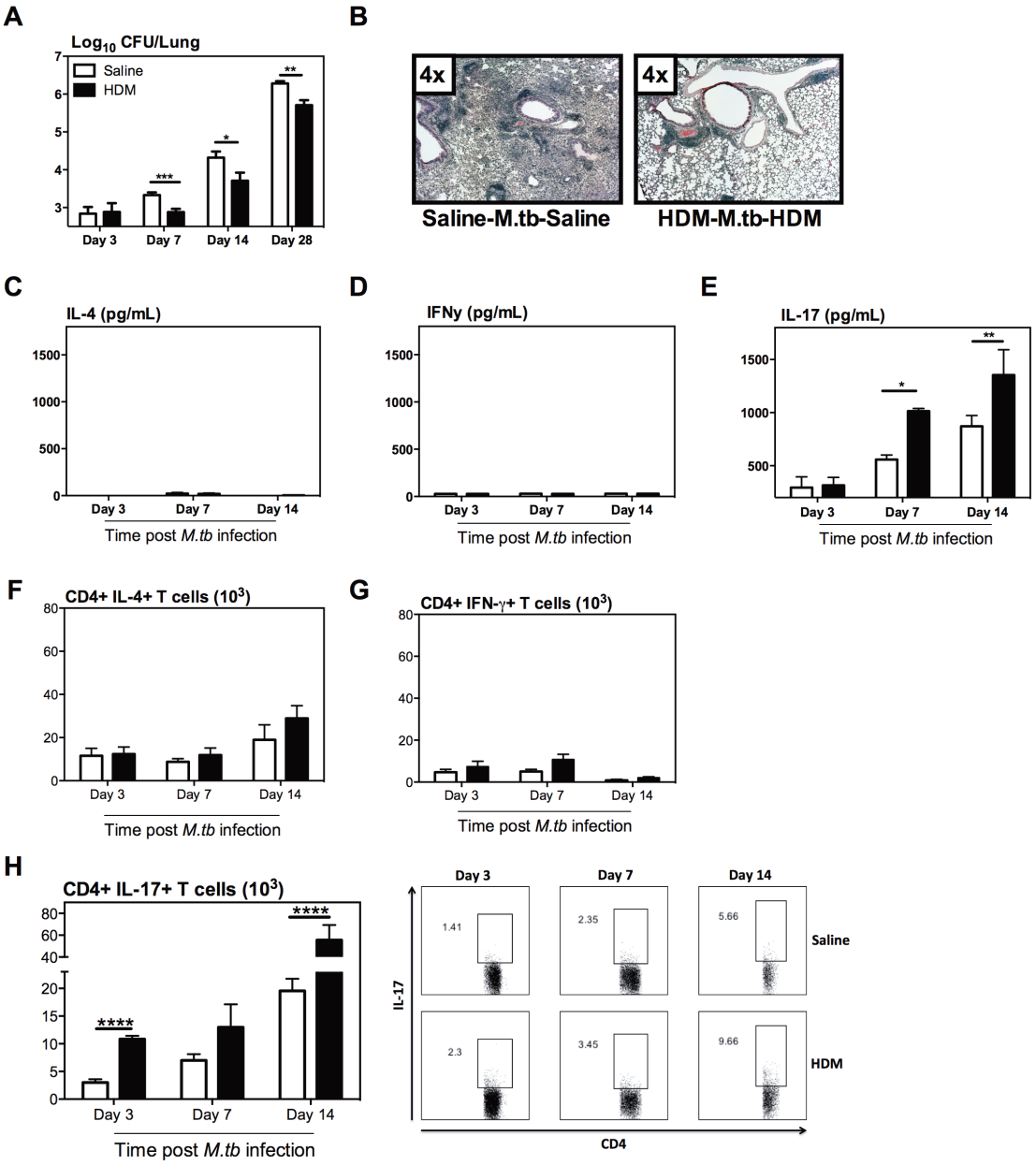


Figure 1: Continuous intranasal allergen delivery provides early and sustained protection against pulmonary *M.tb* infection. Following *M.tb* infection, mice were sacrificed at 3, 7, 14, and 28 days post infection, the lungs of HDM and saline treated mice were collected for the quantification of bacterial burden assessed by colony forming assay (A) and for histology by staining with H&E to assess gross lung pathology at 28 days post infection (B). Lung homogenates were collected at various time points post infection, and the levels of IL-4 (C), IFN-γ (D), and IL-17 (E) were quantified by ELISA. At the time of sacrifice, lung mononuclear cells were isolated from infected lungs and stimulated for 24 hours upon which, cells were stained and the frequency of CD4+IL-4+ (F), CD4+IFN-γ+ (G), and CD4+IL-17+ (H) were obtained. Representative of 2 independent experiments with a minimum of 5 mice per group, challenged with *M.tb* $H_{37}Rv$ and confirmed with *M.tb* $H_{37}Ra$. *$p<0.01$, **$p<0.001$, ***$p<0.0001$, ****$p<0.00001$.

Figure 2: *HDM-primed T cells produce IL-17 in response to M.tb infcetion required for HDM-mediated enhanced protection*

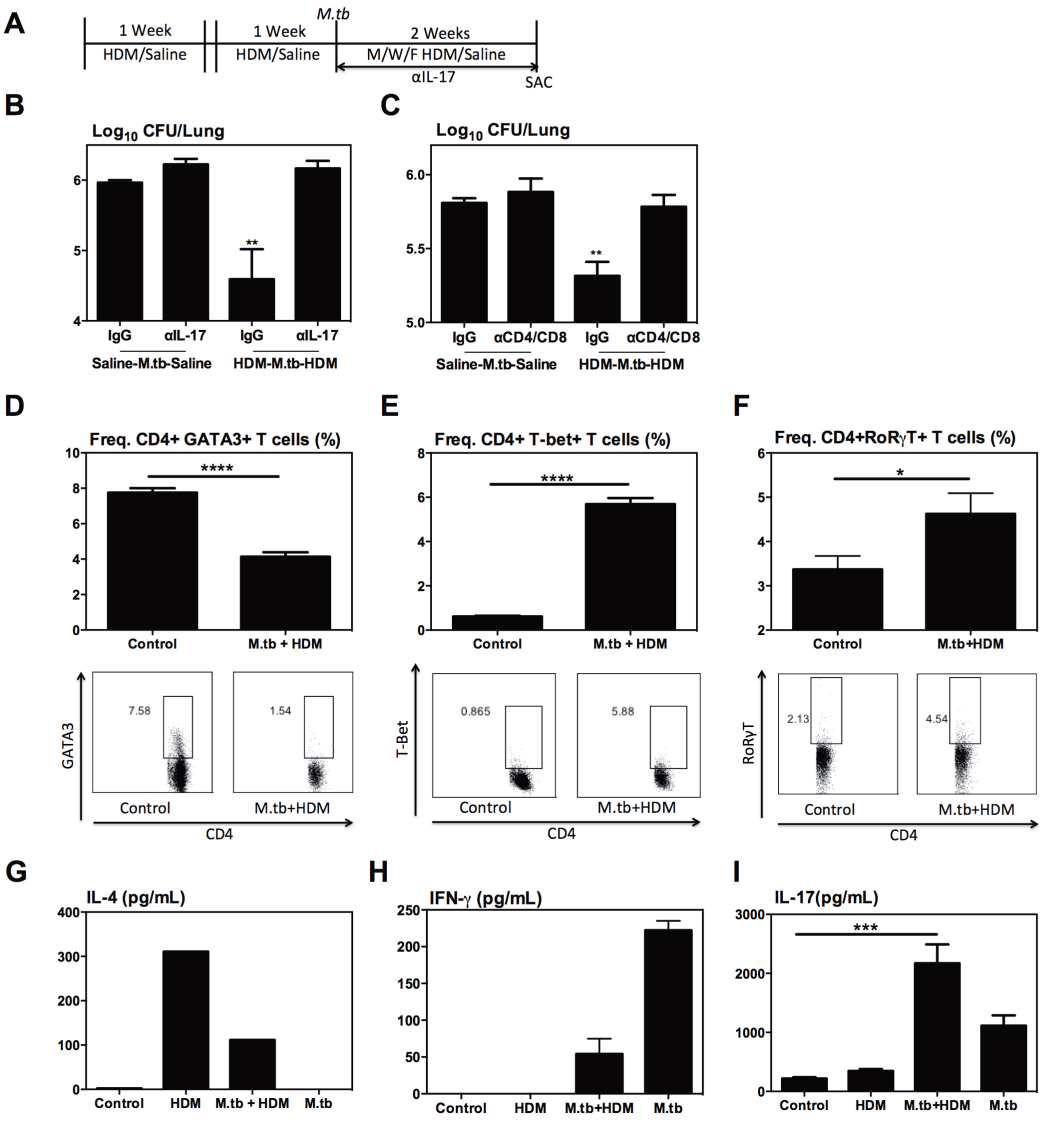


Figure 2: HDM-primed T cells produce IL-17 in response to *M.tb* infection required for HDM-mediated enhanced protection. Mice were exposed to either saline or HDM for 2 weeks to induce an allergic phenotype, then subsequently challenged with *M.tb* after which an IL-17 depleting antibody was administered (A). 2 weeks following challenge, lungs were collected for enumeration of bacterial burden via colony forming assay (B).

Data representative of 2 independent experiments challenged with *M.tb* H_{37}Ra with a minimum of 5 mice per group. Similarly, following 2 weeks of either HDM or saline exposure, CD4 and CD8 T cells were depleted and 4 days prior to challenge with *M.tb*. 4 weeks post infection, mice were sacrificed and collected for bacterial enumeration via colony forming assay (C). Data representative of 2 independent experiments challenged with *M.tb* H_{37}Rv and confirmed using *M.tb* H_{37}Ra with a minimum of 5 mice per group In subsequent experiments, APCs and T cells were purified from mice exposed to HDM for 2 weeks and cultured for 48 hours. Cells were then stained and the frequency of CD4+GATA3+ (D), CD4+T-bet+ (E), and CD4+RORγT+ (F) were quantified. From the same cultures as described above, the level of IL-4 (G), IFN-γ (H), and IL-17 (I) production was quantified by ELISA. Data representative of 4 independent experiments, 2 challenged with *M.tb* H_{37}Rv, and confirmed with 2 *M.tb* H_{37}Ra challenge experiments. All experiments were performed with a minimum of 5 mice per group, plated in triplicate. *$p<0.01$, **$p<0.001$, ***$p<0.0001$, ****$p<0.00001$.

Figure 3: *TLR4 is required for IL-17 protection in HDM exposed mice infected with M.tb*

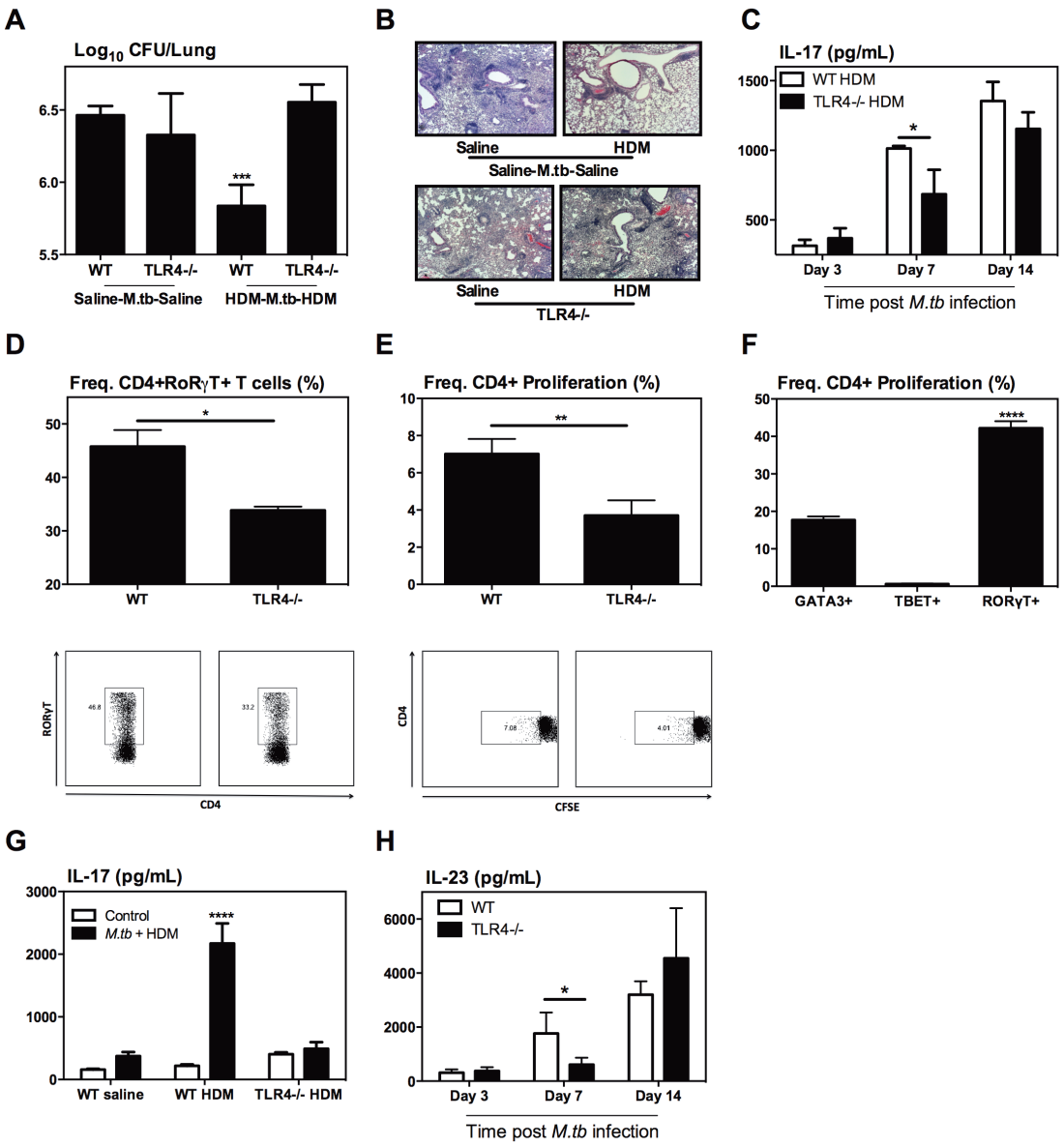


Figure 3: TLR4 is required for IL-17 production in HDM exposed mice infected with *M.tb*. Wild type or TLR4-/- mice were exposed to HDM or saline for 2 weeks, and then infected with virulent *M.tb*. 4 weeks post infection, lungs were collected for enumeration of bacterial burden by colony forming assay (A), and for histology by

staining with H&E to assess gross lung pathology at 28 days post infection (B). Representative of 2 independent experiments with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Rv. In subsequent experiments, wild type and TLR4-/- mice were sacrificed at day 3, 7, and 14 post *M.tb* infection and lung homogenates were collected and the level of IL-17 was quantified (C). Representative of 1 experiment with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Ra. In separate experiments, wild type and TLR4-/- mice were exposed to HDM for 2 weeks, and APCs and T cells were subsequently purified and cultured with *M.tb* and HDM for 48 hours. Cells were stained and the frequency of CD4+RORγT+ cells were quantified (D), and assessed for proliferation via CFSE dilution by flow cytometry (E). Cultured cells were also assessed for the frequency of CFSE diluted CD4+GATA3+, CD4+T-Bet+, and CD4+RORγT+ to determine which T cell subsets had proliferated (F). The level of IL-17 production was also quantified by ELISA in the culture supernatants from the experiments described above (G). Lung homogenates from various time points post *M.tb* infection were also assessed for the level of IL-23 production by ELISA (H). Representative of 3 independent experiments with a minimum of 5 mice per group and plated in triplicate. *$p<0.01$, **$p<0.001$, ***$p<0.0001$, ****$p<0.00001$.

Figure 4: *IL-17 mediated bacterial control is achieved via IFN-γ-independant production of nitric oxide*

