CONTROLLING *M.TB*: A BALANCE OF IMMUNITY AND IMMUNOPATHOLOGY

CONTROLLING *MYCOBACTERIUM TUBERCULOSIS*: A BALANCE OF IMMUNITY AND IMMUNOPATHOLOGY

By CHRISTOPHER R. SHALER, B.SC

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AUTHOR:	CHRISTOPHER R. SHALER, B.Sc (McMaster University)
SUPERVISOR:	Dr. Zhou Xing
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ABSTRACT:

Pulmonary tuberculosis (TB) remains the third leading infectious killer worldwide. It is estimated that tuberculosis latently infects one third of the world's population and is responsible for 1.4 million deaths annually. *Mycobacterium tuberculosis (M.tb),* the bacteria species responsible for tuberculosis, is unique in its ability to evade and survive within the infected host for many years and in some cases the lifetime of the host. The ability of *M.tb* to survive within the host is linked to its ability to evade the host immune response. Following infection there is the establishment of a dynamic equilibrium between bacterial load and active immunity. Central to the establishment of this dynamic equilibrium and survival of the host is the formation of the pulmonary type I 'immune' granuloma. The granuloma is composed of a loose association of innate and adaptive immune cells, where infected macrophages are restricted and contained by activated T cells. The ability of the host to maintain this dynamic equilibrium is responsible for the long-term control of *M.tb* seen in the majority of infected hosts. However, only a small shift in immune activation can detrimentally alter this delicate balance leading to the development of active TB disease. A number of factors have been identified which enhance an individual's risk for disease reactivation. Some such risk factors include the acquisition of HIV-AIDS, exposure of cigarette smoke, and the neutralization of TNF, a key cytokine required for anti-TB immunity. Within this thesis we will investigate the underlying mechanisms associated with the establishment of the granuloma, as well as its disruption following cigarette smoke exposure and in the absence of TNF.

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ABBREVIATIONS AND SYMBOLS:

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
CR - Complement receptors
PRRs – pattern recognition receptors
TLRs- toll-like receptors
NOD - nucleotide-binding oligomerization domain protein
MR- mannose receptor
LAM – lipoarabinomannan
AEII - type II alveolar epithelial cells
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
CIITA - class II, major histocompatibility complex, transactivator
VCAM - vascular cell adhesion molecule
LFA - lymphocyte function-associated 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
MMP - Matrix metalloproteinases
Fas - Apoptosis Stimulating Fragment
M1 – macrophage polarization towards type 1 activation
M2 – macrophage polarization towards type 2 activation

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> **CH –** assisted in the design and execution of experiments and writing the manuscript
> **SM, MJ, AK -** assisted with the execution of experiments
> **AZ, JK –** assisted with animal care and smoke exposure
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CHAPTER 1: INTRODUCTION

1. The global TB epidemic

1.1. TB epidemiology

Pulmonary tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis (M.tb)* is the leading cause of death due to a bacterial pathogen and is responsible for 1.4 million deaths annually [1]. Currently it is estimated that one third of the world's population is latently infected with *Mycobacterium tuberculosis (M.tb)*. With an alarming 9 million new cases reported annually, *M.tb* is the third leading cause of death due to an infectious disease worldwide [1].

Despite the magnitude of cases globally, typically 90% of infected individuals will adequately control, but not clear, *M.tb,* resulting in a chronic latent infection [2]. While the majority of cases result in latency, approximately 10% of infected individuals will develop active TB at some point in their lifetime [Diagram 1]. Adequately controlled by a healthy immune system, a significant suppression of the host immune response is responsible for almost all cases of TB disease [2]. With one third of the world's population latently infected with *M.tb,* and the highest rates of infection overlapping with regions endemic to HIV-AIDS, TB is a major public health concern particularly in the developing world [2]. Augmented by the growing numbers of individuals acquiring an immuno-compromising disease within the TB endemic regions, the current HIV-AIDS pandemic has a significantly impacted the progression of the disease in latently infected hosts [2]. The ability of HIV-AIDS to dramatically suppress cellular immunity has made co-infections with *M.tb* particularly deadly. Of the estimated 1.4 million deaths by TB per year, approximately 400,000, nearly one third, are HIV-TB co-infected individuals,

highlighting the significance of this deadly co-infection [3]. While non-HIV infected individuals have a 10% lifetime risk of progressing to TB, largely due to a loss of CD4 T cell mediated immunity, an HIV co-infected host has estimated 5-10% annual risk of developing tuberculosis disease [4]. This highlights the significance of cellular immune control in the manifestation of disease following infection with *M.tb* infection.