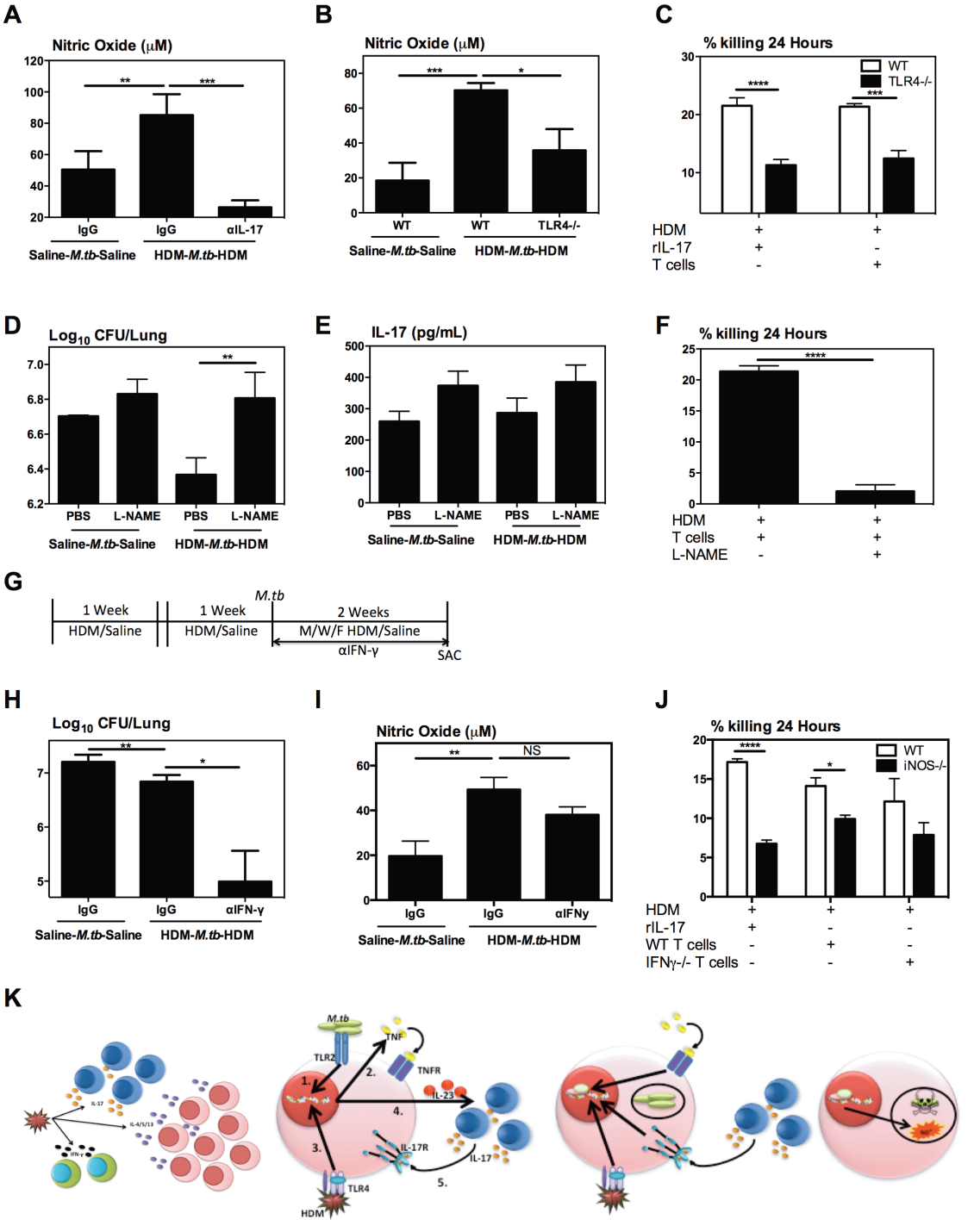
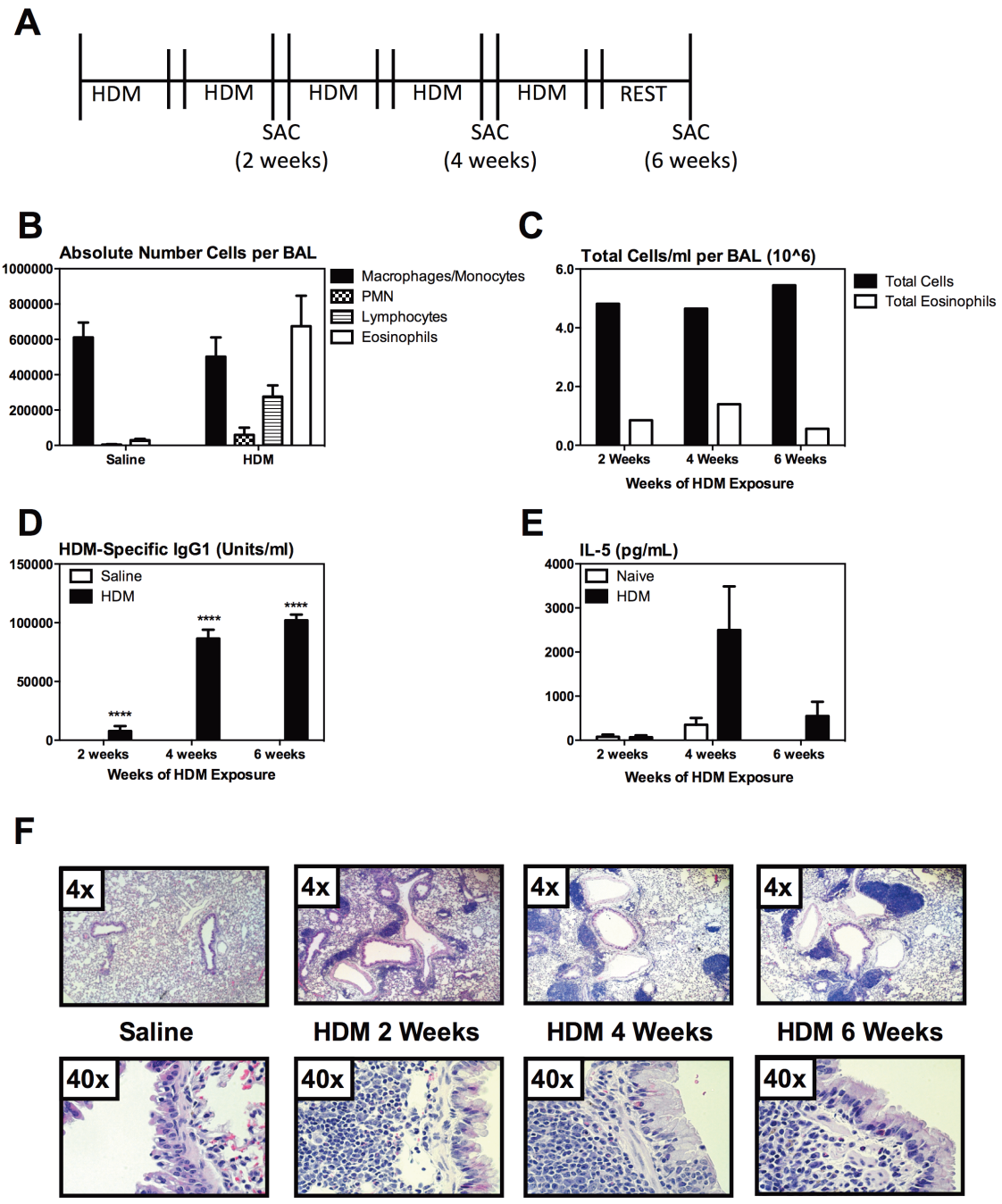


Figure 4: IL-17 mediated bacterial control is achieved via IFN-γ independent production of nitric oxide. In experiments where HDM exposed mice were depleted of IL-17 following *M.tb* infection, lung mononuclear cells were isolated and cultured for 72 hours with *M.tb* antigen. The supernatants were then analyzed for the level of nitric oxide production (A). Representative of 2 independent experiments with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Ra. In additional experiments, wild type or TLR4-/- mice exposed to either saline or HDM were infected with *M.tb* and sacrificed 2 weeks later. Isolated lung mononuclear cells were cultured for 72 hours with *M.tb* antigen and the level of nitric oxide production in the supernatants was quantified (B). Representative of 1 experiment with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Ra. To determine the differential killing capacity of HDM exposed APCs from wild type and TLR4-/- mice, APCs purified from mice following 2 weeks of HDM were infected with *M.tb.* The killing capacity of these cells under different conditions was assessed via colony forming assay 24 hours post infection (C). Representative of 2 independent experiments with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Ra. To ascertain the requirement of nitric oxide for HDM mediated protection, HDM or saline exposed mice were infected with *M.tb* in the presence of a nitric oxide inhibitor, L-NAME. 2 weeks post infection, mice were sacrificed and the level of bacterial burden within the lungs was assessed via colony forming assay (D). The lung homogenates from such experiments were evaluated for the level of IL-17 production at the time of sacrifice (E). In separate experiments, mice exposed to HDM for 2 weeks were sacrificed and APCs and T cells were purified from the lungs. APCs were infected with *M.tb,* and the

total killing capacity of such APCs with and without the presence of L-NAME was assessed via colony forming assay after 24 hours (F). Representative of 1 experiment with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Ra. In experiments designed to determine the role of IFN-γ in HDM-mediated enhanced protection against *M.tb*, mice were exposed to saline or HDM for two weeks and subsequently infected with *M.tb* at which time IFN-γ depleting antibodies were administered for the duration of infection (G). The lungs from these mice were collected 2 weeks post infection and bacterial burden was enumerated via colony forming assay (H). Isolated mononuclear cells from the lungs of infected mice were cultured in the presence of *M.tb* antigen for 72 hours. Supernatants from these cultures were then assessed for nitric oxide production (I). Representative of 2 independent experiments with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Ra. Separate experiments were also conducted to determine the bactericidal capacity of HDM exposed APCs in the absence of either iNOS or IFN-γ. APCs and T cells were purified from the lungs of HDM exposed wild type, IFN-γ-/-, and iNOS-/- mice and infected with *M.tb* under different culture conditions. The percent reduction in bacterial killing was enumerated 24 hours later by colony forming assay (J). Representative of 1 experiment with a minimum of 5 mice per group, challenged with *M.tb* H_{37}Ra. A representative schematic of the proposed mechanism by which HDM provides enhanced protection against *M.tb* infection whereby HDM exposure yields a mixture of T cell subsets with Th2 and Th17 subsets larger than Th1. Upon *M.tb* infection, combined TLR2 and 4 signaling induces the production of TNF-α and IL-23. This IL-23 production stimulates resident Th17 cells to up-regulate RORγT expression

and production of IL-17. This IL-17 production then induces the production of nitric oxide-mediated bacteriocidality independently of IFN-γ (K). Representative of 2-3 independent experiments with a minimum of 5 mice per group or plated in triplicate. *p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001, NS=not significant.

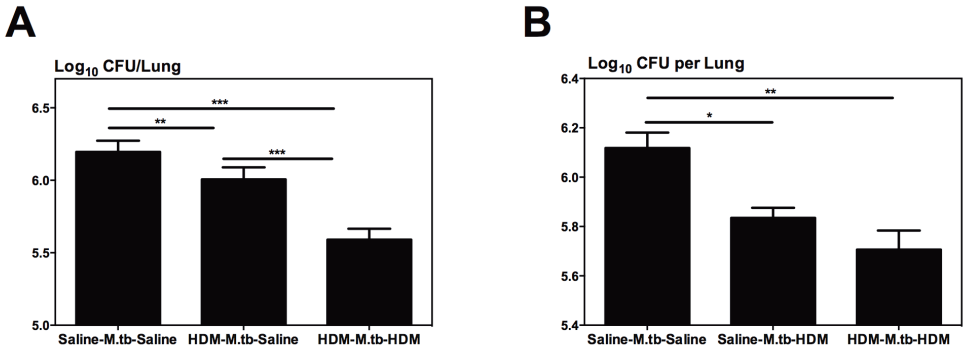
Supp. Fig 1:*Chronic HDM exposure induces an allergic immunity in the lung*



Supplementary Figure 1: Chronic HDM exposure induces allergic immunity within the lung. To confirm an allergic phenotype following chronic administration of HDM, we

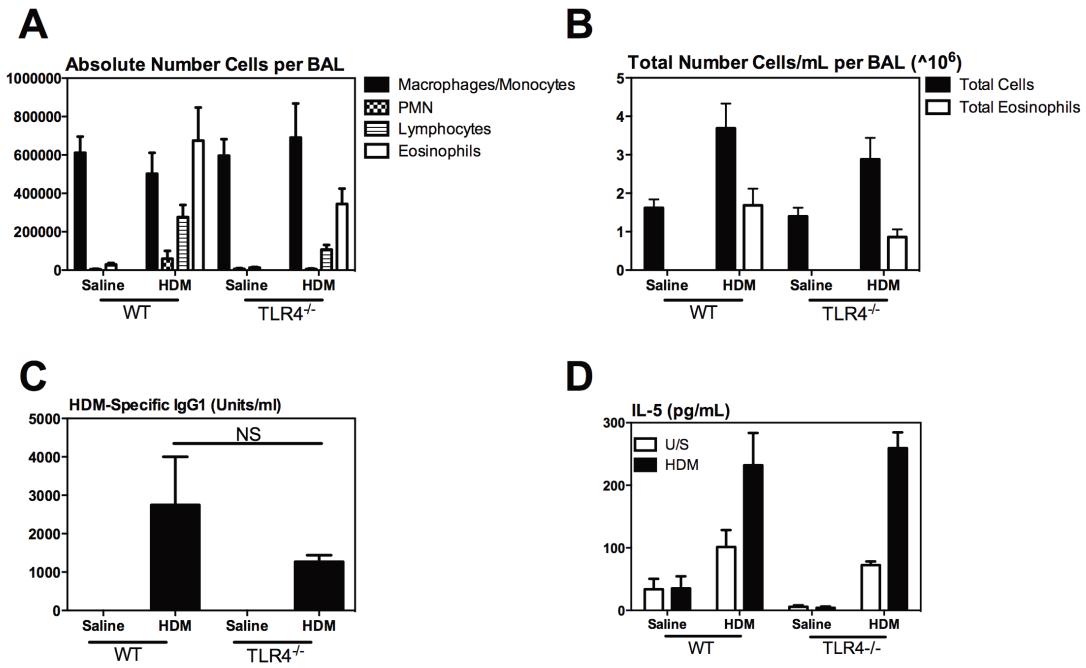
delivered HDM intranasally continuously for 2, 4, or 5 weeks with 1week rest (A). At 2 weeks post HDM administration, total bronchoalveolar lavage (BAL) cells were collected, stained and differential cell counts were conducted to determine the different subsets of cells induced by HDM (B). At each time point post HDM delivery, the total cells and the total number of eosinophils were enumerated by differential cell counts (C). At each time point post-HDM exposure, serum was collected from both HDM and saline exposed mice, and the levels of HDM-specific IgG1 was quantified by ELISA (D). The lung mononuclear cells were collected from each time point and cultured in the presence of HDM stimulation for 48 hours. Supernatants were collected and the production of IL-5 was quantified by ELISA (E). Lungs from HDM or saline exposed mice at each time point were fixed and stained with H&E for visualization of gross histopathological changes (F). Representative of 2 experiments with minimum 5 mice per group. ****p<0.00001.

Supp. Figure 2: *Continuous allergen exposure is required for maximal protection against M.tb infection.*



Supplementary Figure 2: Continuous allergen exposure is required for maximal protection against *M.tb* infection. In order to determine the requirement for continuous HDM exposure for protection against *M.tb* infection, mice were either exposed to saline or HDM for 2 weeks, and then infected with *M.tb* $H_{37}Rv$. After infection, mice received either saline or HDM every other day until the time of sacrifice 4 weeks later. At the time of sacrifice, lungs were collected and bacterial burden was enumerated by colony forming assay. Experiments comparing only prior HDM exposure to continuous is represented in (A), and those comparing only post-infection HDM exposure to continuous are displayed in (B). Representative of 2 independent experiments, with a minimum of 5 mice per group. *p<0.01, **p<0.001, ***p<0.0001

Supp. Figure 3: *Chronic exposure to HDM induces an allergic phenotype in TLR4-/- mice.*



Supplementary Figure 3: Chronic exposure to HDM induces an allergic phenotype in TLR4-/- mice. To determine the phenotype of TLR4-/- mice exposed to HDM, we exposed wild type or TLR4-/- mice to either saline or HDM for 2 weeks and then collected and stained BAL-derived cells for differential cell counting (A). Serum was also collected at the time of sacrifice and the level of HDM-specific IgG1 was measured by ELISA (B). The absolute number of eosinophils from the isolated BAL cells was also quantified by differential cell counts (C). Lung mononuclear cells were cultured in the presence of HDM for 48 hours, and the level of IL-5 production was quantified by ELISA (D). Representative of 2 independent experiments. NS= not significant.

CHAPTER 4: Continuous and discontinuous cigarette smoke exposure differentially affects protective immunity against pulmonary tuberculosis

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Published in *PLOS One*. 2013, 8(3): e59185

Continuous and discontinuous cigarette smoke exposure differentially affects protective immunity against pulmonary tuberculosis

In this study we explored the mechanisms by which cigarette smoke exposure results in impaired immune protection against pulmonary tuberculosis. In addition, we also sought to address the mechanism by which smoke cessation restores protective immunity and is associated with improved prognosis in cases of pulmonary tuberculosis. We found that cigarette smoke exposure prevents the recruitment and activation of Ag-specific T cells into the lung interstitium and airway lumen, resulting in enhanced bacterial replication and dissemination. APC populations were also found to be defective in cytokine production as well as recruitment to the lungs of *M.tb* infected mice. However, upon smoke cessation, the ability of Ag-specific T cells to home into the lung and airway lumen was restored, along with bacterial control, thus providing rationale for cigarette smoke cessation as a first line treatment for cases of active tuberculosis.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

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Continuous and Discontinuous Cigarette Smoke Exposure Differentially Affects Protective Th1 Immunity against Pulmonary Tuberculosis

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Abstract

Pulmonary tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the leading cause of death due to a bacterial pathogen. Emerging epidemiologic evidence suggests that the leading risk factor associated with TB mortality is cigarette smoke exposure. Despite this, it remains poorly understood what is the effect of cigarette smoke exposure on anti-TB immunity and whether its potential detrimental effect can be reversed by cigarette smoking cessation. In our current study, we have investigated the impact of both continuous and discontinuous cigarette smoke exposure on the development of anti-mycobacterial type 1 immunity in murine models. We find that while continuous cigarette smoke exposure severely impairs type 1 immunity in the lung, a short-term smoking cessation allows rapid restoration of anti-mycobacterial immunity. The ability of continuous cigarette smoke exposure to dampen type 1 protective immunity is attributed locally to its affects on innate immune cells in the lung. Continuous cigarette smoke exposure locally, by not systemically, impairs APC accumulation and their production of TNF, IL-12, and RANTES, blunts the recruitment of CD4+IFN-γ+ T cells to the lung, and weakens the formation of granuloma. On the other hand, smoking cessation was found to help restore type 1 immunity by rapidly improving the functionality of lung APCs, enhancing the recruitment of CD4+IFN-γ+ T cells to the lung, and promoting the formation of granuloma. Our study for the first time demonstrates that continuous, but not discontinuous, cigarette smoke exposure severely impedes the lung expression of anti-TB Th1 immunity via inhibiting innate immune activation and lung T cell recruitment. Our findings thus suggest cigarette smoking cessation to be beneficial to the control of pulmonary TB.

Citation: Shaler CR, Horvath CN, McCormick S, Jeyanathan M, Khera A, et al. (2013) Continuous and Discontinuous Cigarette Smoke Exposure Differentially Affects Protective Th1 Immunity against Pulmonary Tuberculosis. PLoS ONE 8(3): e59185. doi:10.1371/journal.pone.0059185

Editor: T. Mark Doherty, Glaxo Smith Kline, Denmark

Received November 30, 2012; **Accepted** February 12, 2013; **Published** March 19, 2013

Funding: This study is supported by funds from the Canadian Institutes of Health Research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Globally, tuberculosis (TB) represents a leading public heath concern with one third of the world's population latently infected [1]. Despite the prevalence of individuals infected with *Mycobacterium tuberculosis (M.tb)*, only 5–10% of them go to develop the active disease [1]. While the majority of TB cases are seen in the developing world, developed nations are not immune. Notably, TB is common among Native American, prison and homeless populations, where incidence rates are very similar to those seen in the developing world [2,3].

The influence of HIV-AIDS on TB has long been acknowledged and is commonly perceived as a leading risk factor in the developing world. Indeed, HIV-AIDS accounts for one quarter of all TB-related deaths [1]. While nutrition, population density, and access to advanced health care are significant factors in the development of active disease, the leading risk factor associated with acquisition, active disease and mortality is the exposure to tobacco smoke [4]. Active smoking and exposure to second hand smoke which is a significant concern especially in children [5,6], account for approximately 60% of all TB related deaths. Alarmingly, the consumption of tobacco products has skyrocketed in the developing world. Current estimates suggest that 85% of all cigarettes are now being consumed in the developing world, with the highest numbers in regions where TB is rampant [7]. The collision of these two epidemics makes unraveling how cigarette smoke exposure impacts TB immunity a particularly relevant challenge.

Protective immunity to *M.tb* largely relies on the generation of a robust type 1 immune response, requiring the elaborate coordination of the innate and adaptive immune systems. Following exposure, *M.tb* primarily infects the alveolar macrophage (AM), utilizing the cell's phagocytic machinery to facilitate its uptake. The infected macrophage detects *M.tb* through the engagement of pattern recognition receptors, specifically toll like receptors (TLRs) 2, 4 & 9, triggering the release of various pro-inflammatory cytokines [8]. Notably, the production of TNF and IL-12 is critical to bridging the innate and adaptive immune systems. Acting as an alarm cytokine TNF plays a central role in coordinating the release of chemokines and the recruitment of innate immune cells to the lung. After acquiring antigen in the lung, recruited APCs (antigen presenting cells) migrate to the

draining lymph node (dLN) to present antigen to naïve T cells, initiating the cellular immune response. At the time of antigen presentation, the release of IL-12 is essential to correctly polarizing Th1 responses, and an absence of IL-12 is detrimental to generating protective immunity [9]. Similarly, a failure to establish strong chemokine gradients prevents T cells from homing to the lung and is equally detrimental to establishment of protective immunity, as has been seen in RANTES deficient mice [10].

Cigarette smoke's impact on immunity is complex; cigarette smoke exerts damaging and pro-inflammatory effects, while suppressing components of both innate and adaptive immunity (reviewed in Stampfli and Anderson [11]). While cigarette smoke activates the AM, cigarette smoke attenuates the expression of key inflammatory mediators such as IL-12, TNF and RANTES that play a critical role in anti-TB host defense. Furthermore, evidence suggests that cigarette smoke impairs the generation of type 1 immunity, leaving infected hosts highly susceptible to certain viral and bacterial pathogens [12,13].

Recently, two groups have begun to address the impact of cigarette smoke exposure on the development of type 1 immunity in the context of *M.tb* or mycobacterial infection in experimental models [14,15]. While these studies have demonstrated a link between cigarette smoke exposure and impaired type 1 immunity in the lung, they have only assessed the impact of prior (discontinued) cigarette smoke exposure on anti-TB immunity. To date no study has evaluated the effect of continuous cigarette smoke exposure, relative to discontinued cigarette smoke exposure, on host defense against pulmonary mycobacterial infection, leaving a critical knowledge gap.

In the current study, we addressed this significant knowledge gap and investigated the impact of both continuous and discontinuous cigarette smoke exposure on the generation of protective immunity following mycobacterial challenge. We have evaluated the effect of cigarette smoke exposure on immune responses generated both locally in the lung and distally in the draining lymph nodes and spleen. Our study has revealed a profound negative effect of continuous, but not prior (discontinuous), cigarette smoke exposure on host defense mechanisms in the lung with a much less effect in the systemic tissue compartments.

Materials and Methods

Ethic Statement

All animal experiments including animal care and procedures were conducted in accordance with the guidelines from the Canadian Council on Animal Care. This study was approved by the Animal Research Ethics Board of McMaster University with an animal utilization protocol number 10-04-23.

Mice

Female C57BL/6 mice (6–8 wk old) were purchased from Charles River Laboratories (Charles River, St Constant, Quebec, Canada) and housed in a specific pathogen-free, level B facility for the duration of cigarette smoke exposure. Following cigarette smoke exposure mice were either infected with *Mycobacterium bovis* Bacille Calmette Guerien (BCG) and housed under level II biohazardous conditions, or *M.tb* H_{37}Rv and housed at level III biohazardous conditions, all mice were maintained in specific pathogen-free environments regardless of containment level. All animals were maintained on a constant light: dark 12:12 cycle and given free access to food and water. For all experiments, mice were euthanized by exsanguination of the abdominal artery under anesthesia.

Cigarette Smoke Exposure

Using a whole body exposure system (SIU-48, Promech Lab AB, Vintrie, Sweden), mice were exposed to cigarette smoke as previously described [16,17,18]. In brief mice were exposed twice daily for 50 mins, 5 days a week to 12 2R4F reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) with filters removed. Mice were exposed to cigarette smoke (or room air) for 6 wks prior to mycobacterial infection. At the time of infection, one group of exposed mice stopped cigarette smoke exposure (cessation), while the other group continued for the duration of the experimental infection, leading to an exposure of 6 or 10 wks, respectively. This protocol of cigarette smoke exposure has been validated and shown to achieve blood carboxyhaemoglobin and cotinine levels that are comparable to those found in regular human smokers [19].

Mycobacterial Preparation and Infectious Dose

Mycobacterium bovis BCG (Connaught strain) was prepared as previously described [9,20]. Briefly, BCG was grown in Middlebrook 7H9 broth (Difco) supplemented with Middlebrook OADC enrichment (Invitrogen), 20% glycerol, and 0.05% Tween 80 for 10 to 15 days, and samples were then divided into aliquots and stored at −70°C. Before each use, a BCG aliquot was washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween 80 and resuspended in PBS. It was then passed through a 27-gauge needle 10 times to disperse clumps and diluted with PBS to the desired concentration. Mice were infected intratracheally with a dose of 0.5×10^6 cfu/mouse for elicitation of strong Th1 immune responses and granuloma formation.

Mycobacterium tuberculosis H_{37}Rv was prepared and processed as described above for BCG. Mice were infected with *M.tb* intranasally by using a dose of 10000 CFU/mouse as previously described by us (depositing 1000±150 CFU into the lung). A higher inoculum of BCG was used to compensate for its attenuated virulence nature, allowing generation of Th1 immune responses highly similar to those seen following *M.tb* challenge. These doses have previously been shown to elicit significant Th1 immunity and the formation of robust granulomas [21,22].

Cell Culture and Cytokine Measurement

Total airway luminal, lung interstitial, spleen or mononuclear cells (MLN) (0.25×10^6/well) were seeded into a 96-well flat bottom plate and cultured at 37°C and 5% CO_2 with or without mycobacterial antigen stimulation for 48 hrs. The antigens used for stimulation were *M. tuberculosis* culture filtrate proteins (*M.tb*-CF) (2 μg/well). Cells were cultured in a total volume of 250 μl of cRPMI. Culture supernatants were collected at 48 hours and stored at −20°C until cytokine/chemokine measurement. TNF-α, IFN-γ IL-12p40 and IL-10 concentrations were measured by using duoset ELISA kits (R&D systems).

Nitric Oxide Production Measurement

The release of nitric oxide (NO) by lung derived cells was determined by measuring the end product of NO, nitrite, as previously described [23]. Briefly, diluted supernatants were added at a 1:1 ratio with Griess reagent buffer (Sigma-Aldrich). The absorbance was measured at 540 nm by a spectrophotometer. The final concentration of nitrite was calculated by referring to a standard curve prepared from 0 to 100 μM of sodium nitrite concentrations.

Cell Surface Immunostaining and Intracellular Cytokine Staining (ICCS)

All monoclonal antibodies (mAbs) used were purchased from BD Pharmingen. Immunostaining and FACS were carried out as previously described [9,24,25]. Briefly, cells were blocked for non-specific binding of their Fc receptors with anti-CD16/CD32 antibodies for 15 min and then stained for 30 min on ice with the appropriate combinations of fluorochrome-conjugated mAbs. Fluorochrome-conjugated mAbs to CD11b, CD11c, CD3, CD4, and CD8 were used. Appropriate controls were used for each antibody. For intracellular cytokine staining (ICCS), single cell suspensions from airway lumen, lung, spleen and MLN were cultured and stained as previously described [26]. Briefly, cells were cultured for 24 hours with or without mycobacterial antigens (*M.tb*-Culture Filtrate-CF and crude BCG), Golgi Plug (5 μg/ml brefeldin A BD Bioscience, Burlington, Ontario, Canada) was added 18 hours after stimulation. After culture, cells were washed and blocked with CD16/CD32 for 15 min on ice and stained with cell surface Abs. In some experiments, cells were then washed, permeabilized and stained with IFN-γ and IL-4, or TNF and IL-12, Abs according to the manufacturer's instructions included in the ICCS kit (BD Pharmingen). Stained cells were run on the LSRII (BD Biosciences) flow cytometer using FACSDiva software and data was analyzed with Flowjo software (Tree Star, Ashland, OR). Depending on the number of cells available, 100,000 to 250,000 events per sample were analyzed.

Bacterial Enumeration and Lung Histology

The bacterial load in the lung and spleen were enumerated as previously described. Briefly, half lungs and whole spleens were sterilely collected at the time of sacrifice and homogenized in PBS. Lung and spleen homogenates were subjected to serial dilution and plated on Middlebrook 7H10 agar plates, supplemented with Middlebrook OADC enrichment (Invitrogen). Bacterial plates were incubated at 37°C for 15–17 days until colonies were visible, at which time colonies were enumerated and the bacterial burden at time of sacrifice calculated.

For the evaluation of histological changes the left lungs of infected mice were isolated sectioned and stained with haematoxylin and eosin. Stained sections were mounted to slides and histological evaluation was performed by conventional light microscopy at various magnification (5×, 10×, 20×), looking for structurally and morphological changes associated with cigarette smoke exposure and/or mycobacterial infection. Histological sections were blindly scored for lung inflammation, cellular infiltration and granuloma formation using 5× magnification H&E stained lung sections. Three sections were evaluated per mouse lung, with 4–5 mice evaluated per group.

Statistical Analysis

Statistical analysis was performed using either one-way anova, or unpaired two-tailed student's *t* test using the statistical analysis component of GraphPad Prism software. Values of $p<0.05$ were considered statistically significant.

Results

Continuous, but not Discontinued, Cigarette Smoke Exposure Significantly Impairs Bacterial Control Following Pulmonary Mycobacterial Infection

In support of epidemiological data suggests that cigarette smoke significantly impacts the host's ability to control *M.tb* [5,6,14,15], experimental models have shown that mice exposed to cigarette smoke prior to *M.tb* infection reduces the host's ability to control bacterial growth and prevent dissemination [14,15]. However, to date no model has compared the impact of both prior and continuous cigarette smoke exposure on the development of anti-mycobacterial immunity and bacterial control. To address this question, we first established and characterized a 6-wk continuous cigarette smoke exposure model (Figure S1A). Cigarette smoke exposure significantly increased alveolar macrophages, neutrophils and lymphocytes in the airway lumen (Figure S1B/S1C). Consistent with increased inflammatory cells in the airway lumen was increased inflammatory cellularity in the bronchial epithelium and alveolar septa (Figure S1D–G). By using FACS, compared to sham, room air-exposed animals, the total lung mononuclear cells from cigarette smoke-exposed animals, contained a marked increased number of activated macrophages (CD11b+CD11c+) and neutrophils (CD11b+GR1+) and a F4/80+ cell population consistent with the phenotype of newly recruited macrophages (Figure S2A/S2B).

To investigate the impact of continuous and discontinuous cigarette smoke exposure on anti-mycobacterial host defense, mice are exposed to cigarette smoke or room air for a period of 6 wks and subsequently infected with mycobacterial BCG (Figure 1A), at which time cigarette smoke was discontinued (cessation model) for one group, while being continued until sacrifice in another (continuous exposure model). Continuous cigarette smoke exposure most significantly impaired bacterial control both locally in lung and systemically in the spleen (Figure 1B and 1C). However, by comparison cigarette smoke cessation (prior cigarette smoke exposure) partially restored mycobacterial control in both the lung and spleen (Figure 1B and 1C). Noting that cigarette smoke cessation for 4 wks significantly improved bacterial control, we sought to evaluate whether prolonged cessation would allow for prior cigarette smoke exposed mice to regain further improved bacterial control similar to that seen in room air exposed mice (Figure 2A). Indeed, compared to a 4-wk smoke cessation interval, at a 6-wk interval post-*M.tb* infection the bacterial control of prior cigarette smoke exposed mice was comparable to that of room air controls (Figure 2B/2C). The profound ability of cigarette smoking cessation to improve bacterial control indicates that continuous cigarette smoke exposure is required to maintain a robust suppressive effect on anti-mycobacterial immunity.

BCG immunization is implemented in most of the developing countries and it enhances anti-TB immunity in the lung of experimental animals [21]. As we have seen the improved protective immunity following cigarette smoking cessation (Figures 1 and 2), we examined whether this could also be the case in prior BCG-immunized animals. Using the model outlined in Figure S3A, BCG-immunized mice that had previously been exposed to cigarette smoke, demonstrated a level of enhanced protection in the lung and spleen from *M.tb* challenge similar to that in *M.tb*-challenged room air (sham) exposed animals (Figure S3B/S3C). However, prior cigarette smoke exposure increased lung pathology in response to *M.tb* infection (Figure S3D–F). These results suggest that like in unimmunized hosts, cigarette smoking cessation helps restore protective immunity in the lung and spleen but at the expense of causing more pronounced lung pathology.

Continuous Cigarette Smoke Exposure Abrogates the Generation of Granuloma Formation and T Cell Immunity in the Lung during Pulmonary Mycobacterial Infection

To date little is known about how continuous cigarette smoke exposure influences the development of lung pathology during mycobacterial infection. Using the above described exposure

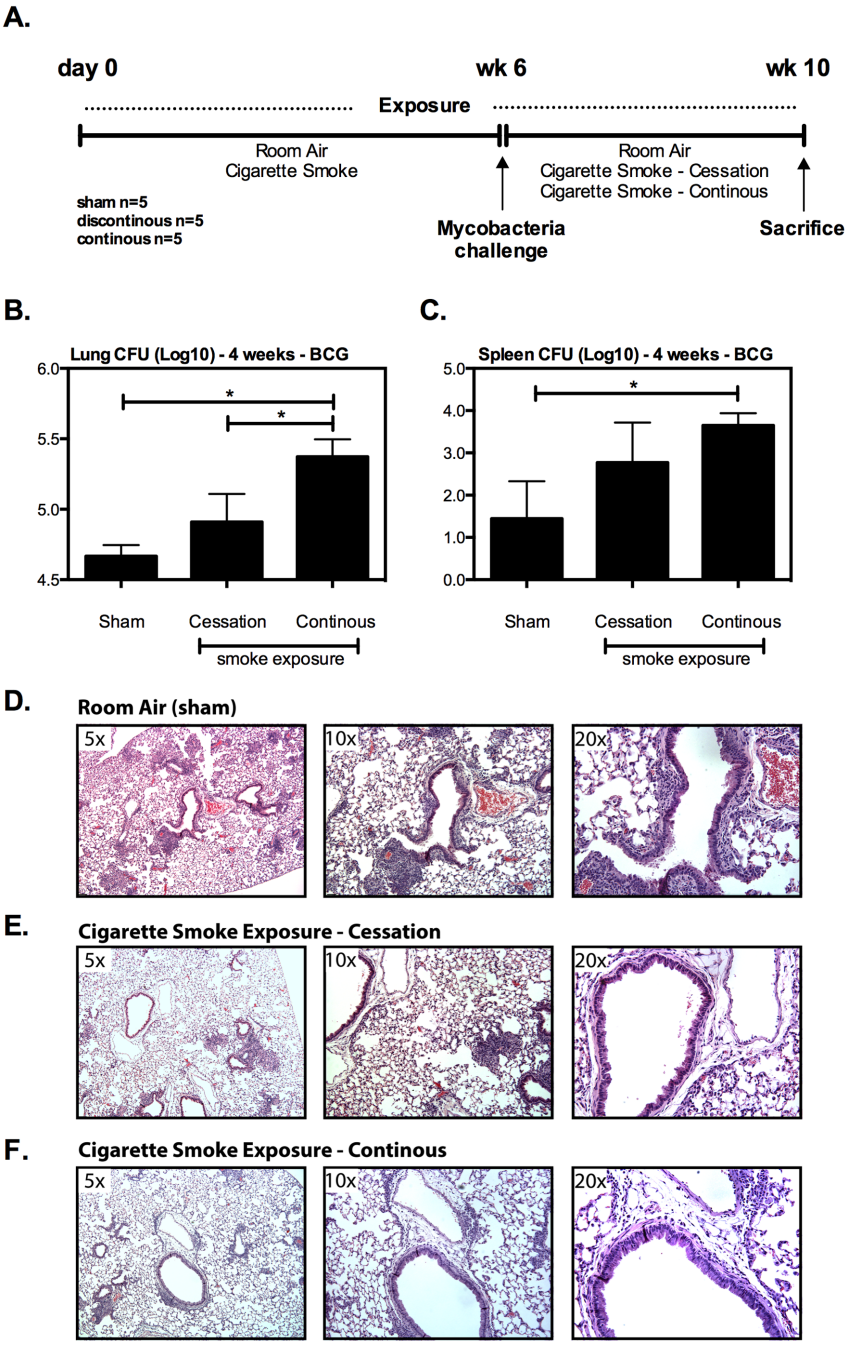
A.
day 0
wk 6
wk 10
Exposure
Room Air
Cigarette Smoke
Room Air
Cigarette Smoke - Cessation
Cigarette Smoke - Continous
sham n=5
discontinous n=5
continous n=5
Mycobacteria challenge
Sacrifice
B.
Lung CFU (Log10) - 4 weeks - BCG
6.0
5.5
5.0
4.5
Sham
Cessation
Continous
smoke exposure
C.
Spleen CFU (Log10) - 4 weeks - BCG
5.0
4.0
3.0
2.0
1.0
0.0
Sham
Cessation
Continous
smoke exposure
D.
Room Air (sham)
5x
10x
20x
E.
Cigarette Smoke Exposure - Cessation
5x
10x
20x
F.
Cigarette Smoke Exposure - Continous
5x
10x
20x

Figure 1. Continuous cigarette smoke exposure alters lung pathology and decreases bacterial control following pulmonary mycobacterial infection. Following 6 wks of cigarette smoke (or room air) exposure, mice were subjected to Bacillus Calmette–Guérin - *M. bovis* challenge (A). At the time of challenge one group of previously cs exposed mice was discontinued from cigarette smoke exposure to determine the impact of cessation of mycobacterial immunity, while another continued exposure for the duration of infection. At 4 wks post-infection, the bacterial burden following the various exposure protocols was determined by colony formation assay in the lung and spleen of mycobacterial infected mice (B&C), and the histological impact on lung pathology by H&E staining of lung sections isolated from infected mice (D–F). CFU numbers represents the mean and standard error of 5 mice per exposure protocol. Selected histological sections are representative of their exposure protocol. *p≤0.05.
doi:10.1371/journal.pone.0059185.g001

models (Figure 1A) we set out to determine the effect of cigarette smoke exposure on the development of lung granuloma and tissue inflammatory responses. At 4 wks following mycobacterial infection compared to vigorous tissue inflammation seen in the lung of room air exposure (Figure 1D), continuous cigarette smoke exposure markedly depressed cellular infiltration and granuloma formation in the lungs of mycobacterial-infected mice (Figure 1F; Table 1). Cigarette smoke cessation was found to increase cellular infiltration compared to continuous cigarette smoke exposure although the extent of infiltration did not reach what was seen in room air exposed mice (Figure 1D/1E; Figure 2D/2E; Tables 1 and 2).