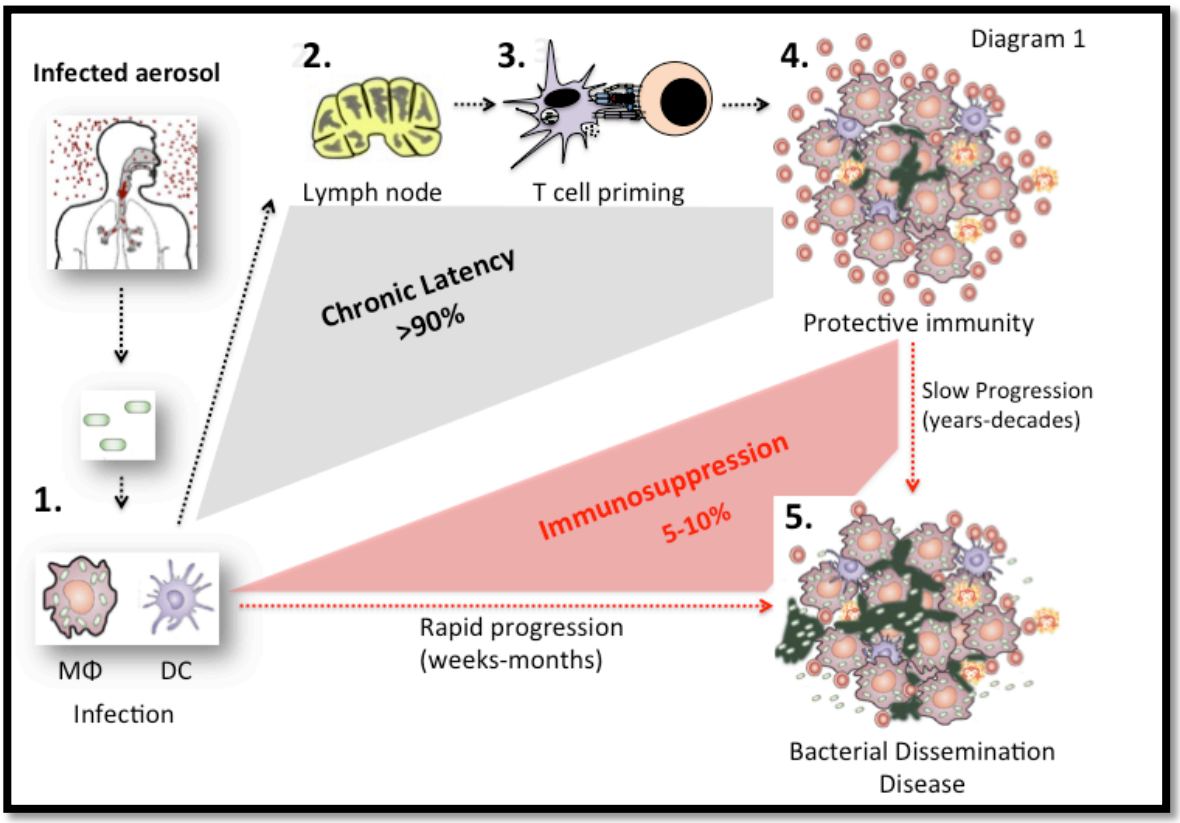

Diagram 1: The course of an *M.tb* infection.
Aerosol infection leads to uptake of APC populations within the lung (1). APCs migrate to the local draining lymph node (2) and facilitate T cell priming (3). Primed T cells migrate to lung where they form protective granulomas (4). In 5-10% of cases, an infected individual will transition to active disease within their lifetime (5).

M.tb, a facultative intracellular pathogen, is spread person-to-person through infected aerosols generated by coughing or sneezing. Once deposited in the airways, *M.tb* primarily infects the alveolar macrophage, the resident macrophage of the airway lumen [5,6]. While *M.tb* has a relatively low transmission rate, commonly considered to be 30% or less, although susceptibility is highly dependent upon living conditions, contact with infected individual(s), as well as nutrition and immune status [Diagram 2] [7,8,9,10]. Alterations to these variables can dramatically impact the susceptibility of an exposed individual to infection and it is estimated that under the correct conditions, a single bacillus could establish a successful infection [5]. The fact that *M.tb* is spread via aerosols, and can be infectious in low numbers makes it a major health concern in regions with high population densities, poor living conditions, and immune compromising diseases, such as those commonly seen in developing world.