Having noted that cigarette smoke cessation restored bacterial control by 6 wks post-*M.tb* challenge (Figure 2B/2C), we

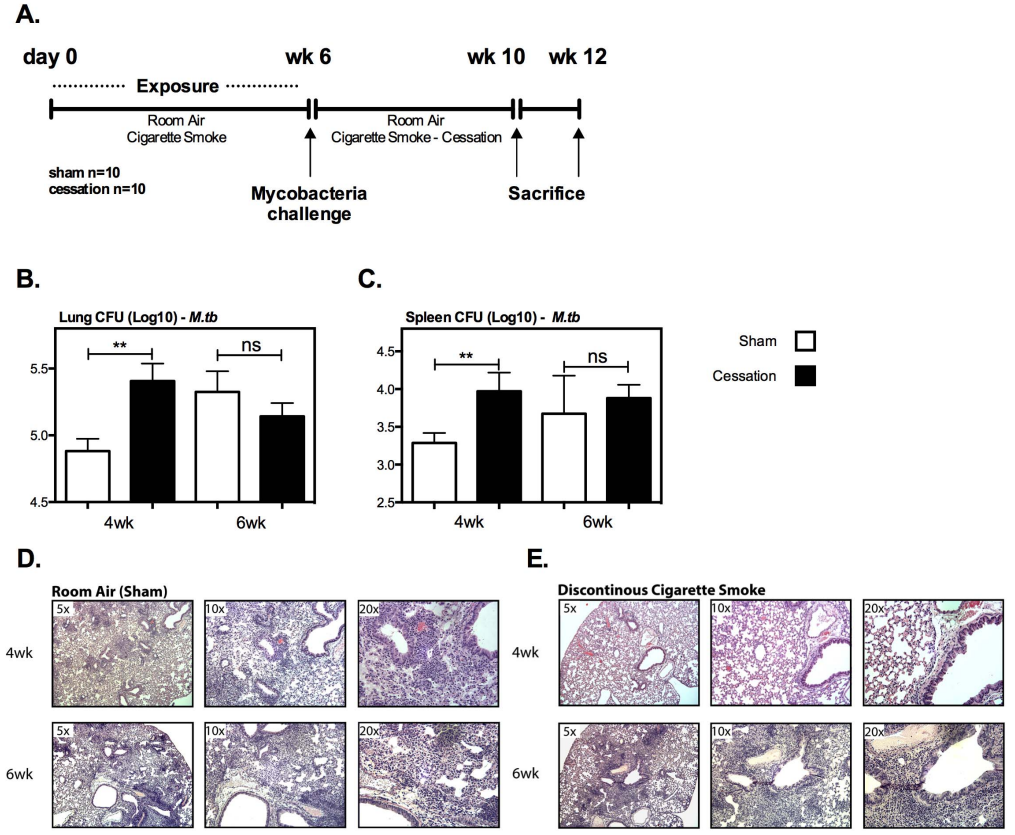

Figure 2. Prolonged cigarette smoke cessation enhances cellular infiltration, granuloma formation and bacterial control following mycobacterial challenge. Mice where either exposed to cigarette smoke or room air for a period of 6 wks, at which time both groups were subjected to challenge with *M.tb* $H_{37}Rv$ (A). The bacterial burden was determined in the lung (B), and spleen (C) by colony formation assay of organ homogenates from infected mice at 4 and 6 wk post infection. The histological impact on lung pathology was determined by H&E staining of lung sections isolated 4 and 6 wks post infection with *M.tb* (D&E). CFU numbers represent the mean and standard error of 5 mice per exposure protocol. The 4 wk challenge data is representative of two independent experiments. Selected histological sections are representative of their exposure protocol. *p≤0.05; **p≤0.01.
doi:10.1371/journal.pone.0059185.g002

Table 1. Assessment of Histopathological Changes in the Lung following BCG challenge.

	Room Air (sham)	Discontinuous	Continuous
size granuloma	+++	+++	++
number of granuloma	+++	+++	++
cellular infiltration	++++	+++	++

Granuloma size, granuloma number, and lung mononuclear cell infiltration were scored.
Results are representative of $n = 5$ mice/exposure/time point.
+, minimal; ++, slight; +++, moderate; ++++, marked; (+ half point).
doi:10.1371/journal.pone.0059185.t001

wondered whether cellular infiltration and granuloma formation was similarly restored. As anticipated, by 6 wks of cigarette smoke cessation the mice showed similar levels of lung cellular infiltration to room air controls, with notable granuloma formation (Figure 2D/2E; Table 2).

Given that continuous cigarette smoke exposure led to significantly impaired granulomatous inflammation in the lung following mycobacterial infection (Figure 1F), we examined whether it impacted the development of T cell immunity. In accordance with their severely impaired lung protection, the mice that were continuously exposed to cigarette smoke showed a pronounced defect in the accumulation of T cells in the lung. Continuous cigarette smoke exposure resulted in profound lymphopenia (lack of total CD4 T cells) in the lung (Figure 3A/3D), with virtually undetectable CD4+ IFN-γ+ T cell responses in both the airway lumen and lung interstitium (Figure 3B/3E/3C/3F). In contrast to continuous cigarette smoke exposure, by 4 wks cigarette smoking cessation had partially restored the recruitment of CD4+ IFN-γ+ T cells into the lung, resulting in an increase in both the frequency (Figure 3C/3F) and total numbers of CD4+IFN-γ+ T cells (Figure 3B/3E). Previously we have documented that cigarette smoke exposure significantly hampers the production of a critical T cell chemokine, RANTES (CCL5), by alveolar macrophages exposed to cigarette smoke [18]. Further, RANTES has been shown to play an essential role in the recruitment of antigen specific T cells to lung following *M.tb* infection [10] as the absence of RANTES delayed T cell entry into the lung and impaired bacterial control [10]. Based on these data, we opted to evaluate whether cigarette smoke exposure had attenuated the level of RANTES produced in our mycobacterial infection model. To do so, bronchoalveolar lavage fluids (BALF) were collected at the time of sacrifice, and a specific ELISA for RANTES was conducted. In keeping with T cell data, continuous exposure to cigarette smoke significantly attenuated levels of RANTES by >70% (Figure 3G). On the other hand, cigarette smoke cessation partially restored the levels of RANTES although they did not reach the levels seen room air exposed mice (Figure 3G), correlating closely with the relative levels of T cell responses in the lung (Figure 3A–F).

In contrast with the observed T cell deficiency in the lung (Figure 3A–G), continuous cigarette smoke exposure resulted in an increased number of total CD4+ (Figure 4A) and activated CD4+IFN-γ+ T cells (Figure 4B) in the spleen, while having a minimal effect on numbers of T cells in the MLN (Figure 4C/4D), indicating that cigarette smoke exposure impairs the recruitment of CD4+IFN-γ+ to the lung, rather than suppressing their priming in the peripheral lymphoid tissues.

Table 2. Assessment of Histopathological Changes in the Lung following *M.tb* challenge.

	4 wk		6 wk	
	Sham	**Discontinuous**	**Sham**	**Discontinuous**
size granuloma	++++	+++	+++++	++++
number of granuloma	+++++	+++	+++++	++++
cellular infiltration	+++++	+++	+++++	+++++

Granuloma size, granuloma number, and lung mononuclear cell infiltration were scored.
Results are representative of $n = 5$/exposure/time point.
+++, moderate; ++++, marked; +++++, severe. (+ half point).
doi:10.1371/journal.pone.0059185.t002

Continuous Cigarette Smoke Exposure Reduces Type 1 but Enhances Type 2 Cytokine Responses in the Lung

Given the remarkable impact of cigarette smoke exposure, particularly the continuous cigarette smoke exposure, on the recruitment of Th1 polarized cells to the lung, we set out to evaluate whether cigarette smoke exposure had altered the balance of Th1 and Th2 T cells in the lung. Of significant interest, while continuous cigarette smoke exposure inhibited the recruitment of CD4+IFN-γ+ T cells to the lung (Figure 5A), it enhanced Th2 CD4+IL-4+ (Figure 5B) responses in the lung, suggesting that cigarette smoke exposure may alter the specific polarization of T cells that enter the lung.

To further investigate the effect of cigarette smoke exposure on the balance between Th1 and Th2 polarization during mycobacterial infection, lung mononuclear cells (MNC) were isolated from the different exposure groups and subjected to *ex vivo* recall stimulation with crude mycobacterial antigens and following 48 hr culture, supernatants were collected and the production of specific cytokines determine in collected supernatants. Continuous cigarette smoke exposure, but not cigarette smoking cessation, significantly impaired the production of TNF (Figure 6A), and Th1 cytokines IL-12 (Figure 6B), and IFN-γ (Figure 6C), while enhancing the production of Th2 cytokine IL-4 (Figure 6D). Given its critical mycobactericidal activities in infected macrophages [27], we also examined the levels of nitric oxide production. Consistent with severely diminished Th1 cytokine production, continuous cigarette smoke exposure severely hindered the ability of lung MNC to produce nitric oxide (Figure 6E). On the other hand, correlating with relatively unaltered Th1 cytokine responses, cigarette smoking cessation only minimally affected the production of nitric oxide (Figure 6E). Together, these data indicate that continuous cigarette smoke exposure, but not cigarette smoking cessation, markedly dampens the production of Th1 cytokines and bactericidal products in the lung. Thus severely blunted Th1 immunity in the lung by continuous cigarette smoke exposure is the mechanism for weakened mycobacterial control in the lung.

Continuous Cigarette Smoke Exposure Dampens the Functionality of APC Populations in the Lung, but not in the Spleen or MLN

Thus far we have observed that cigarette smoke exposure, particularly continuous cigarette smoke exposure, suppressed T cell responses in the lung, but had little impact on T cell priming in the mediastinal draining lymph nodes (MLN). In order to understand the potential mechanisms for this divergence in T cell

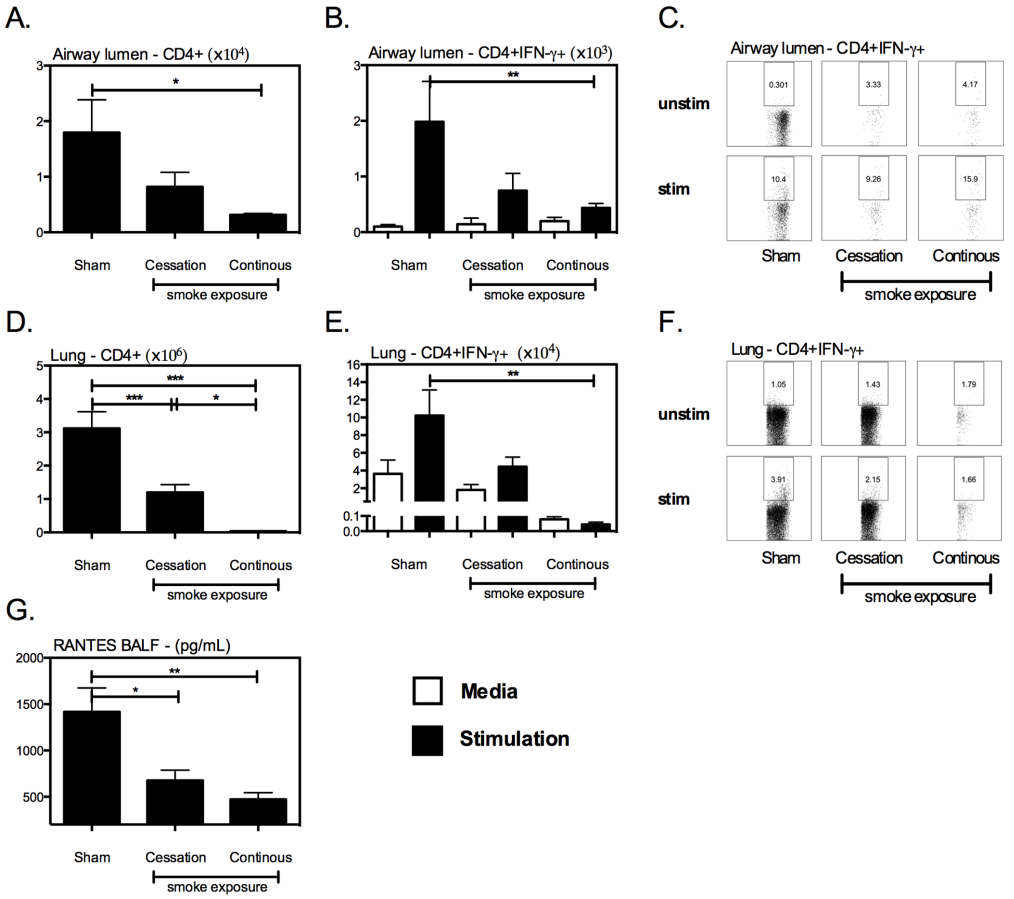

Figure 3. Continuous cigarette smoke exposure impairs the establishment of type 1 immunity in the lung of mycobacteria infected mice. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the establishment of type 1 immune response in the lung of mycobacterial infected mice. The numbers or frequencies of CD4+ and CD4+IFN-γ+ T cells were evaluated in the airway lumen (A/B/C), and the lung interstitium (D/E/F). The levels of RANTES were assessed in bronchoalveolar lavage fluids (BALFs) (G). Values represent the mean and standard error for 5 mice per exposure protocol. The dotplots are representative images from their respective groups. *p≤0.05; **p≤0.01; ***p≤0.001.
doi:10.1371/journal.pone.0059185.g003

responses, we sought to evaluate the phenotype of various APC populations in the lung, MLN and spleen following mycobacterial infection. Compared to sham room air exposure or cigarette smoking cessation groups, continuous cigarette smoke exposure dramatically reduced the frequency and total numbers of CD11b+CD11c−, CD11b+CD11c+, and CD11b−CD11c+ APCs, indicating a global reduction in the number of APCs in the lungs of these animals following mycobacterial infection (Figure 7A). Of interest, this effect was not seen in the spleen or MLN of continuous cigarette smoke-exposed mice, and the distributions of APC populations in these compartments were similar (Figure 7B/7C). In keeping with the T cell responses, cigarette smoke cessation partially restored the distribution of lung APC populations (Figure 7A), with no notable differences seen in the MLN or spleen (Figure 7B/7C) of the mice of cigarette smoking cessation.

To determine the functionality of lung APCs, we examined their production of Th1-polarizing cytokines. Following mycobacterial infection, continuous cigarette smoke exposure markedly reduced the numbers of IL-12-producing cells in all lung APC populations analyzed (Figure 8A). Continuous cigarette smoke exposure also similarly reduced IL-12 producers in all APC populations in the MLN (Figure 8B). In comparison, in keeping with T cell responses, cigarette smoking cessation did not reduce IL-12 producers in the lung and only mildly decreased it in the MLN (Figure 8B). Contrast to its profound effect on lung APCs, continuous cigarette smoke exposure had little impact on IL-12-producing APC populations in the spleen while cigarette smoke cessation even

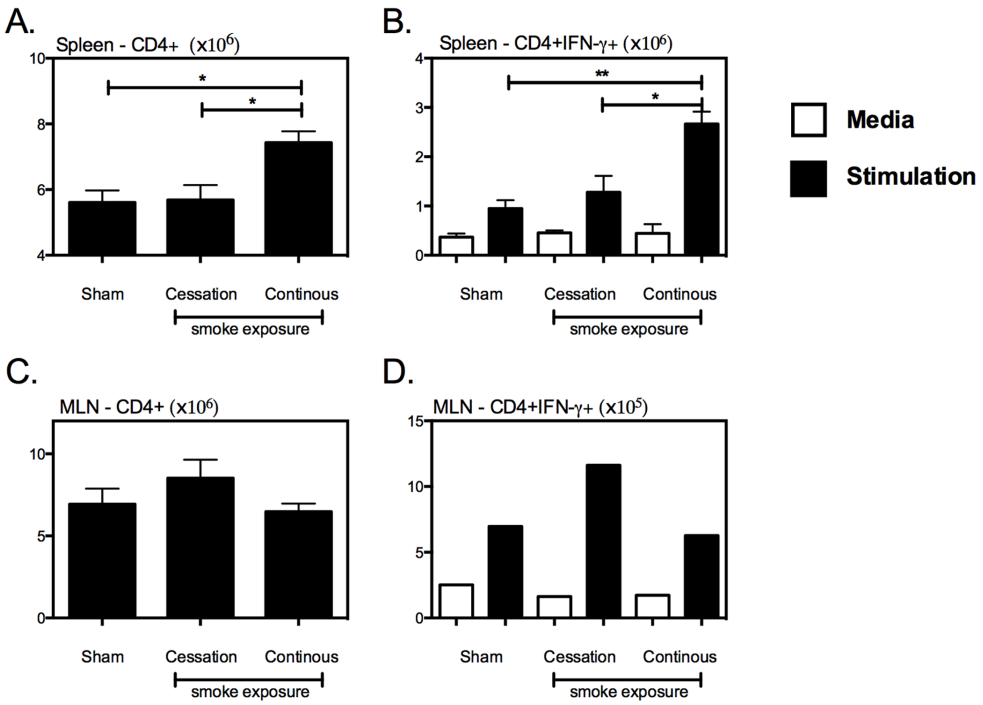

Figure 4. Continuous cigarette smoke exposure does not impair the generation of type 1 immunity in the in the spleen or MLN. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the establishment of type 1 immune response in the spleen and MLN of mycobacterial infected mice. The numbers of CD4+ and CD4+IFN-γ+ T cells were evaluated in the spleen (A&B), and the MLN (C&D). Values represent the mean and standard error for 5 mice per exposure protocol. *p≤0.05; **p≤0.01.
doi:10.1371/journal.pone.0059185.g004

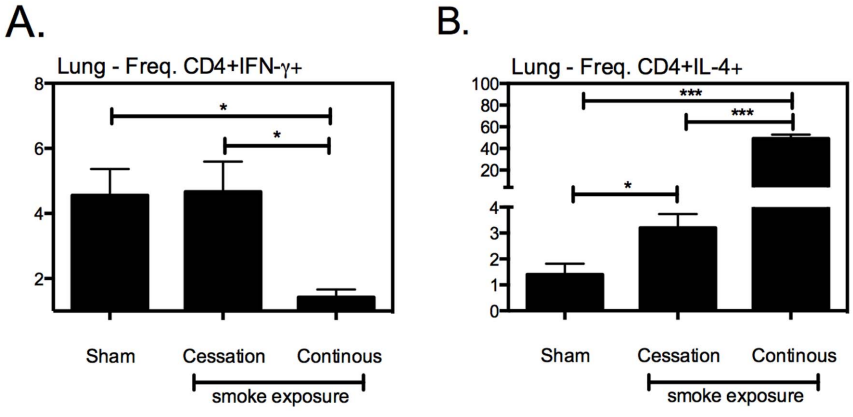

Figure 5. Continuous cigarette smoke exposure alters the balance of Th1 and Th2 CD4 T cells in the lung. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the frequency of CD4+IFN-γ+ (A) and CD4+IL-4+ (B) T cells in the airway lumen of mycobacterial infected mice. Values represent the mean and standard error for 5 mice per exposure protocol. *p≤0.05; **p≤0.01; ***p≤0.001.
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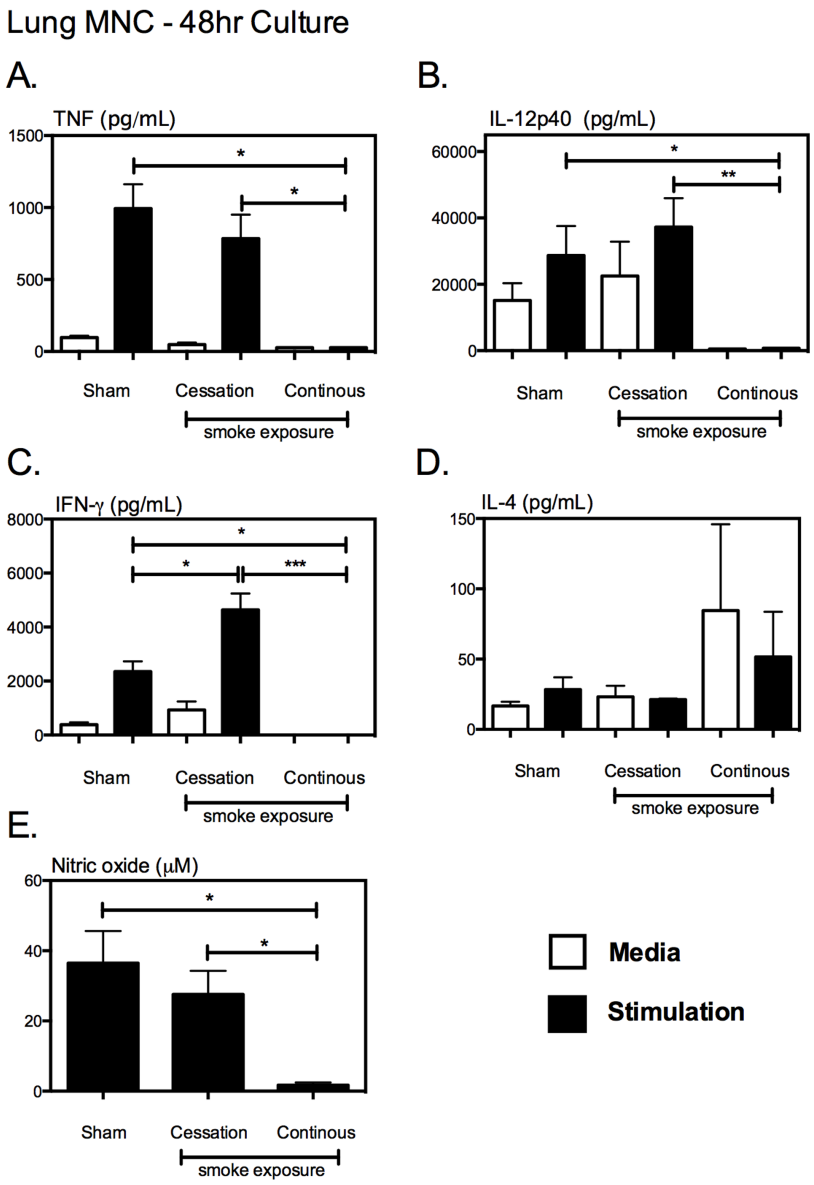