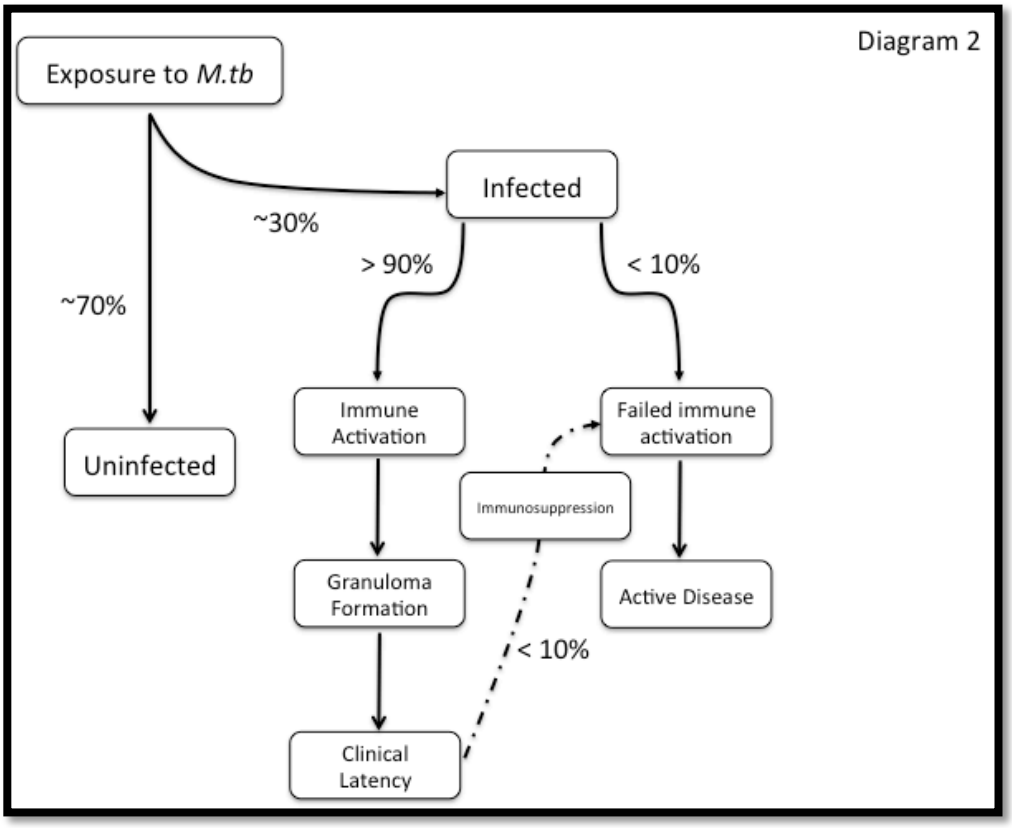

Diagram 2: Flow chart of TB disease progression and major events leading to protection. Major steps are outlined for the progression of an infected or uninfected hosts from the point of exposure to development of active disease or clinical latency (protection). The relative percentage of individuals to progress between steps is shown beside the appropriate progression line.

1.2. The current status of TB vaccines

Currently, vaccination against *M.tb* is carried out worldwide, with the exception of North America and recently some European countries. The only licensed TB vaccine is an attenuated *Mycobacterium bovis* strain, Bacillus Calmette-Guérin (BCG) that has been utilized extensively for more than 80 years. However, regardless of widespread implementation, BCG has exceedingly variable efficacy (0-80%) [11]. Even with the highest efficacy, BCG is only effective in limiting severe disseminated forms of TB in

children, not preventing infection or providing sterile immunity [11]. Moreover, the usefulness of BCG is further limited as protective immunity typically wanes by adolescence [12] and cannot be boosted by repeated BCG vaccination [13]. Despite extensive vaccine coverage in endemic areas, the incidence of TB has not significantly decreased since the implementation of BCG.

Due to the apparent inability of BCG to provide lifelong immunity, current research is focused on the development of a new vaccine to replace or more likely, to boost BCG. Given our limited understanding and the complexity of natural immunity there have been significant roadblocks in the efforts to develop an effective vaccine. Although much has been learned about the immune response to *M.tb* a number of major questions remain unanswered, in particular, why *M.tb* is not cleared in immune competent hosts despite the generation of significant cell mediated immunity.

Over the past decade, the development of novel TB vaccine candidates has produced a wealth of knowledge on the ways in which TB vaccination can be improved. Arguably the most significant finding has been the repeated observation that vaccinating mucosally provides enhanced protection over parenteral immunization against pulmonary *M.tb* infection. In particular, work from our laboratory has demonstrated that a recombinant adenoviral vector expressing *M.tb* antigen 85a (AdAg85a) when delivered intranasally was seen to provide enhanced protection against virulent *M.tb* challenge compared to intramuscular administration [14]. Furthermore, intranasal vaccination was able to provide superior protection over the 'Gold Standard' subcutaneous BCG immunization, with at least an additional log reduction in bacterial burden following *M.tb*

infection [14]. However, AdAg85a does not stand alone in this observation, as several others have reported that mucosal immunization provides greater protection against *M.tb* infection compared to parenteral vaccination [15,16].

The discrepancy between the protection against TB afforded by both mucosal and parenteral immunization stems from the fact that the lung can be divided into two main compartments: the interstitial tissue residing in between the alveoli, and the mucosal surface of the lung known as the airway lumen [Diagram 3].

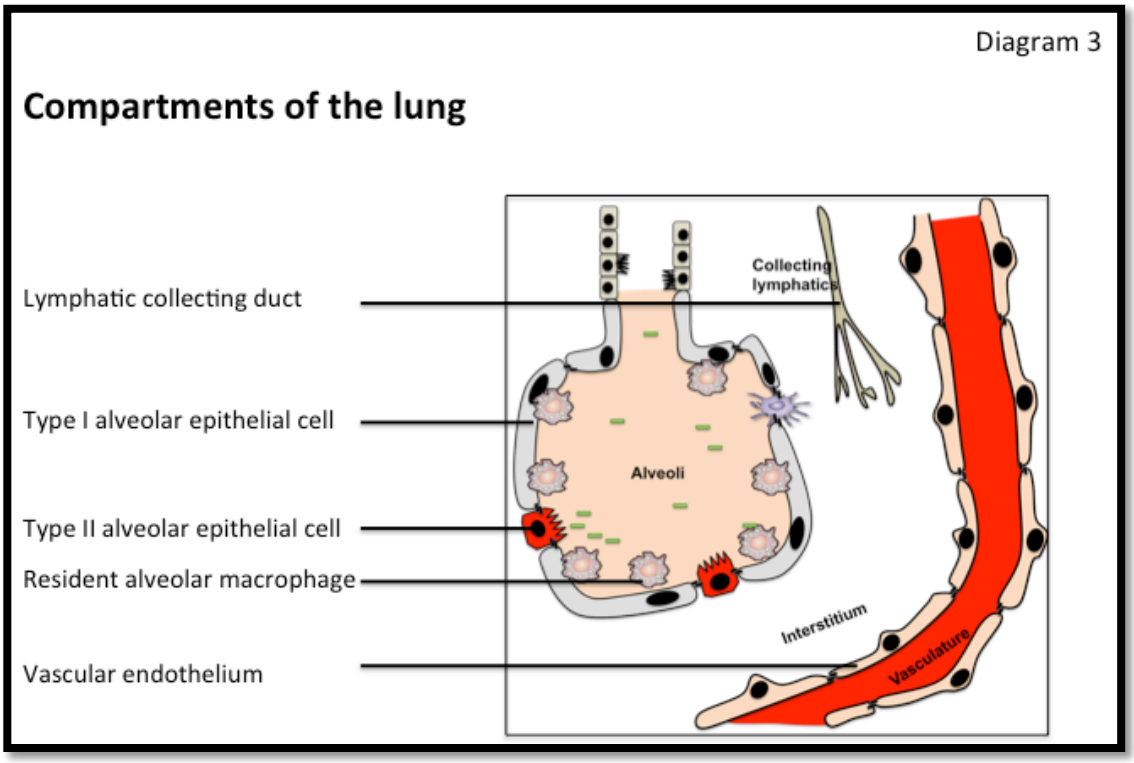

Diagram 3: Compartments of the lung. A diagram depicting the major lung compartments. The lung can be divided into airway lumen (alveoli), interstitial space, and the vasculature.

Although the current literature describes the kinetics of effector T cell responses in the lung interstitium, the role of airway luminal T cells (ALTs) in the airway lumen

have been largely neglected to date [17,18]. Recently our group and others have begun to characterize the T cell kinetics in these two lung tissue compartments. Moreover, despite BCG's ability to mobilize an earlier arrival of effector T cells in the lung interstitium, following *M.tb* infection it is the arrival of ALTs to the airway lumen that is associated with the plateau of bacterial replication [19]. Furthermore, investigation into the mechanisms by which this enhanced protection is achieved has revealed that mucosal but not parental vaccination resulted in the generation of a population of antigen-specific T cells that reside within the airway lumen [20]. The failure of parenteral vaccination to generate a population of ALTs can be largely attributed to the lack of an inflammatory response generated in the airway [20]. This defect is particularly evident in experiments where the delivery of soluble mycobacterial antigens to the airway prior to infection elicits a potent inflammatory response capable of overcoming the defects associated with parenteral vaccination [21,22]. The production of these inflammatory mediators function to draw in peripherally primed T cells, enhancing protection to a level comparable to that of mucosally vaccinated animals [21,22]. Moreover, the neutralization of IP-10 or MIP-1α at the time of soluble protein delivery significantly impaired the recruitment of peripherally primed T cells into the airway, ablating protection and thereby demonstrating the critical role of these chemokines in populating this compartment [21,22]. Specifically, ALTs were found to be critical for protection as they are capable of responding quickly upon *M.tb* infection, eliminating the delay of effector responses [21,22]. These findings indicate that both the timing and geographic localization of T cell responses is critical to the efficiency of bacterial control. 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 insight into the mechanisms of delayed or impaired T cell trafficking to the lung, and thus provide ways by which protection against *M.tb* can be enhanced.

1.3. Historical and current TB chemotherapeutics

In 1946, the first effective chemotherapeutic, streptomycin (SM) was discovered for treatment of TB [23]. While highly effective at first, within 5 years the majority of TB patients treated with SM died with similar kinetics to untreated individuals and it was determined that resistance to SM had been acquired by the majority of *M.tb* strains worldwide [23]. Shortly after the wide spread resistance to SM, it was determined that resistance could be prevented if SM was co-administered with para-aminosalicylic acid (PAS) [23]. In 1952 isoniazid (INH) was introduced and has been highly effective at preventing SM resistance and the treatment of TB. Isoniazid efficacy was largely due to the low minimum inhibitory concentration, and minimal toxicity [23]. However, similarly to SM, regions that were treated with INH alone rapidly developed resistance, and it was proposed that a combination therapy using SM, PAS and INH be widely adopted for the treatment of TB [23]. While highly effective, a duration of one year was required for the complete clearance of *M.tb* to be achieved. The length of treatment and the cost of drugs precluded the use of this strategy in most TB endemic regions [23]. Further studies determined that rifampicin (RMP) could effectively accelerate the bactericidal effects of the current treatment strategies, shortening the treatment period [23]. While some advancement has been made over the last 6 decades, the currently accepted treatment

involves co-administration of INH, RMP and or pyrazinaminde (PZA) and still requires 6 months to be effective [23]. Complicating this issue, the majority of antibiotics require replicating bacteria to be effective, and with *M.tb* having a very long half-life, which is increased when it enters into long-term latency, it takes several months to successfully clear *M.tb* [5,23]. Multidrug resistance is thought to be due to a failure of individuals to complete the entire treatment period, emphasizing the requirement for new more effective anti-TB drugs to be developed [23].