Figure 6. Continuous, but not discontinuous smoke exposure, impairs the production of type 1 cytokines while enhancing the production of IL-4, and reducing the production of bactericidal nitric oxide by lung MNCs following mycobacterial infection. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the production of type 1 &2 cytokines and nitric oxide by mycobacteria infected lung MNCs. Following 48 hr lung MNC culture, the levels of TNF (A), IL-12p40 (B), IFN-γ (C), IL-4 (D) were evaluated by cytokine ELISA, and production of nitric oxide (E) by a modified Griess assay. Values represent the mean and standard error for 5 mice per exposure protocol. *p≤0.05; **p≤0.01; ***p≤0.001.
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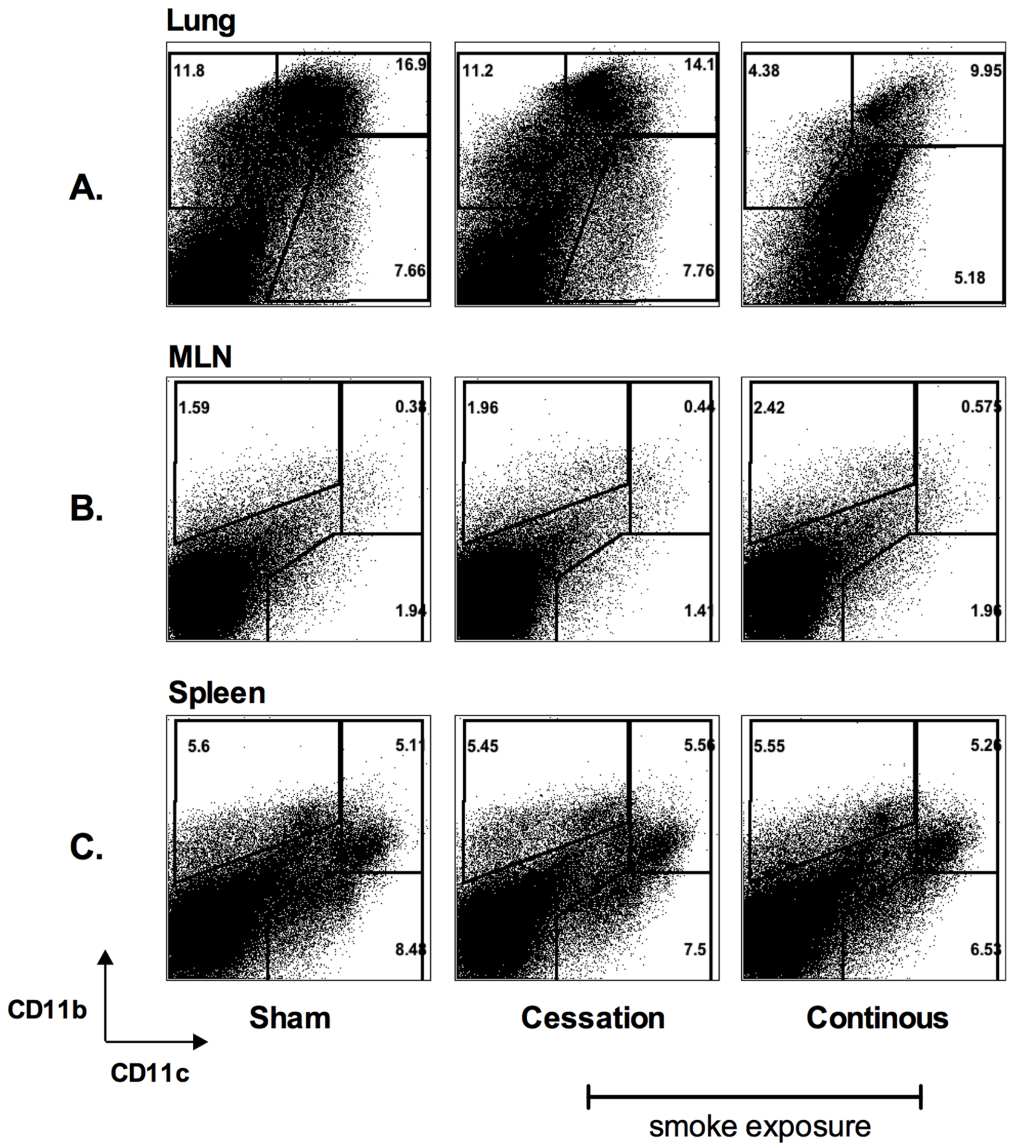

Figure 7. Continuous cigarette smoke exposure alters the surface marker expression of lung, but not spleen or MLN APC populations following mycobacterial infection. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the expression of common APC markers, CD11b+ and CD11c+ in the lung of mycobacterial infected mice. The distribution of CD11b+ and CD11c+ by APC populations of the lung (A), MLN (B) and spleen (C) were evaluated. Panels are representative flow plots for lung mononuclear cells isolated from 5 mice for each exposure protocol.
doi:10.1371/journal.pone.0059185.g007

somewhat increased such cells in the spleen (Figure 8C). The impact of cigarette smoke exposure on TNF-producing APCs in various tissue compartments was less pronounced (data not shown). Taken together, these data indicate that continuous cigarette smoke exposure, but not cigarette smoking cessation, severely reduces the number and activation of APC populations

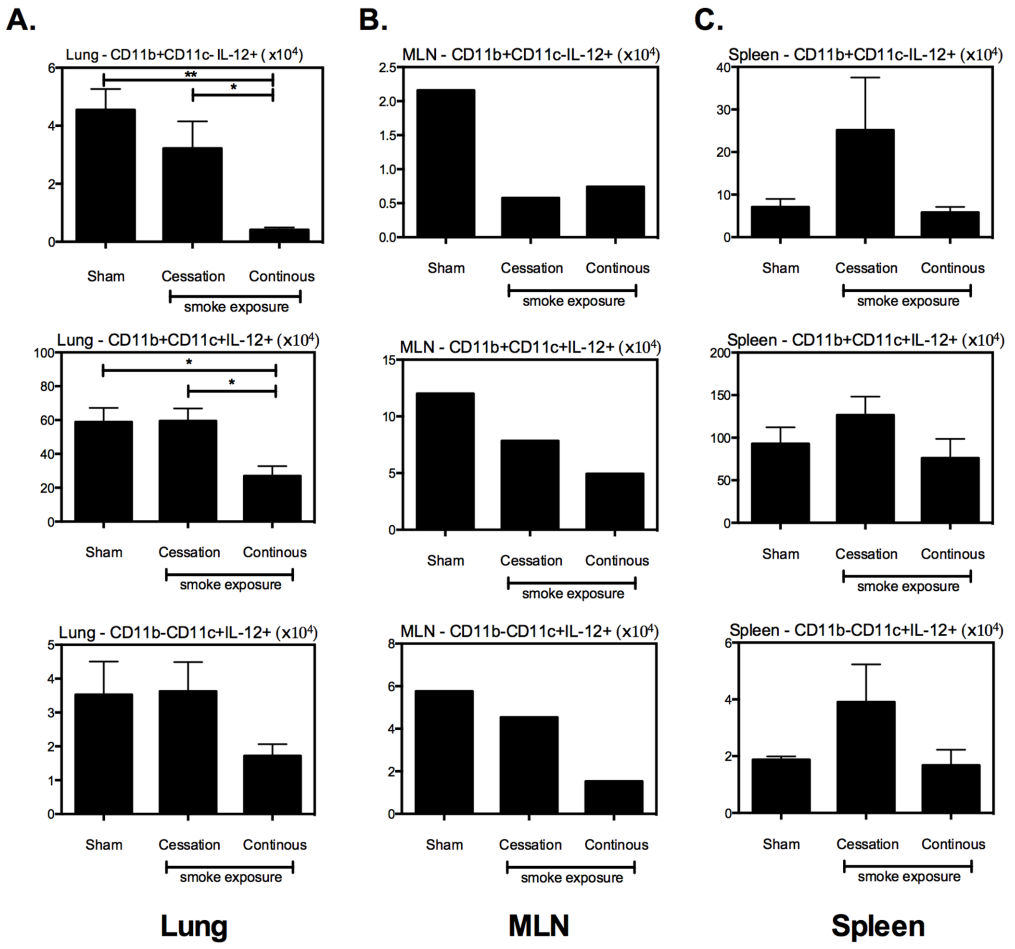

Figure 8. Continuous cigarette smoke exposure suppresses the ability of local, but not systemic, APCs to produce Th1 polarizing cytokines following mycobacterial infection. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the total number of IL-12 & TNF producing APCs in the lung (A), MLN (B), and spleen (C). Values represent the mean and standard error for 5 mice per exposure protocol. *p≤0.05; **p≤0.01.
doi:10.1371/journal.pone.0059185.g008

primarily in the lung with a much less effect in the systemic tissue compartments. These are likely the mechanisms accounting for the blunted T cell responses in the lung and unaltered T cells in the MLN and spleen in the animals that were continuously exposed to cigarette smoke.

Discussion

Despite strong epidemiological data linking cigarette smoke exposure to the development of active pulmonary TB, the role of cigarette smoke exposure in TB remains to be established. Recent experimental studies have only begun to dissect the relationship of prior cigarette smoke exposure to host anti-mycobacterial immunity [14,15]. In these studies, the cigarette smoke exposure was discontinued throughout the course of pulmonary mycobacterial infection. Thus to date it has remained completely unknown whether host defense against mycobacterial infection is differentially affected by continuous and discontinuous cigarette smoke exposure, and if so, whether cigarette smoking cessation may help restore the altered host defense.

We set out to address these questions in the models of pulmonary mycobacterial infection established by using both attenuated and virulent strains of mycobacteria. The use of attenuated *M. bovis* BCG strain allowed us to compare the effects of continuous and discontinuous cigarette smoke exposure on anti-mycobacterial immunity. Specifically, using this model, after

mycobacterial challenge the prior cigarette smoke-exposed animals were continuously exposed to cigarette smoke on a daily basis. Continuous cigarette smoke exposure post-*M.tb* infection is unfeasible within the P3 biohazard confinement facility and is made possible only when an attenuated mycobacterial species is used for challenge. However, we used a virulent strain of *M.tb* ($H_{37}Rv$) to verify the protection result that compared to continuous exposure, a 4-wk cigarette smoking cessation improved immune protection from pulmonary TB. Of importance, we extended this observation and found that a 6-wk cigarette smoking cessation completely restored anti-TB immune protection to the level seen in sham room air-exposed animals. Despite the virulent nature of *M.tb* $H_{37}Rv$, there is significant variability in the virulence and inflammatory responses mounted by various region-specific clinical strains. Future studies may examine the potentially differential impact of cigarette smoke on the outcome associated with these strains [28]. Regardless, our findings hold significant implications to anti-cigarette smoking campaign, suggesting that smoking cessation is beneficial to restoring lung host defense mechanisms against pulmonary TB.

While continuous cigarette smoke exposure profoundly impacts local immunity within the lung, we did not note any alteration to the generation of CD4+IFN-γ+ T cells in the peripheral lymphoid organs of mycobacterial-*infected* mice. We demonstrate that whereas continuous cigarette smoke exposure profoundly alters the local lung immune environment attenuating the release of critical anti-mycobacterial cytokines, IL-12, TNF and IFN-γ and the T cell chemokine RANTES, cigarette smoking cessation moderated these affects. Given that a loss of any one of these cytokines can severely compromised mycobacterial immunity, it is interesting that the phenotype seen following cigarette smoke exposure is unique to any one deficiency model. To draw comparison, in the absence of IL-12, mycobacterial infected mice fail to mount Th1 responses, fail to control bacterial growth, and fail to localize T cells to lung, an observation shared with cigarette smoke exposure [9]. Similarly, TNF and IFN-γ are synergistically required for the production of nitric oxide without which bacterial dissemination rapidly occurs. Moreover, the absence of RANTES severely attenuates the recruitment of T cells to lung, impairing bacterial control [10]. Despite their similarities it should be noted that the development of lung pathology significantly differs between these deficiencies, with IL-12 and RANTES deficient mice displaying less lung pathology, while TNF and IFN-γ deficient mice displaying exaggerated lung pathology. The difference lies in the recruitment of T cells to the lung, where IL-12 and RANTES deficient mice recruit far fewer active T cells, TNF and IFN-γ deficient mice recruit far more, likely in an attempt to compensate for the impaired ability of the infected APCs to produce nitric oxide. While cigarette smoke impairs bacterial control we attribute the decreased lung pathology to the reduced recruitment of T cells to lung, similar to what has been documented with IL-12 and RANTES deficiencies. It is interesting that cigarette smoke so profoundly influences multiple anti-mycobacterial immune pathways, reducing APC's ability to recruit and maintain protective CD4+IFN-γ+ T cells in the lung, while simultaneously attenuating pathology. Furthermore, it should be noted that while cigarette smoke exposure significantly blocked T cell recruitment to lung, those T cells that did get recruited displayed enhanced Th2 responses, promoting an increased frequency of Th2 CD4+ IL-4+ T cells, and higher levels of IL-4. Not only did continuous cigarette smoking attenuate the establishment of Th1 immunity but it also augmented Th2 immunity, thus likely further impairing the host's ability to control mycobacteria. While the reduced production of RANTES likely contributed to the defective accumulation of T cells in the lung, it remains plausible that the unique environment created by cigarette smoke may have negatively impacted the survival of recruited T cells causing them to undergo apoptosis or necrosis. Moreover, while not addressed in this study cigarette smoke may have generated a suppressive population of T regulatory cells capable of attenuating Th1 immunity in the lung. Taken together, the unique influence of cigarette exposure on the development of mycobacterial immunity cannot be attributed to its effect on a single component of the host immune response, but rather it is due to its broad impact on the innate, and ensuing adaptive immune cells locally residing in the lung.

Our study for the first time demonstrates that sufficient cigarette smoke cessation restores protective immunity to *M.tb* challenge by reestablishing APC functionality, and promoting the recruitment of CD4+IFN-γ+ T cells to the lung. Conceivably, the enhanced recruitment of CD4+IFN-γ+ T cells can be attributed to increased levels of RANTES produced following smoking cessation. Moreover, the increased presence of IFN-γ-producing T cells likely contributed to the production of nitric oxide and enhanced bacterial control. These observations provide an explanation for the rapid recovery and restoration of TB immunity seen clinically in humans following cigarette smoking cessation [29]. Improved TB protective immunity in the lung by cigarette smoking cessation was further demonstrated in our BCG immunization model. Of note, such improvement appears even more robust in BCG-immunized animals than in unimmunized counterparts as the *M.tb*-challenged BCG-vaccinated mice of 4 wk smoking cessation had similarly improved protection as those of sham room air exposure. Although it is unfeasible to carry out such a study, conceivably continuous cigarette smoke exposure in *M.tb*-challenged BCG-immunized animals would have produced a different outcome, as has been documented clinically [4,5,6]. Nonetheless, the observation that cigarette smoking cessation allows the BCG-immunized hosts to even more quickly restore lung protection is highly relevant to TB endemic areas where BCG vaccination is routinely carried out in childhood. These findings together further support the view that cigarette smoking cessation will help control the global TB epidemic.

Our study further reveals that continuous cigarette smoke exposure results in much less lung granulomatous inflammation, in keeping with impaired innate and adaptive immune responses in the lung. This observation is highly significant as the majority of TB symptoms are due to the inflammatory responses generated by the host. Particularly alarming is the notion that cigarette smoke mediated - inflammatory suppression may allow for the infected host to remain asymptomatic despite active bacterial growth. Indeed, epidemiological data suggests that smokers are 9 times more likely to die of active TB than non-smokers, with the vast majority (83%) having no TB-like symptoms prior to the onset of disease [29]. This sharply contrasts non-smokers where mild TB-like symptoms are generally reported significantly before the onset of disease [29]. The notion that cigarette smoking may mask TB symptoms, allowing a critical bacterial threshold to be reached before diagnosis, may explain why the likelihood of mortality is so much higher in TB-infected smokers. Together, our findings imply that cigarette smoke exposure has the capacity to augment the lethality of this deadly pathogen by impairing host mechanisms of bacterial control.

In summary, our data demonstrates that cigarette smoke impacts anti-TB immunity largely through impairing the recruitment and maintenance of Th1 T cells in the lung, rather than impairing systemic T cell priming. Furthermore we have shown that cigarette smoke exposure must be maintained for its

immunosuppressive effects to persist, where cigarette smoke cessation restores chemotactic signals, promoting the recruitment of T cells to lung, vastly improving bacterial control. Therefore, we have experimentally provided novel information on how cigarette smoke exposure impacts the establishment of anti-mycobacterial immunity in the lung, and why protective immunity to *M.tb* can be rapidly restored following cigarette smoking cessation. Our findings suggest that one of the effective ways to avoid/combat active TB is to stop cigarette smoking. Such recommendation is further supported by clinical observation that continued cigarette smoke exposure not only suppresses host immunity but also hinders the effect of anti-TB antibiotic therapy [30].

Supporting Information

Figure S1 Cigarette smoke exposure recruits various immune cell populations and causes pronounced alterations to the lung structure. Following 6 wks of cigarette smoke (or room air) exposure mice were sacrificed and their lungs removed and bronchoalveolar lavage performed (A). One lobe of the collected lung was used for mononuclear cell isolation, and the remaining were sectioned and stained with H&E for the assessment of gross pathology. Cigarette smoke exposure altered the percentage (B) and absolute numbers (C) of various immune cells infiltrating the airway lumen. Lung histological sections revealed pronounced structural changes were induced by cigarette smoke (cs) exposure (D). Specifically, cs resulted in increased alveolar space (E), inflammation of the alveolar septum (F), and moderate epithelial damage (G). Differential cell counts represent the mean frequencies and total numbers of 5 room air and 5 cigarette smoke exposed mice. Specific sections displayed for the assessment of gross pathology are representative of each exposure group.
(TIFF)

Figure S2 Cigarette smoke exposure alters surface marker expression on lung APC populations. Following 6 wks of cigarette smoke (or room air) exposure mice were sacrificed and their lungs removed and processed for mononuclear cell isolation. Specific changes in the expression of APC markers were determined by flow cytometry. Representative flow plots for lung mononuclear cells isolated from 5 individual room air (A) or cs exposed mice (B). Samples were stained for CD11b, CD11c, GR1 and F4/80 to determine the change in specific lung APC populations.
(TIFF)

Figure S3 Prior cigarette smoke exposure does not impair BCG vaccine efficacy following *M.tb* challenge. Following subcutaneous BCG immunization, mice were exposed for a period of 6 wks to cigarette smoke (or room air). Following cigarette smoke exposure immunized and unimmunized mice were subjected to *M.tb* $H_{37}Rv$ challenge. At the time of challenge cigarette smoke exposure was discontinued (A). *M.tb* infected, prior cigarette smoke exposed BCG vaccinated mice were compared to room air- unimmunized and immunization controls. The bacterial burden following the various exposure protocols was determined by colony formation assay in the lung and spleen of infected mice (B&C), and the histological impact on lung pathology determined by H&E staining of lung sections (D–F). CFU numbers represent the mean and standard error of 5 mice exposed to either, continuous cigarette smoke, or room air and BCG immunized. Selected histological sections are representative of the independent groups with 5 mice per exposure protocol. Values *$p \leq 0.05$; **$p \leq 0.01$.
(TIFF)

Author Contributions

Conceived and designed the experiments: CRS MRS ZX. Performed the experiments: CRS CNH SM MJ AK AZ JK. Analyzed the data: CRS MRS ZX. Wrote the paper: CRS MRS ZX.

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CHAPTER 5: DISCUSSION:

[1.0] Discussion of key findings

Tuberculosis has plagued mankind throughout human evolution (175,176,177,178) and remains a serious global health concern. Despite the high vaccination coverage within TB endemic areas, there is still a failure to control adult pulmonary tuberculosis. This is largely due to the delayed initiation of the immune response at the site of *M.tb* infection: the respiratory mucosa. Furthermore, it remains unknown what effects specific environmental influences in the lungs have on subsequent host defenses against *M.tb,* and by what mechanisms these processes mediate such effects.

In each of the studies described above, we evaluated the impact of three different environmental influences affecting the respiratory mucosa on the protection against pulmonary tuberculosis. While many of the risk factors for tuberculosis have been well characterized, mechanistic insight into the failure of *M.tb* control following cigarette smoke exposure remains to be elucidated. In contrast, the impact of other environmental factors such as the chronic exposure to common allergens have not been investigated to date. It is this knowledge that is necessary to gain insight into mechanisms of *M.tb* control, and how the local microenvironment of the lung can influence such control in the hopes of increasing the protective efficacy of vaccines against *M.tb,* and fuel the development of novel vaccine and therapeutic initiatives.

In our first study, we sought to determine the T cell distribution following BCG parenteral immunization, and whether T cell distribution could be manipulated to enhance the protective efficacy of the BCG. We demonstrated for the first time that following

parenteral BCG immunization, there was a complete absence of Ag-specific T cells residing within the airway lumen. In addition, we found that this population does not appear within the airway luminal compartment until at least 10 days following *M.tb* infection, and the arrival of such cells is associated with bacterial control. Further supporting the importance of Ag-specific T cells within the airway lumen, we also found that following *M.tb* infection in unimmunized mice, the recruitment of Ag-specific cells into the airway was even further delayed compared to BCG immunized mice, and bacterial control was not achieved until such recruitment. Given the association between Ag-specific airway luminal T cells and protection against *M.tb,* we then sought to increase the efficacy of BCG immunized animals through the installation of Ag-specific T cells to the airway lumen. To this end, we demonstrated that by delivering soluble *M.tb* culture filtrate proteins intranasally to BCG immunized mice, BCG-primed T cells could be recruited and maintained within the airway lumen, resulting in enhance protection against virulent *M.tb* challenge. Indeed, it was the presence of these Ag-specific cells at the site of *M.tb* infection that was demonstrated to be the mechanism by which earlier bacterial control could be achieved, with BCG/CF treated mice able to control bacterial replication as early as two weeks post infection. This was demonstrated by both the earlier airway luminal T cell responses following *M.tb* infection, and that when Ag-specific T cells but not naïve T cells were intratracheally delivered to BCG immunized mice, earlier and enhanced protection was observed. Therefore, collectively we were able to profile the Ag-specific T cell responses in BCG immunized and unimmunized mice

within the airway lumen, and enhance the protective efficacy of the BCG by installing T cells within this compartment.

In our second study, we aimed to investigate the impact of HDM-induced allergic immunity on host anti-mycobacterial control. We found that contrary to our expectations, a pre-existing allergic environment and continued allergen exposure resulted in enhanced protection against virulent *M.tb* infection. Moreover, we found that the enhanced protection against *M.tb* following continuous HDM exposure was dependent on TLR4-mediated production of IL-17. We demonstrated that following *M.tb* infection in HDM-treated animals, the T cells residing within the lung underwent a phenotypic change from that of a predominantly Th2/Th17 phenotype to that of a Th17/Th1 phenotype in a TLR4-dependent manner. These T cells, and the production of IL-17 following *M.tb* infection were shown to be absolutely required for HDM-mediated enhanced protection, as both T cell and IL-17 depletion via monoclonal antibodies ablated the protective response against pulmonary tuberculosis. Interestingly, we also found that such protective responses were independent of IFN-γ production, despite enhanced production of nitric oxide and bacteriocidal capacity in macrophages isolated from HDM-exposed mice. Indeed, when we investigated further, we found that IL-17 was responsible for the induction of enhanced nitric oxide production, and bactericidal capability following HDM exposure. IL-17-dependant nitric oxide was shown to be absolutely required for HDM-mediated enhanced protection against *M.tb* infection, as selective inhibition of the iNOS pathway resulted in ablated protective responses as well as impaired killing in isolated macrophages. Thus taken together, we have found a protective role of allergic immunity

against *M.tb* infection and that this protection is achieved via a TLR4-dependant induction of IL-17 and nitric oxide.

In our final study, we explored the mechanisms by which cigarette smoke alters the host immune response to mycobacterial infection, and impairs pulmonary bacterial control. To address this question, we employed a model of cigarette exposure for 6 weeks, at which point mice were infected with *M.tb* and received either continued or discontinued smoke exposure. We found that mice that continued to be smoke exposed were impaired in the ability to control *M.tb* infection compared to those that were never smoked and those that were discontinued from smoking. When we investigated the mechanism by which cigarette smoke impairs immune function during pulmonary *M.tb* infection, we found several aspects of the host immune response to *M.tb* was impaired. We discovered that despite Ag-specific T cell responses in the peripheral organs such as the spleen, and in the local draining lymph nodes, there was a significant impairment in the number of Ag-specific T cells recruited into the lung interstitium and airway lumen of *M.tb* infected smoke exposed animals. This impaired recruitment of T cells into the lungs was associated with a decrease in the production of the Th1 chemo-attractant cytokine RANTES.

In addition to T cell impairment, we found that there was also decreased functionality and recruitment of the APC populations within the lung. We demonstrated that such APC populations were deficient in the capacity to produce IL-12 and there was an overall impairment of the production of TNF-α, IL-12, IFN-γ, and nitric oxide by lung mononuclear cells. In contrast to the lack of Th1 cytokine production, there was an

increase in the production of IL-4 from airway luminal and lung interstitial Ag-specific T cells as well as isolated lung mononuclear cells from smoke exposed animals. Overall, we concluded that smoke exposure significantly impaired immune control following *M.tb* infection, and that this impairment was attributed to both the reduction in T cell and APC recruitment and functionality in the lung and airway lumen. It was also evident that upon cessation of smoke exposure, immune function was restored within the lung and airway lumen and bacterial control could eventually be achieved.

[2.0] Common themes

The primary objective of our work was to evaluate if our central hypothesis that T cell geography is critical to host defense against mycobacterial infection. We further sought to determine specifically if the longer the delay in T cell arrival to the airway lumen following infection, the greater the delay in bacterial control. Finally, we proposed that environmental modulation of the respiratory lung mucosa would result in an altered host immune response and subsequent bacterial control following pulmonary mycobacterial challenge. Therefore, each of the three studies described test this hypothesis and address this common theme.

[2.1] The importance of T cell geography in protection against *M.tb*

Throughout each study, we investigated the role of T cell geography in host defense against pulmonary *M.tb* infection. While previous work from our lab has demonstrated the differential location of Ag-specific T cells following mucosal and parenteral immunization with the human AdAg85a TB vaccine (56), the location of T cells following parenteral BCG immunization had never been fully characterized. Thus

we found that similar to other parenterally administered TB vaccines, parenteral BCG immunization results in Ag-specific T cells populating the peripheral locations of the lung interstitium and the spleen, but not the airway mucosa. This lack of T cells within the respiratory mucosa is associated with a delay in bacterial control until such T cells arrive. However, as we found, T cell geography is not a fixed entity. Peripherally located T cells can be relocated into the respiratory mucosa by the intranasal administration of *M.tb* culture filtrate proteins.

Similar to parenterally BCG immunized animals prior to *M.tb* infection, cigarette smoke exposed animals display peripherally located Ag-specific T cells following *M.tb* infection. However, while parenterally immunized hosts exhibit delayed recruitment of Ag-specific T cells into the airway, cigarette smoke exposure significantly impairs the recruitment process. Thus, the impaired recruitment of T cells into both the lung interstitium and the airway lumen following cigarette exposure results in uncontrolled bacterial replication and severe outcome from the infection. The association between T cell geography and bacterial control is further cemented by the observation that smoke cessation restores the T cell homing into the lung and with it, immune control of bacterial replication.

Indeed, our study of allergic immunity on the protection of pulmonary TB demonstrated that protective T cell responses within the respiratory mucosa were crucial to bacterial control. While such T cells were not Ag-specific in nature, the ability of these T cells to mediate protective effector responses immediately following infection was a direct result of the proximity to the infection. The importance of T cell location is

particularly reflected in the early bacterial control that is seen in animals chronically exposed to HDM and infected with *M.tb*.

Therefore collectively these studies demonstrate the association between T cell geography and protection against pulmonary *M.tb* infection. In particular, the closer the T cells reside in relation to the site of *M.tb* infection, the greater the immune control, and the lower the bacterial burden within the lung. Thus these results provide justification for the evaluation of T cell geography in the context of TB vaccination, and for the redistrubution of such geography in the effort to enhance protection against *M.tb* infection.

[2.2] The contribution of airway luminal T cells

As an extension of the importance of T cell geography in protection against pulmonary *M.tb* infection, we determined that the most critical location for protective T cell responses is to reside at the site of infection; the airway lumen. Indeed, all three studies collectively support the critical role of airway luminal T cells in protection against *M.tb.* In our BGC/CF study, the absence of airway luminal T cells following parenteral BCG vaccination was associated with a delay in bacterial control until their arrival within this compartment at least 10 days post *M.tb* infection despite the presence of Ag-specific T cells within the lung interstitium. This delay was even further exacerbated in unimmunized mice following *M.tb* infection, as T cells failed to infiltrate the airway lumen for at least two weeks post infection, and bacterial control was once again associated with their arrival within this compartment. The association between airway luminal T cells and protection was further solidified by the fact that both adoptive transfer

and recruiting T cells from the periphery using *M.tb* culture filtrate proteins to install airway luminal T cells in parenterally BCG vaccinated mice resulted in early pronounced bacterial control compared to BCG immunization alone.

The importance of T cells within the airway lumen at the time of *M.tb* infection is critical to enhanced bacterial control. While this was demonstrated in the BGC/CF study by adoptive transfer and the use of *M.tb* culture filtrate protein delivery, it is also evident in our study of chronic exposure to HDM. In this study, the allergic inflammation that exists within the lungs and airways prior to challenge results in a robust population of protective HDM-specific T cells at the site of *M.tb* infection. However, if these T cells are depleted just prior to the time of *M.tb* challenge, HDM-mediated enhanced protection against *M.tb* is not observed despite continued inflammation within the lung.

Furthermore, if the recruitment of Ag-specific T cells into the airway lumen is further impaired such as that seen with smoke exposure, uncontrolled bacterial growth ensues until their eventual arrival. Indeed, in our model of smoke cessation we again observed the associated between the arrival of airway luminal T cells and *M.tb* protection. The arrival of Ag-specific T cells responses in this compartment was associated with an increase in IFN-γ production and subsequent APC activation, thus establishing control of bacterial replication within the lung.

[2.3] T cell kinetics within the lung following *M.tb* infection

In addition to establishing the requirement for airway luminal T cells, the arrival of such T cells within this lung compartment is an equally important consideration. In our BCG/CF study, we found that there existed an "immunological gap" in BCG

immunized mice, whereby there was still a significant delay in the homing of Ag-specific T cells into the airway following *M.tb* infection. While this delay was not as substantial as in unimmunized mice, unchecked bacterial replication was permitted until T cell arrival. Therefore, we found that when this delay could be overcome, protective T cell responses could be initiated earlier, and thus bacterial control could be obtained earlier. Indeed, we were able to overcome the substantial delay in bacterial control by manipulating T cell geography within BCG immunized mice through the intranasal delivery of *M.tb* culture filtrate proteins. When T cells are located within the airway prior to *M.tb* infection, we observed that they respond immediately, and such effector responses are associated with a reduction in the lung bacterial burden 2 weeks prior to that of BCG immunization alone.

Further supporting this conclusion is the immediate protective responses that are also seen in HDM exposed mice following *M.tb* infection. Continued HDM exposure results in immune control of bacterial replication within the first week of infection, which persists throughout the course of infection. This critical early control of bacterial replication requires protective T cells within the site of infection, as when they are depleted prior to *M.tb* challenge, such protection is ablated. Moreover, if the delay in protective T cell recruitment is even further delayed such as following cigarette smoke exposure, we find that bacterial control is as expected, further delayed. Therefore collectively, the data from all three studies support the conclusion that T cell kinetics and bacterial burden within the lung are undoubtedly linked. Specifically, the arrival of T

cells into the airway lumen is associated with the cessation of bacterial growth, and the earlier the arrival, the earlier bacterial control is achieved.

[2.4] Environmental modulation of respiratory mucosal immunity

The association between the presence of airway luminal T cells and bacterial control is critical to our understanding of mycobacterial host defense and exposure to environmental factors have implications for altering the kinetics of bacterial control. We found that modifying the airway lumen with *M.tb* culture filtrate proteins was able to effectively attract peripherally located Ag-specific T cells in parenterally BCG immunized mice. Thus by altering the local microenvironment to support the retention of protective Ag-specific T cells within the airway, we were able to achieve earlier and greater bacterial control within the lung. Another method by which we were able to alter the local environment in favor of immune protection was by the chronic exposure to the common aeroallergen, HDM. Unlike intranasal *M.tb* culture filtrate delivery, environmental manipulation by HDM did not enhance or induce Ag-specific T cell responses, but the local allergic inflammation was able to generate protective T cells within the site of *M.tb* infection.

In contrast however, cigarette smoke exposure severely impaired immune protection against *M.tb* infection due to the immunosuppressive effect exerted on the lung as a whole. Therefore, by impairing immune function in both the airway lumen and the lung interstitium, bacterial replication ensued unchecked. Indeed, only by lifting this environmental immune suppression with smoke cessation was immune function restored, and bacterial control achieved. Thus collectively, environmental factors have a profound

impact on immune control against pulmonary *M.tb* infection. It is obvious from our data that the outcome of *M.tb* infection is dependent on the nature of the environmental exposure and that importantly; resulting inflammation does not require antigen-specificity to be protective.

[2.5] Protective T cell responses and the requirement of nitric oxide

Our work investigating the impact of environmental modulation on the outcome of pulmonary *M.tb* infection has additionally provided insight into novel mechanisms of protection against *M.tb*. In this respect we have enhanced knowledge on the requirements of IFN-γ, IL-17, and nitric oxide in protection against pulmonary tuberculosis. It is apparent from both our BCG/CF and cigarette smoke exposure studies that in accordance with the literature, IFN-γ is essential to bacterial control and the induction of nitric oxide following *M.tb* infection. Indeed, we find that in both instances, potent IFN-γ responses associated with the influx of Ag-specific T cells into the site of infection is associated with a plateau of bacterial growth within the lung. IFN-γ production is also associated with the classical activation of infected macrophages within the lung, characterized by the production of nitric oxide. Thus our work has both confirmed the requirement of IFN-γ and nitric oxide in the control of *M.tb* replication, and established that the earlier these protective mediators are present, the earlier bacterial control is achieved.

However, we have also discovered a situation where IFN-γ is uniquely dispensable for immune protection against *M.tb* infection. In our chronic HDM exposure model we found that nitric oxide can be induced in the absence of IFN-γ by the production of IL-17. In this case, similar to IFN-γ producing T cells; IL-17 production by

T cells is also required early following *M.tb* infection in order to be protective. Indeed the kinetics of bacterial growth are comparable when either IFN-γ or IL-17 producing T cells are present within the airway lumen at the time of *M.tb* infection. This highlights the requirement of the downstream production of the bacteriostatic nitric oxide for bacterial control, and redundancies in the mechanism by which it can be induced. Therefore, collectively our data indicates that there is a critical requirement for the classical activation of *M.tb* infected macrophages, and while most often activated by IFN-γ, alteration of the immune environment can result in macrophage activation by other means.

[3.0] Significance of work

Our study on geographical redistribution of peripheral T cells in parenterally BCG immunized mice by intranasal delivery of *M.tb* culture filtrate proteins adds crucial knowledge to the field. For the first time, we characterized the T cell geography in BCG immunized mice by separating the lung into two separate compartments. We were able to show that despite a significant population of Ag-specific T cells within the lung interstitium, the airway lumen remains devoid of T cells. Furthermore, we characterized the Ag-specific T cell recruitment into the airway lumen for the first time following pulmonary *M.tb* infection in both BCG immunized and unimmunized mice. Our findings have provided significant insight into the mechanism of bacterial control following *M.tb* infection in that it is the arrival of T cells within the airway lumen, and not the lung interstitium that is associated with gain of bacterial control. This is significant in two respects: 1) that it is important to recognize and continue to characterize the lung as two

compartments and 2) the airway lumen is a better indication of both vaccine efficacy and bacterial control following *M.tb* infection.