2. Mechanisms of immune mediated control of pulmonary TB

As one of the most persistent global health concerns, the success of *M.tb* as a human pathogen can be attributed to its ability to parasitize the host-pathogen microenvironment. Studies of ancient DNA and skeletal remains have traced the co-evolution of *M.tb* and pre-human lineages for nearly 3 million years [24,25,26,27,28]. As such, *M.tb* has evolved multiple mechanisms to evade, elude, and subvert the host immune system. For instance, compared to many other respiratory pathogens, *M.tb* infection slows adaptive T cell activation by eliciting much delayed T cell priming and subsequent recruitment to the lung [29,30]. Temporally, *M.tb* targets both early immune initiation as well as chronic bacterial control effectively preventing the host from ever achieving sterile immunity. While much research has been done to understand the various ways *M.tb* suppresses established immunity, little progress has been made in understanding the mechanisms underlying delayed early adaptive immune activation.

2.1. Infection and innate immune activation

Infection*: Mycobacteria tuberculosis* (*M.tb*) is a facultative intracellular pathogen, which primarily infects the resident macrophage of the airway lumen, and replicates intracellularly, shielding it from the host humoral response [31]. *M.tb* is spread through aerosols generated by an infected individual [32] which are deposited in the lung airways [5,6]. Once infected aerosols are taken into the lung and deposited in the alveolar space, *M.tb* is actively taken up by the resident alveolar macrophage (AM) via phagocytosis [33]. The mycobacteria can then replicate unchecked within the lung until the arrival of T cells, upon which time *M.tb* can enter into latency and live intracellularly within the alveolar macrophage for several decades. This is achieved by evading immune detection and elimination through a variety of evasion strategies, including blocking phago-lysosome fusion and detoxifying oxygen and nitrogen radicals [34]. Although the primary cell type to be infected is the AM, *M.tb* can also actively infect and replicate within recruited neutrophils [35], dendritic cells [36] and alveolar type II epithelial cells [37] generating a number of reservoirs for *M.tb* hide within.

Structure of M.tb: *M.tb* has a unique lipid structure that does not retain conventional biological dyes, and therefore cannot be classified as a gram negative or positive based by classical measures. The ability to detect *M.tb* was developed through a technique of acid-fast staining also referred to as Ziehl-Neelsen staining. The ability of *M.tb* to be stained in this manner allows it to be classified as acid-fast gram-positive bacteria owing to high amounts of peptidoglycan in the cell wall. One of the first line treatments is isoniazid, which functions to restrict the transfer of mycolic acids to the cell wall. Mycolic acids afford *M.tb* resistance to the harsh lysosome compartment, and failure to continually

produce mycolic acids makes rapidly growing *M.tb* susceptible to killing [38,39]. However, due to *M.tb's* long half-life, isoniazid is only effective if given for several months. The ability of *M.tb* to become resistant to isoniazid is high if given alone and usually is given in conjunction with rifampicin, a bacterial RNA polymerase inhibitor [39].

Innate recognition: Upon entering the airway lumen, *M.tb* is thought to 'silently' enter the resident AM. Infection of the AM occurs through receptor-mediated phagocytosis. Utilizing the complement receptors (CR3 & CR4), the mannose binding receptor, surfactant molecules, and DC-SIGN, *M.tb* rapidly facilitates its uptake by the AM [40,41,42]. Upon entry, recognition of *M.tb* is mediated through the engagement of pattern recognition receptors (PRRs). While the toll-like receptors (TLRs), specifically TLR-2, 4 & 9, have long been recognized as the primary PRRs required for the detection of *M.tb* [43,44], recently a member of the NOD family of receptors, NOD2, has been shown to play a critical role in the intracellular recognition and activation by the *M.tb* infected macrophage [45]. Typically, the AM acts as a sentinel, detecting and alerting surrounding cells to the presence of an invader. However, in the case of *M.tb,* this function is thought to be impaired. In particular, *M.tb* has been shown to uniquely engage the mannose receptor (MR) of responding macrophages. A major cell wall component of *M.tb,* lipoarabinomannan (LAM), is alternatively capped with mannose, which signals through the MR, inducing an anti-inflammatory program; impairing the secretion of both pro-inflammatory cytokines (TNF-α & IL-1β) and chemokines (MCP-1 & IP-10) [42,46], thereby delaying the recruitment of innate immune cells to the lung. A systematic

diagram outlining the major steps in the induction of anti-TB immunity is shown in diagram 4.