Another significant result from this study is the novel insight into the failure of BCG in protecting against *M.tb* infection. Our data indicate that one of the major means by which BCG fails to provide adequate pulmonary protection against *M.tb* infection is the failure to install a population of Ag-specific T cells within the airway lumen; at the site of infection. Moreover, the airway luminal compartment does not become populated for at least ten days post infection despite the maintenance of a population of Ag-specific T cells within the lung. Thus, by demonstrating that when Ag-specific T cells are installed into the airway lumen, we were able to reinforce the importance of T cell geography following vaccination and the requirement of such T cells at the airway mucosa at the time of *M.tb* infection. This observation also applies to the future of TB vaccine design and the justification for mucosal immunization against TB. Therefore, preclinical and clinical research should focus on the location of Ag-specific T cells following immunization, and how such populations are correlated with protective efficacy.

Lastly, our use of intranasal delivery of *M.tb* culture filtrate proteins to parenterally BCG immunized animals provides a novel method by which the current vaccine platform can be enhanced without the introduction of a novel heterologous boost regime. This is especially significant as there is a large proportion of the world is currently vaccinated with BCG and continues to be vaccinated shortly after birth. The intranasal delivery of soluble *M.tb* culture filtrate proteins provides an extremely feasible

solution to the lack of efficacy of the BCG in controlling pulmonary TB. Specifically, this method allows for a cheap, easily mass produced, and practical means of enhancing the efficacy of the current "Gold Standard" by the simple redistribution of T cell geography. The feasibility of this potential solution would mean that intranasal administration would be more readily available and would not necessarily require delivery by a health care professional compared to that of a novel vaccine platform.

Our study on the impact of chronic allergen exposure on *M.tb* host defense has provided unquestionable novel insight into both *M.tb* immunity and T cell biology in the context of two opposing diseases. For the first time, we have demonstrated the immunological paradox of allergic immunity to pulmonary tuberculosis. In this respect, our study challenges the notion that prior Th2 responses in the lungs are detrimental to subsequent infections that require a dominant Th1 response for protection. Therefore this novel finding demonstrates that two diseases with opposing immune responses are in fact mutually beneficial and protective to the other. We must re-evaluate our dogmatic approach to the T helper cell paradigm and recognize that T cells in fact demonstrate a high degree of plasticity. This is especially important in our understanding of how the immune system works, and adapts to changing conditions. This knowledge should be applied to other co-infections and situations with opposing immune environments, in order to further our understanding of such immune responses, but also for the future development of vaccines and therapeutics.

In particular, this study has highlighted a novel mechanistic role of Th17 cells and IL-17 production in the protection against *M.tb* that can be applied to future TB vaccine

design. This knowledge will provide justification for using IL-17 responses as a marker for protection in the absence or in association with the typical association with IFN-γ. Indeed, our study provides a mechanism by which several studies investigating novel TB vaccine candidates have found that protection against *M.tb* was not associated with IFN-γ, but rather IL-17 responses; especially in studies involving respiratory mucosal vaccination (72). Therefore, our study challenges the current dogma in the field that IFN-γ is essential and always required for protection against *M.tb* infection. While this appears to be the case in unimmunized primary infection models, alterations to the local airway luminal environment by intranasal immunization or by environmental modulation with chronic allergen exposure can overcome the requirement for IFN-γ. Caution should be taken in the future when evaluating markers of immune protection against *M.tb*, and the use of IFN-γ as the only marker.

Our other novel finding from this study is the ultimate mechanism by which IL-17 is able to mediate protection against pulmonary *M.tb* infection. The observation that nitric oxide could be produced in the absence of IFN-γ by IL-17 following *M.tb* infection is a novel finding in the field of TB immunology. This adds to the current understanding of macrophage biology not only specifically in host defense against TB, but also in the broader context of classical macrophage activation. It is of particular importance that it is recognized that pathways of immune activation are not fixed, but are in fact plastic, and redundancies exist when the local immune environment changes.

Our study on the impact of cigarette smoke exposure on the outcome of pulmonary *M.tb* infection has added significant insight into the understanding of how

smoke exposure impairs host defense against pulmonary TB. Specifically, we were able to provide convincing mechanistic rationale as to the benefit of smoke cessation as a method of pulmonary TB treatment measures. In areas of the developing world that experience both the highest rates of pulmonary TB cases as well as the highest frequency of smokers, there is great concern and uncertainty as to the validity of tobacco control and smoking cessation programs as a mechanism of TB control and treatment. Our study therefore provides evidence that such measures should most defiantly be employed, as smoking cessation in the context of pulmonary TB is able to lift the veil of immunosuppression and support the regain of immune function within the lung. These findings are also importantly in line with epidemiological data from TB endemic areas that demonstrate a significant correlation between cigarette smoke exposure and outcome of a TB infection. Whereby smoke exposure is associated with increased rates of TB infection, active cases, and death; whereas smoke cessation and never-smokers have significantly decreased TB-associated risks.

In addition to real-world applications of our data in the human population, our findings are also particularly important in furthering our understanding of how smoke exposure impairs immune function specifically to pulmonary TB, as well as our general understanding of host defense mechanisms against *M.tb* infection as a whole. In this respect, we were able to demonstrate that cigarette smoke exposure not only impairs cellular immune function such as cytokine secretion critical to protection against *M.tb,* but it additionally affects APC and lymphocyte recruitment into the lungs following infection. This observation is of particular importance in the context of pulmonary *M.tb*

infection as discussed earlier, there is already a significant delay in immune activation within the lung interstitium, and airway luminal compartments due to direct suppression of the pathogen itself. Therefore in the case of cigarette exposure, the problem of delayed immune activation and recruitment into the site of *M.tb* infection becomes further augmented. This creates an environment whereby *M.tb* can replicate uninhibited within the lungs for an extended period of time and gain an even greater "foothold" than under normal conditions. The repercussions are severe as the risk of dissemination, lack of response to treatment, and therefore mortality is severely enhanced.

The important role of Ag-specific T cells in the lung, specifically within the airway lumen is also further reinforced by this study. In the case of cigarette exposure, there is a severe impairment in the recruitment of protective Ag-specific T cells into both the lung and especially the airway lumen despite adequate T cell priming and population of the periphery. Thus, the importance of T cell geography in pulmonary *M.tb* infection is critical to the outcome of the disease, as exemplified by the return of T cell homing in the event of smoke cessation, and subsequent bacterial control which does not occur in continuously smoked animals.

[4.0] Future directions

The findings from our first study on the manipulation of T cell geography in BCG immunized mice create the potential for several avenues of future pursuit. In a preclinical setting, the evaluation of optimal delivery time and dose of the soluble culture filtrate proteins should be elucidated. In this respect, investigating the efficacy of BCG/CF treatment at shorter or longer intervals post BCG vaccination would provide rationale for

a particular dose and delivery regimen for potential human clinical trials in the future. In addition, it should be confirmed that the same T cell responses in the lungs and airways occur when mice are challenged with virulent *M.tb* as with the avirulent strain. While the two strains normally yield comparable results, it is important that virulent *M.tb* displays similar T cell kinetics if this work is to be extrapolated for use within humans.

Future directions should also include further investigation into the mechanism by which Ag-specific T cells are recruited and maintained within the airway lumen following *M.tb* culture filtrate delivery in parenterally BCG immunized animals. Similarly, it would also be warranted to determine if recruited airway luminal T cells following BCG/CF treatment are subject to the same problem of waning over time as those found within the periphery, and if so, could this problem be reconciled by repeated *M.tb* CF administrations. Another potential investigative direction is to determine if BCG/CF treatment provides a superior foundation for which a heterologous vaccine could be used to boost prior BCG immunity, especially one that is administered intranasally. Such a regime would increase booster vaccine efficacy without the requirement for an intranasal BCG delivery, which is not feasible due to safety concerns. Indeed, further research should be conducted into the safety of intranasal *M.tb* CF delivery, especially in humans. The ultimate goal would be to utilize this novel strategy as a cheaper, easier, and effective way to enhance the efficacy of the current TB vaccine platform and thus clinical trials should be initiated in order to verify such advantages in humans.

The exciting findings from our study on the protective role of chronic allergen exposure on subsequent pulmonary tuberculosis provide a foundation for several novel

research directions. Given the wide geographic distribution of house dust mite, and the high proportion of cases of allergic asthma that can be attributed to this common aeroallergen, we chose this as our model. However, it would be worthwhile to investigate whether the same protective effect holds true with the use of other allergens such as chronic exposure to cockroach or ragweed. In addition, the impact of chronic allergen exposure on the efficacy of TB vaccines would also be a potential research direction. In this respect, knowledge would be gained on both the impact of allergic asthma on vaccine efficacy; in particular the BCG, and the immunologic profile of allergic asthma in prior BCG immunized mice. An extension of this avenue would be to investigate the impact of chronic allergen exposure on mucosal vaccination efficacy, and the impact of prior mucosal immunization on the development of allergic asthma.

One of the most important findings from this study was the indispensible role of IL-17, while IFN-γ was dispensable for HDM-mediated protection against *M.tb*. This is especially useful information to consider when evaluating what is considered to be a "protective" immune response generated by potential TB vaccine candidates. Therefore, future investigation into the role of IL-17 in TB vaccine efficacy should be used in conjunction with that of IFN-γ to establish a more accurate measure of protective immunity. Finally, there is a lack of epidemiological data investigating the association between allergic asthma and tuberculosis outcome. It would be highly beneficial to determine whether there is a real-world association between the two diseases in humans.

Although our study on the impact of cigarette smoke demonstrated that the impairment of host immunity towards pulmonary tuberculosis could be overcome by

smoke cessation, there are several areas that warrant further study. Firstly, due to the constraints of our bio-containment level 3 facility, we were unable to evaluate the effects of continuous smoke exposure following virulent *M.tb* infection. While it is presumed that a similar effect will be observed as with an avirulent infection, the results from our study should be confirmed with a virulent *M.tb* challenge that compares continued and discontinued smoke exposure. In addition, while we conducted some preliminary APC functionality characterization, future work could focus on furthering our understanding of smoke exposure on APC functionality, and the regain of such function following smoke cessation. In particular, defects in recruitment and homing molecules, antigen presentation, and activation are all potential directions.

One area of research that has yet to be explored is the effect of cigarette smoke exposure on tuberculosis vaccine efficacy. In particular, given the high proportion of individuals that smoke in TB endemic areas, and the waning immunity of BCG over time; it would be warranted to study the effect of cigarette smoke exposure on the protective efficacy of the BCG. Similarly, vaccines currently in clinical trials that are hoping to be used to boost prior BCG immunity should be evaluated in the context of individuals who smoke. In particular, the relationship between cigarette smoke and respiratory mucosal vaccination would be of primary concern. Specifically, the role of cigarette smoke exposure on the generation and maintenance of airway luminal T cells following intranasal immunization should be investigated. This is especially justified given the suppressive effect of cigarette smoke on the recruitment of T cells into the lung and airway lumen following *M.tb* infection. Furthermore, the effect on such airway luminal T

cell populations in regards to their functionality and therefore vaccine efficacy following *M.tb* infection should also be an area of pursuit.

[5.0] Summary and conclusions

Collectively, this body of scientific work has provided the field of immunology insight into mechanisms of host defense against pulmonary *M.tb* infection, and the impact of environmental modulation within the respiratory mucosa. Specifically, we have addressed the imperative role of airway luminal T cells in the control of *M.tb* replication within the lung, and the association of such T cell kinetics and immune control. Moreover, T cell geography was found to be a critical factor in both BCG vaccine efficacy, as well as under the environmental stress of smoke exposure following pulmonary *M.tb* infection.

In addition to the importance of T cell geography in protection against *M.tb* infection, the "protective" T cell phenotype in the context of TB was also addressed. Our research challenged the current dogma within the field of tuberculosis immunology with regards to the essential requirement of IFN-γ for protection. It is apparent, that while this cytokine is critical in naïve infection models, the role of IFN-γ is dispensable under different environmental conditions within the lung. Therefore, our finding that IL-17 can replace the requirement of IFN-γ for classical macrophage activation of iNOS following *M.tb* infection is both novel and advantageous in its potential for future applications. This knowledge will undoubtedly serve to benefit future TB vaccine and therapeutic design.

We also highlighted the impact of environmental conditions within the respiratory mucosa on the outcome of subsequent pulmonary *M.tb* infection. In the case of chronic

allergen exposure, we were able to demonstrate the paradoxical protective capacity of allergic immunity to TB, whereas cigarette smoke exposure had dire consequences on host immune defense. In both instances we were able to effectively demonstrate the resilient nature and the plasticity of the immune system. The observation that T cell phenotype is not a fixed entity, but an extremely flexible characteristic with the ability to adapt to changing immune environments is critical to both our understanding of T cell biology and immunology as a whole. We must resist the urge to label and "pigeon-hole" the constituents of the immune system, and instead recognize the system as a plastic and complex organization if we are to further our knowledge within the field, especially in the context of heterologous models.

Lastly, the resiliency of the immune response was particularly highlighted in our study of cigarette smoke exposure and *M.tb* infection. While smoke exposure results in the loss of immune function within the local respiratory mucosa, such function can be regained. This observation serves not only to benefit TB control efforts in support of tobacco control as part of first-line treatment efforts for tuberculosis, but can be extrapolated to the control of other respiratory infections. Dedicated resources and effort into promoting smoking cessation as part of a standard treatment for all respiratory infections should continue to be promoted and implemented on a global scale.

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APPENDIX I

Immunization strategies against pulmonary tuberculosis: considerations of T cell geography

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Published in *Advances in Experimental Medicine and Biology,* 2013, 783:267-278

Immunization Strategies Against Pulmonary Tuberculosis: Considerations of T Cell Geography

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Abstract Pulmonary tuberculosis (TB) remains a global health concern with an astounding 9 million new cases and 2 million deaths per year. This leading infectious cause of death remains highly prevalent with one third of the world's population latently infected with *Mycobacterium tuberculosis (M.tb)* despite routine vaccination against TB in endemic areas. The only approved TB vaccine is the Bacille Calmette-Guerin (BCG), which provides protection against childhood miliary tuberculosis and has been administered intradermally in humans for almost a century. While effective in preventing disseminated forms of TB, the BCG has variable efficacy in providing protection against pulmonary TB. Therefore, the BCG has been unable to control the instance of adult pulmonary TB which constitutes the global disease burden. Despite the fact that mechanisms underlying the lack of pulmonary protection provided by the BCG remain poorly understood, it remains the "Gold Standard" for vaccine-mediated protection against *M.tb* and will continue to be used for the foreseeable future. Therefore, continued effort has been placed on understanding the mechanisms behind the failure of BCG to provide sufficient protection against *M.tb* in the lung and to design new vaccines to be used in conjunction with the BCG as boost strategies to install protective immunity at the site of infection. Growing evidence supports that the route of immunization dictates the geographical location of TB-reactive T cells, and it is this distribution which predicts the protective outcome of such vaccine-elicited immunity. Such vaccines that are able to localize TB-reactive T cells to the lung and airway mucosa are thought to fill the "immunological gap" in the lung that is required for enhanced protection against *M.tb* infection. This chapter focuses on

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M. Divangahi (ed.), *The New Paradigm of Immunity to Tuberculosis*, Advances in Experimental Medicine and Biology 783, DOI: 10.1007/978-1-4614-6111-1_14,

the critical importance of T cell geography when designing new immunization strategies against pulmonary TB.

Keywords *Mycobacterium tuberculosis* (M.tb) · Bacille Calmette Guerin (BCG) vaccine · Effector T cells · T cell geography · Lung interstitium · Subunit vaccines · Genetic-based vaccines · Human type 5 adenovirus (Ad5-vectored) vaccines · Vesicular stomatitis virus-based vaccines · "Vaccineless" boost strategies · Chemokines · Viral-vectored TB vaccines

1 Introduction

Pulmonary tuberculosis (TB) is one of the leading infectious causes of morbidity and mortality, responsible for the deaths of approximately 2 million annually [1]. It is estimated that one-third of the world's population is currently latently infected with *Mycobacterium tuberculosis* (*M.tb*), of which 9 million individuals develop active disease each year [1]. The only TB vaccine, Bacille Calmette Guerin (BCG), is an attenuated strain of *Mycobacterium bovis* typically administered intradermally shortly after birth in TB endemic areas, and has been used in humans since the 1920s. However, while BCG is effective in protecting against severe forms of disseminated *M.tb* infections in children, it is largely ineffective in protecting against adult pulmonary TB of which constitutes the majority of the global burden of disease [2–8]. As such, a large proportion of individuals with active TB are BCG immunized, resulting in highly variable efficacy ranging from 0 to 80 %. The immunologic mechanisms underlying poor lung protection by BCG still remain poorly understood although it has been suggested that the variable efficacy of BCG can be attributed to a wide variety of factors including host genetics, the presence of environmental mycobacteria, and the type of memory T cells generated from the immunization [2, 4, 7]. However, despite its shortcoming, BCG is the "Gold Standard" for vaccine-mediated protection against *M.tb* infection, and BCG or an improved BCG vaccine will continue to be used in the foreseeable future. Thus, the continuing effort is required to better understand the immune mechanisms behind poor lung protection by parenteral BCG immunization and develop effective boost immunization strategies to fill up such "immunologic gap" for enhanced lung protection. Recent growing evidence has suggested that the route of immunization dictates the geographical distribution of TB-reactive T cells which determines the immune protective outcome in the lung following pulmonary *M.tb* infection. This chapter will focus on the important consideration of T cell geography in the design of immunization strategies against pulmonary TB.

2 T Cell Geography Following *M.tb* Infection in Non-Immunized and BCG-Immunized Hosts

It has now been widely accepted that one of the critical defects in the immune response following pulmonary *M.tb* infection is the delay in the initiation of T cell priming in the local draining lymph nodes [2]. This delay in T cell priming results in delayed recruitment of effector T cells to the airway lumen and lung interstitium, the principal site of infection. Thus, *M.tb* is permitted to increase at a logarithmic rate within the lungs of the infected host for approximately 20 days, creating a robust "foothold" before the arrival of antigen-specific effector T cells to the site of infection [2, 4, 9]. Concurrent with the mass arrival and abundance of effector T cells into the lungs approximately 18–20 days post-infection is the control of bacterial growth (a plateau) [2, 10].