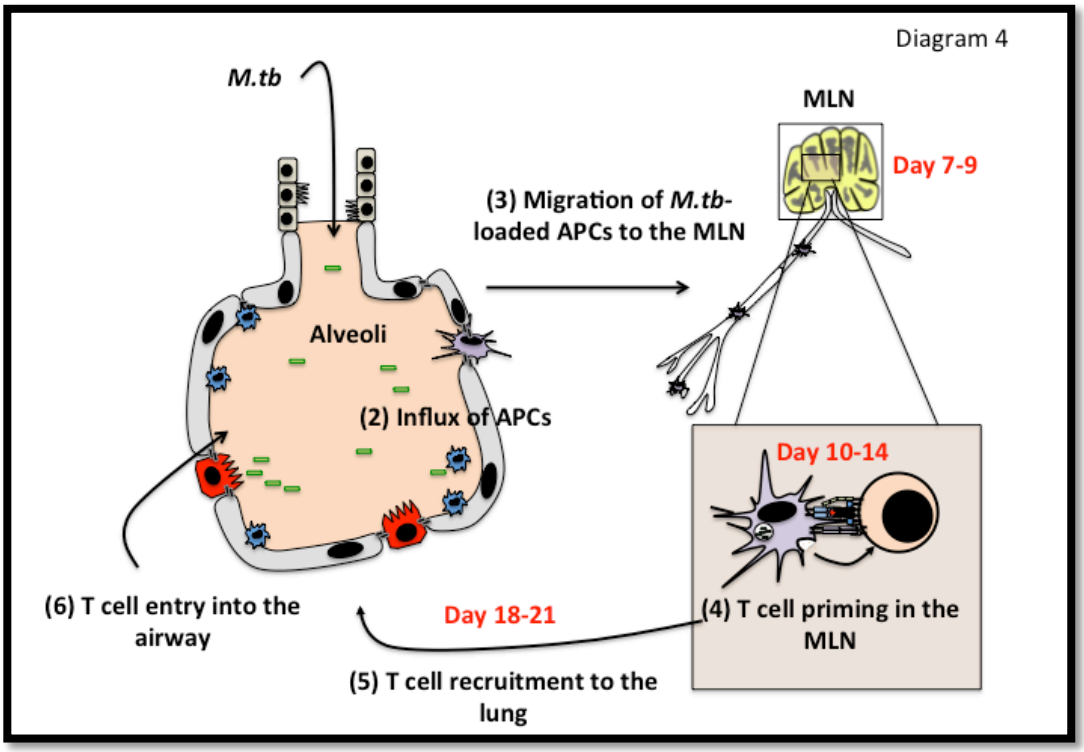

Diagram 4: Illustration of the speculated major immunologic setbacks seen in the early course of pulmonary *M.tb* infection. The major defects are numbered in the diagram according to the sequence of events. APC: antigen-presenting cells; MLN: mediastinal lymph nodes.

Antigen presenting cell (APC) recruitment and antigen acquisition: Under non-inflammatory conditions, immune surveillance of the airway lumen is passive and mediated primarily by a limited number of intraepithelial dendritic cells (DCs) [47,48]. Upon infection, there is a rapid recruitment of DCs to the lung interstitium [47]. Recruitment of immune cells from the vasculature to the airway lumen is a two-step process. Recruited cells first exit the vasculature into the interstitium, migrating through the interstitial matrix to the alveolar epithelium [49,50]. Rather than fully entering the

airway lumen, recruited dendritic cells interdigitate into the epithelial wall, extending their dendrites into the luminal space where they acquire antigen, while maintaining access to the collecting lymphatics located in the interstitium [47,51]. AM derived TNF-α commences the recruitment process by activating the type II alveolar epithelial cell (AEII), which initiates the production of specific chemokines, up-regulates critical adhesion molecules, and reduces tight junction adhesion [52,53,54]. While TNF has many functions, it is one of the earliest identified molecules considered central to the appropriate control of an *M.tb* infection. During the initial stages of *M.tb* infection, TNF acts primarily as an alarm cytokine alerting surrounding cells to the presence of an infection. It is believed that AM-derived TNF commences the recruitment of innate immune cells by activating the type II alveolar epithelial cells (AEII). This initiates the production of chemokines such as MCP-1, up-regulates critical adhesion molecules, and reduces tight junction adhesion [Diagram 5] [52,53,54].

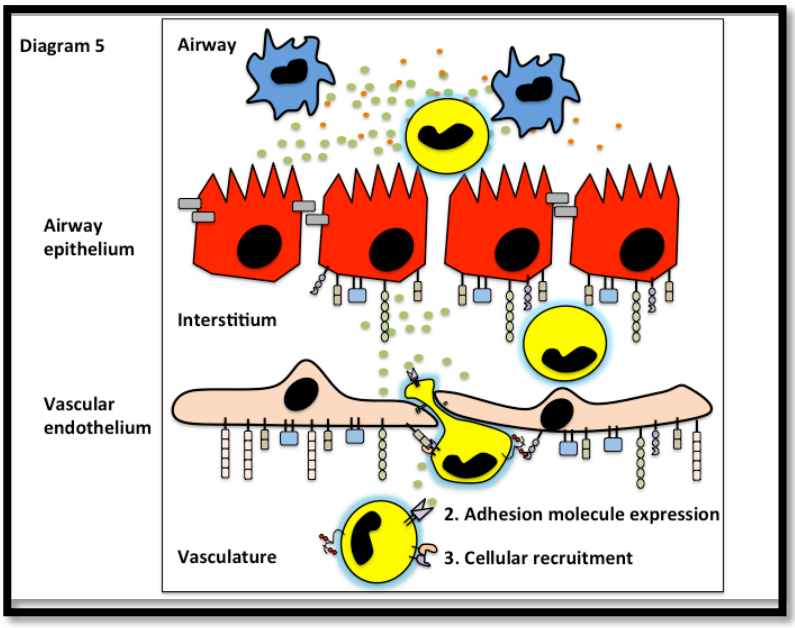

Diagram 5: A diagrammatic depiction of the path of immune cell migration into the airway lumen. This diagram depicts the migration of an immune cell from the vasculature to the interstitium and then into the airway lumen. The first stage of cellular recruitment is the production of chemokines by type II alveolar epithelial cells (1), followed by the up-regulation of critical adhesion molecules and the reduction of tight junction adhesion (2). Finally, cells recruited by the induced chemokine gradient are able to navigate into the airway through adhesion molecule interactions (3).

AEII are central to the initial recruitment of APCs to the lung, functioning as the 'gatekeepers' of the airway lumen controlling the production of chemokines such as MCP-1 and regulating the expression of addressin molecules [48,55]. The early mobilization of APCs to the lung is critical to the timely control of an *M.tb* infection as it has been shown that in the absence of the MCP-1 receptor CCR2, APC recruitment to the lung is significantly delayed, impairing the downstream adaptive immune activation and bacterial control [56]. Further confounding the issue of delayed APC migration to the site of infection, due to *M.tb's* slow replication, it has been speculated that there may be a limited amount of antigen available for APC loading, delaying migration to the lymph node [57]. While the intraepithelial DC represents the primary APC responsible for antigen uptake in the airway, it has been proposed that the alveolar macrophage may egress from the airway lumen into the interstitium, gaining access to the collecting lymphatics, to transport *M.tb* to the MLN [58].

2.2. Adaptive immune activation

APC migration to the draining lymph node and T cell priming: While much controversy surrounds the generation of adaptive immune responses following *M.tb*, it is now widely accepted that the earliest responses are seen 10 to 14 days post infection in the MLNs [59]. This delay is highly significant when compared to other lung pathogens,

such as influenza or *legionella pneumophila,* where adaptive responses are seen in the MLN 2-3 days [60,61] and in the lung 6-7 days post infection [30,62,63]. This may suggest that such delay is due to insufficient bacteria or bacterial products in the lymph node required for T cell priming [64]. Regardless of the underlying cause, considered central to this delay, *M.tb* directly infects DCs, impairing both DC migration to the lymph node and maturation [36,65]. However, the precise arrival of *M.tb* to the MLN is still debated in the field with some groups having identified *M.tb* in the MLN as early as 4hrs post-infection, while others cannot detect *M.tb* for 7-9 days post infection [64,66]. Nevertheless, it is now well established that viable *M.tb* resides within the MLN several days prior to the emergence of the effector T cells with greatest influx of DCs to the lymph node between 7 and 10 days post infection. [36,64].

Migration of DCs to the MLN: The ability of antigen-loaded DCs to home to the MLN is largely due to the up-regulated expression of chemokine receptor CCR7. CCR7 expression allows activated DCs to rapidly migrate towards the CCL19/21 chemokine gradients generated by the MLN [67]. Khader *et al* [68] have demonstrated the dependence of IL-12p40 for the expression of CCR7 by *M.tb*-infected DCs. To this end, impairing the production of IL-12p40 was found to delay the migration of infected DCs to the MLN, thus deferring T cell priming by several days [68]. Furthermore, it has also been demonstrated that *M.tb* directly induces a splice variant of the IL-12 receptor, significantly enhancing the responsiveness of infected DCs to IL-12p40, augmenting the migratory capacity of these populations [69]. It is proposed that *M.tb* may utilize the induction of this high efficacy receptor to facilitate its dissemination away from the lung

in the manner similar to a Trojan horse [69]. These data suggest that that the delay in T cell priming is a result of impaired DC functionality in the lymph node rather than the impaired migration of DCs into this compartment [31,70].

Migrating APC subsets: While the intraepithelial DC may be the primary APC sampling mycobacteria or mycobacterial antigen in the airway lumen, it has been proposed that the AM may egress from the airway lumen into the interstitium, accessing the collecting lymphatics and transporting *M.tb* to the MLN [58]. Within the lung it is difficult to appropriately classify the APC populations based on a single cell surface marker. To this end, the expression of conventional markers such as CD11c must be used in conjunction with other makers such as MHC class II expression to denote activated DC populations [67,71]. In recent years there has been a movement to classify the migratory potential of specific DC cell populations in the lung based on cell surface marker expression. While far from resolved, two distinct DC populations have been identified based on their potential to migrate to the MLN. The expression of $CD103^+$ ($CD11c^{hi}$ $CD11b^-$ $MHC\text{-}II^{hi}$ $CD103^+$) has recently been shown to be important in transporting apoptotic bodies and mediating Ag cross-presentation to $CD8^+$ T cells during many viral infections [72]. The expression of $CD11b^+$ ($CD11c^{hi}$ $CD11b^{hi}$ $MHC\text{-}II^{hi}$ $CD103^-$) has been shown to be key to the delivery of the majority of viable mycobacteria to the MLN [36]. From our preliminary studies we have seen a surge in both DC subsets in the MLN following pulmonary mycobacterial infection. As described above, complement represents one of the major mechanisms responsible for the uptake of mycobacteria by recruited APC populations. As such, it can be considered that the expression of both