Similar to their unimmunized counterparts, BCG vaccinated hosts also suffer delayed recruitment of antigen-specific effector T cells to the airway lumen and lung interstitium, albeit somewhat accelerated due to the peripheral presence of BCG-primed T cells poised for action. Given its intradermal route of administration, the BCG vaccine generates a population of T cells which reside in peripheral tissues. Although a moderate number of such T cells can also be found within the lung interstitium of BCG-immunized animals, several studies have demonstrated that these cells do not immediately undergo expansion upon *M.tb* infection [2, 10, 11] and there is still a lack of airway luminal T cells at least for up to 10 days [10]. Such a delay in effector T cell responses in the airway lumen of parenteral BCG hosts has been identified to be a critical immune mechanism for the lack of immune protection for the initial 14 days after *M.tb* challenge [10]. However, given the fact that parenteral BCG immunization does provide enhanced protection over unimmunized hosts around 4 weeks post-infection, BCG-primed T cells are functionally important. Increased effector T cell responses in the lung interstitium and appearance of airway luminal T cells are generally accelerated by approximately 5 days in parenteral BCG-immunized hosts challenged with *M.tb* when compared to the unimmunized controls [10]. Thus, antigen-specific T cells begin to increase within the lung interstitium and appear in the airway lumen around 12–14 days post-infection, which is associated with an earlier plateau of bacterial growth and a 1 log reduction in bacterial burden over unimmunized controls observed around 4 weeks post-infection [2, 10]. Therefore, in spite of a few days of acceleration in T cell responses both in the lung interstitium and airway luminal compartment of parenteral BCG-immunized hosts, there is a very significant "immunologic gap" (at least 10 days) present primarily within the airway lumen, which leaves the lung unprotected from *M.tb* in the early stage of infection. It is believed that this critical shortfall in T cell geography in parenteral BCG immunized hosts is one of the reasons behind the inability of BCG vaccination to effectively protect from pulmonary TB while it fares reasonably well in controlling disseminated forms of TB in children.

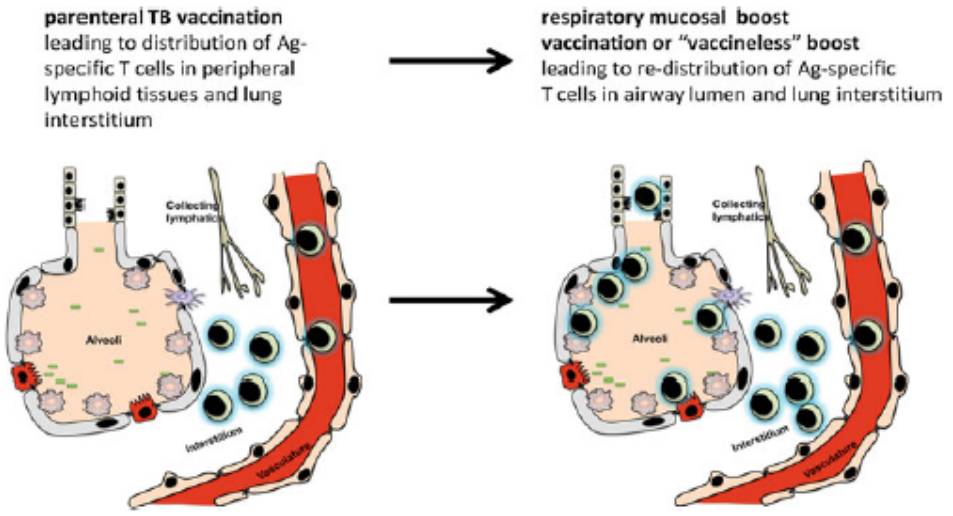

Fig. 1 Respiratory mucosal boost strategies used to install *M.tb* antigen-specific T cells on the surface of the respiratory mucosa for enhanced lung protection in parenterally TB immunized hosts

Thus, the emerging evidence establishes the importance of dividing the lung into the two major anatomic compartments for consideration of T geography and TB vaccination (Fig. 1): the lung interstitium present between the alveoli or between the airway and vasculature, and the mucosal surface of the lung identified as the airway lumen including both the conducting airway and alveoli [4, 5]. It is recognized that to date the majority of studies on pulmonary *M.tb* infection in naive or BCG-vaccinated host have focused on examining antigen-specific T cell responses in the whole lung without separately examining these cells in the lung interstitium and airway lumen [4]. Recent evidence gained from comparing parenteral and respiratory mucosal vaccination protocols has lent further support to the relevance of separately studying antigen-specific T cells both in the airway luminal and lung interstitial compartments [4] (Fig. 1).

3 Importance of T Cell Geography: Comparison of Primary Parenteral and Respiratory Mucosal Immunization Strategies

Immune control of *M.tb* in the lung following primary infection does not begin until the arrival of Ag-specific T cells in the lung following T cell priming in the local draining lymph nodes [2]. Although they bypass the initial priming stage in parenterally TB vaccinated hosts, peripherally located T cells still must enter the lung, particularly the airway lumen, in order to carry out their effector activities. While early vaccine studies primarily focused on the T cell distribution in the spleen and

lymph nodes [12–15], more recent studies have begun to examine T cell responses in both the lung interstitium and the spleen following parenteral TB immunization [16–19]. It is evident from such studies that parenteral immunization elicits robust T cell responses in the lung interstitium and peripheral lymphoid tissues such as the spleen, although the lung population varies with each vaccine candidate. However, when parenteral vaccination is compared head-to-head with mucosal administration with the same vaccine, it becomes apparent that robust T cell responses in the lung interstitium and spleen are not always correlated with protection against *M.tb* infection locally in the lung to the same extent as the presence of an airway luminal T cell population [4]. Thus, respiratory mucosal immunization fills up the "immunologic gap" associated with the parenteral immunization by installing abundant Ag-specific immune protective T cells on the respiratory mucosal surface and in the lung interstitium prior to pulmonary *M.tb* exposure.

Organism-based vaccines. While unlikely to be used as an intranasal vaccine in humans, BCG can be delivered parenterally, intranasally, or by aerosol. Intranasal immunization in mice was shown to provide better protection against pulmonary *M.tb* challenge than the subcutaneous route [20, 21]. Such improved protection was associated with more rapid T cell responses in response to mycobacterial challenge [20, 21]. While it is unknown whether mucosal administration of BCG resulted in a population of airway luminal T cells, there was an increase in the lung interstitial T cell population over that by parenteral administration [20]. The observation that respiratory mucosal delivery of BCG results in enhanced protection is not only limited to murine models, as aerosol-delivered BCG was also found to provide better protection than standard subcutaneous or intradermal BCG administration in guinea pigs [22].

Subunit vaccines. Due to the lack of immunogenic properties of purified or recombinant *M.tb* proteins alone, they require the addition of immune adjuvants as well as repeated deliveries in order to generate effective T cell responses [23, 24]. Nevertheless, several protein-based TB vaccines have been formulated and tested either via a parenteral or respiratory mucosal route. However, the head-to-head comparison studies are still lacking due to the fact that the immune adjuvant used for parenteral and respiratory mucosal deliveries is often different. One study [25] has compared the effect of subcutaneous with intranasal inoculation of a protein-based TB vaccine consisting of *M.tb* culture filtrate proteins and the adjuvant lipophilic quaternary ammonium salt, dimethyldioctadecylammonium bromide (DDA). It was found that the intranasal, but not subcutaneous, delivery resulted in elevated levels of antigen-specific T cell proliferation, IFN-γ, IL-12 in the cervical lymph nodes, and a greater induction of IgA in the nasal lavage [25]. Furthermore, the mice vaccinated intranasally exhibited enhanced protection in the lungs of *M.tb* challenged mice when compared to those immunized subcutaneously [25]. While this study did not fully characterize the T cell geography following the two vaccination routes, the results clearly support the advantage in vaccinating mucosally against pulmonary TB.

Ad5-vectored vaccines. Human type 5 adenovirus (Ad5)-based vector represents a robust *M.tb* antigen-expressing system due to its high level of transgene expression, excellent safety record in humans, high immunogenicity, and its

suitability for both parenteral and intranasal immunization [26, 27]. The first replication-defective Ad5-vectored TB vaccine engineered to express the *M.tb* Ag85A (AdAg85A) was evaluated both as a parenteral and mucosal vaccine candidate [16]. It was found that although intramuscular immunization with AdAg85A induced robust antigen-specific T cell responses in the spleen and lung interstitium, it failed to provide sufficient lung protection against *M.tb* challenge murine models [16]. It was further found that the peripherally distributed T cells activated by intramuscular AdAg85A immunization were not immunologically impaired as upon adoptively transferred into the airway lumen of na SCID mice, they were capable of potent immune protection [28]. On the other hand, respiratory mucosal administration of this vaccine elicited lower systemic levels of antigen-specific T cell responses, but much elevated T cell responses in the lung which correlated with superior protection following pulmonary mycobacterial infection, even surpassing that by parenteral BCG immunization [16]. Upon closer examination into the immune mechanisms, it was found that single respiratory mucosal immunization with AdAg85A elicited a significant antigen-specific CD8 T cell population in the airway lumen which was completely absent in the intramuscularly immunized group [28]. These T cells were of a long-lasting effector memory phenotype and were capable of self-renewing via continuing in situ proliferation in a specific Ag-dependent manner [29]. They remained immune-protective even in the absence of peripheral T cell supply [29] or in the absence of CD4 T cells [30]. These lines of evidence together indicate that following genetic-based TB immunization, the geographical distribution of antigen-specific T cells is closely associated with the route of immunization and ultimately the immune protective outcome against pulmonary *M.tb* challenge.

In addition to Ad5-based TB vaccines, other viral-vectored TB vaccines have also been developed and these include Ad35-based and vesicular stomatitis virus-based vaccines. However, following single intranasal immunization in murine models, these platforms were not as immune protective as the Ad5-based counterpart [31, 32].

4 Respiratory Mucosal Boost Immunization to Alter T Cell Geography in Parenteral BCG-Primed Hosts

The primary purpose of developing novel subunit- and genetic-based TB vaccines is to identify the candidate vaccines that can be used to boost protective immunity in the lung of parenteral BCG prime-immunized hosts [7]. As primary respiratory mucosal vaccination, by installing T cells on the surface of respiratory mucosa, consistently provides better protection against pulmonary *M.tb* exposure than parenteral vaccination [4], boost immunization via the respiratory tract shall most effectively enhance protective immunity in the lung of parenteral BCG prime-immunized hosts. Such respiratory mucosal boost immunization strategy enhances lung protection both by directly mobilizing the peripherally distributed pan-T cells

activated by parenteral BCG immunization into the lung and airway lumen and by activating the T cells specific to the *M.tb* antigen expressed by mucosally delivered vaccine and subsequently installing these cells in the lung.

Although most of the protein-based TB vaccine formulations are used parenterally, several studies have successfully used such vaccines intranasally to boost parenteral BCG immunization. Repeated intranasal deliveries of the fusion protein vaccine consisting of Ag85B-ESAT6 in LTK63 [33] or CTA1-DD/ISCOMs [34] induced potent T cell responses which served to boost the protection by parenteral BCG immunization. However, intranasal administration of an arabinomannan-tetanus toxoid conjugate (AM-TT) combined with a Eurocine adjuvant only enhanced protection in the spleen and not in the lungs of parenteral BCG-immunized mice [35]. Therefore, it appears that the efficacy of intranasal protein-based boost immunization in parenteral BCG hosts is dependent on the formulation of such boost vaccines.

The natural tropism of viral-vectored TB vaccines to the respiratory epithelium, their unique ability to derive robust transgenic *M.tb* Ag expression and their built-in immune adjuvant properties, make viral-vectored TB vaccines preferred candidates over protein- or plasmid DNA-based counterparts for respiratory mucosal boost immunization in parenteral BCG-immunized hosts [7, 26]. While the MVA-vectored TB vaccines when used as stand-alone vaccines do not induce potent T cell activation, when used for boosting parenteral BCG- or DNA plasmid-based prime immunization, these vectors are able to boost immune activation and protection against pulmonary *M.tb* challenge [12, 36]. Intranasal administration of MVAAg85A boosted BCG-primed T cell responses and protection to pulmonary *M.tb* infection [17], and is currently in clinical trials in South Africa based on an intramuscular immunization protocol. It remains to be seen whether this vaccine will also be amenable to respiratory mucosal immunization in humans [8].

The replication-defective Ad5-based vector, when delivered alone via the respiratory tract, has a record of proven safety and efficacy both in humans and animals [26, 27]. Thus, it makes an ideal respiratory boost vaccine candidate for parenteral BCG prime-immunized hosts. While respiratory mucosal delivery of AdAg85A alone provided a great level of protection following pulmonary *M.tb* challenge in mice, when delivered as a respiratory booster the immune protection in the lung was even further enhanced in parenteral BCG-immunized animals [37–39]. This was in contrast to the poorly enhanced lung protection by intramuscular boost immunization in the parenteral BCG hosts [37, 38]. This disparity between the respiratory and parenteral boosting was again attributed to the lack of Ag-specific airway luminal T cells by parenteral AdAg85A boosting despite its enhancing effects on lung interstitial and splenic T cells in parenteral BCG animals [4, 28]. In addition to murine models, the superior protection by respiratory mucosal boosting over the parenteral boosting with AdAg85A has also been demonstrated in parenteral BCG-immunized guinea pigs [40]. AdAg85A is currently under the evaluation in phase 1 clinical trials in both BCG-na and BCG-immunized healthy human volunteers in Canada but like all other TB vaccines currently in clinical trials, it is given parenterally. It is of importance to further

evaluate the effect of Ad5-based TB vaccine delivered via the respiratory tract in humans. This will allow us to appraise whether the pre-existence of anti-Ad5 immunity in most human populations may interfere with the safety and efficacy of Ad5-based TB vaccine. Apart from parenteral BCG priming and respiratory mucosal boosting strategies, a new immunization regimen involving simultaneous parenteral BCG and respiratory mucosal AdAg85A delivery has recently been investigated with promising results [41].

5 Respiratory Mucosal Manipulations of T Cell Geography Using "Vaccineless" Boost Strategies in Parenteral BCG-Primed Hosts

The "vaccineless" creation of airway luminal T cells in models of parenteral genetic-based immunization. The concept that respiratory mucosal boost immunization with various vaccine vectors, via installing T cells at the site of *M.tb* entry, provides the most robust protection in the lung, prompted the inquiry into whether the "vaccineless" boost strategies can be employed in place of boost vaccines. This concept is built in part on the surmise that early pulmonary *M.tb* exposure itself triggers insufficient innate immune responses within the airway luminal compartment, thus incapable of quickly mobilizing the peripherally spawned T cells by parenteral immunization into the airway lumen and lung interstitium [9]. Indeed, the concept of respiratory mucosal "vaccineless" boost strategy has been validated in murine models of viral- or plasmid DNA-based parenteral immunization. In this regard, intranasal delivery of CpG or the "empty" adenovirus elicited airway luminal T cells and restored the otherwise missing lung protection in intramuscularly AdAg85A-immunized mice [42]. However, such pro-inflammatory treatment does not deposit the specific *M.tb* antigens to the respiratory mucosa and thus can only confer a transient restoration of airway T cells and protection. On the other hand, intranasal delivery of soluble, nonadjuvanted Ag85 complex proteins creates a protracted restoration in either intramuscularly AdAg85A- or DNAAg85A-immunized animals [18, 42]. It was found that intranasal delivery of soluble mycobacterial antigens was sufficient to trigger the necessary responses of cytokines including TNF-α, MIP-1α, MCP-1, and IP-10 while keeping undesired tissue immunopathology to a minimum [18]. Thus, the blockade of either IP-10 or MIP-1α at the time of soluble antigen-delivery impaired the migration of peripherally primed antigen-specific T cells into the airway, associated with the loss of enhanced protection, indicating the essential role of chemokines for T cells to populate this lung compartment [18]. These data lend the support to using the "vaccineless" soluble, unadjuvanted *M.tb* culture filtrate proteins to elicit airway luminal T cells in parenteral BCG-immunized hosts.

The "vaccineless" creation of airway luminal T cells in parenteral BCG-immunized hosts. Parenteral BCG-immunized hosts fail to develop the airway luminal T cells in the initial 10–14 days after pulmonary *M.tb* exposure which is

responsible for the lack of lung protection in the first 2 weeks of time after *M.tb* challenge [10]. The arrival of T cells in the airway lumen ramps up to increased protection by 4 weeks post-challenge. Of note, in the same study it was observed that the T cells present within the lung interstitium were a poor correlate with lung protection [10]. These findings suggest that the "vaccineless" strategy capable of increasing airway luminal T cells would be expected to enhance lung protection by parenteral BCG immunization. Indeed, the intranasal delivery of soluble, unadjuvanted purified *M.tb* culture filtrate proteins artificially established a population of airway luminal T cells prior to *M.tb* infection in parenteral BCG-immunized mice. This airway luminal T cell population rapidly expanded upon pulmonary *M.tb* challenge whereas the lung interstitial T cell population remained unaltered [10]. Thus, the installation of airway luminal T cells prior to *M.tb* challenge in parenteral BCG-immunized hosts restored early lung protection that is otherwise missing in these animals [10]. Extrapolating from these findings, future research should focus on enhancing the speed at which T cell priming and migration to the airway luminal space occurs. Following primary *M.tb* infection it is essential to understand the role that the various immune molecules play in the recruitment of T cells both to the lung interstitium, and most importantly to the airway lumen. Such knowledge will provide further insights into the mechanisms of delayed or impaired T cell trafficking to the lung, and thus help design the strategies by which lung protection by parenteral BCG immunization can be enhanced.

Pros and cons of respiratory mucosal "vaccineless" boost strategies. The "vaccineless" purified *M.tb* antigen preparations (Ag85 complex or *M.tb* culture filtrate proteins) are not adjuvant-formulated, thus unable to prime T cells on their own. Compared to respiratory mucosal boost vaccination strategies, the "vaccineless" approach has several advantages. It is simple and cheap and potentially less pro-inflammatory, hence the safety for respiratory mucosal inoculation. Indeed, following intranasal inoculation of *M.tb* culture filtrate proteins, there was only a very mild inflammatory infiltrate seen around the bronchi [10, 18]. On the other hand, at least two repeated intranasal deliveries of *M.tb* antigen preparation are required to elicit robust airway luminal T cell responses in parenterally immunized animals [10, 18, 42]. In comparison, when a mucosal TB vaccine is used, a single administration suffices [16, 37]. It is also likely that vaccine-based strategies can most robustly create long-lasting memory T cells on the respiratory mucosal surface. Notwithstanding, the mucosal "vaccineless" boost strategy provides a new way to manipulate T cell geography for the benefit of enhancing local lung immune protection in parenterally immunized hosts.

6 Final Remarks

It is now widely accepted that parenteral BCG immunization fails to provide much needed mucosal protection against adult pulmonary TB. However, such parenteral immunization with BCG or an improved BCG vaccine is expected to continue in

human immunization programs. Emerging evidence suggests that the key to mend unsatisfactory lung protection by parenteral BCG is to increase immune protective T cells in the lung, particularly on the surface of the respiratory mucosa (Fig. 1). In this regard, there have been a few frontrunners including MVA- and adenovirus-based TB vaccines which are amenable to robust respiratory mucosal boost immunization in experimental models. These candidates have entered various phases of human trials but noticeably they are being given parenterally [43]. The challenge is to take the next step to evaluate the safety and efficacy of these vaccines following respiratory mucosal inoculation in humans. In order for this to happen, it is urgently needed to identify the reliable immunologic signatures in the peripheral blood that can be used to calibrate the levels of beneficial T cell responses occurring in the lung following respiratory mucosal immunization.

Acknowledgments The work from authors' laboratory is supported by funds from the Canadian Institutes for Health Research. Authors are grateful to Christopher Shaler for his assistance in graphic design.

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