CD11b and CD11c, components of complement receptors 3 and 4 respectively, may allow for more efficient uptake of mycobacteria by these APC subsets. An enhanced capacity to uptake mycobacteria may provide a plausible, yet unconfirmed, explanation for why CD11c+CD11b+ DCs represent the dominant APC population during *M.tb* infection. The relevance of these DC populations with regard to the efficiency of antigen presentation and subsequent T cell priming is still currently unknown in the context of *M.tb* infection. Furthermore, it remains to be understood whether some of the T cell-priming APCs in the MLN are actually AMs, AMs that have differentiated into DCs, or *M.tb*-loaded neutrophils.

Passive transport of M.tb to the MLN: In addition to the active transport of *M.tb/M.tb* antigen to the MLN by migratory DC or AM populations, it has been suggested that the passive transport of antigen could be accomplished via the lymphatic drainage of the lung. Whether the *M.tb* organism actively utilizes this system to mediate its "cell-free" dissemination from the lung to the MLN is unknown. It remains plausible that discrepancy in the time of bacterial arrival to the MLN and the time of T cell priming could be attributed to cell-free transport of *M.tb*. Regardless of how *M.tb* arrives in the lymph node, the appropriate activation of naïve T cells depends on the interaction between the antigen-loaded DCs and their cognate naïve T cell. Critical to this interaction is the expression of sufficient levels of co-stimulatory molecules, a high density of MHC loaded with the cognate antigen, and the production of polarizing cytokines. The inflammatory microenvironment during the acquisition of antigen plays an integral role in the maturation of DC populations and subsequent T cell priming.

T cell priming within the MLN: Following the arrival of APCs in the lymph node, appropriate to the intracellular intraphagosomal nature of *M.tb*, a dominant CD4+IFN-γ+ Th1 response is generated [64] The ability of an infected DC to prime a type I dominated response is owed to its ability to produce high levels of IL-12 [73]. IL-12 mediates type I polarization and is considered to be critical to the generation of protective immunity towards intracellular bacterial, but not viral, infection [73]. The intracellular intraphagosomal nature of mycobacteria makes it a target of MHC class II loading and the dominant immune cells are type I CD4 T cells. Despite the primary induction of the type I CD4 T cells, cross presentation or phagosomal escape by mycobacteria leads to the loading of mycobacterial antigen on MHC class I and the priming of CD8 T cells [74,75,76]. Differential cytokine microenviroments have been shown to induce the priming of mycobacterial specific T helper-17 CD4 T cells (Th17) [77]. Although Th17 cells have been shown to be present in the mycobacterial infected lung, their precise role is not fully understood [77,78,79,80,81]. Studies using MHC class II and class I deficient mice showed that while a deficiency in MHC class I had a very limited impairment on early immunity and bacterial control, a deficiency in MHC class II results in extensive impairment with limited to no control of bacterial load [76]. Nevertheless, the greatest impairment was seen when iNOS knockout mice were used, indicating that IFN-γ from both type I CD4 and CD8 T cells play a role in protection [76]. This was further confirmed by the immunohistochemical staining for NOS2 in MHC class I and II knockout mice [82]

Mechanisms of delayed T cell priming: It has long been recognized that *M.tb* utilizes the induction of IL-10 as a means to suppress effector cell function. Specifically, it has been demonstrated that infected macrophage and DC populations can produce high levels of IL-10 in response to live, but not heat-killed, *M.tb* [83,84]. It has been demonstrated that upon infection, *M.tb* employs multiple secreted virulence factors to subvert host recognition, many of which actively impair antigen processing and loading, and the surface expression of MHC class II [85,86,87]. Most notably, the 19 kDa protein secreted by *M.tb* has been shown to inhibit the activation of several genes involved in antigen presentation, including the down-regulation of MHC class II, HLA-DM, and CIITA [85,86,88]. In addition to impairing antigen processing, *M.tb's* major cell wall component, cord factor (trehalose 6,6′-dimycolate) has been shown to significantly impair the up-regulation and appropriate expression of co-stimulatory molecules such as CD28 [89]. Together, these impairments are thought to alter or delay T cell priming [90]. Furthermore, the expression of high levels of IL-10 results in the preferential induction of an early T regulatory cell population that serves to delay the initiation of protective type 1 immune responses [91].

T cell migration to the lung: As expected, delayed T cell priming in the lymph nodes of *M.tb*-infected animals results in delayed arrival of effector T cells at the lung, the primary site of infection. This permits *M.tb* to increase logarithmically within the lungs of the host for approximately 20 days, thus establishing a robust 'foothold' prior to the arrival and abundant presence of antigen-specific T cells at the site of infection [92,93,94].

Following priming in the MLN, T cell responses begin to be seen in the lung interstitium around day 14 and are maximal by day 28 [95,96]. The recruitment of cells into the airway lumen is not seen until day 21 and becomes maximal by day 28. The efficient recruitment of T cells into the lung requires the coordinated up-regulation of specific adhesion molecules on both the endothelium/epithelium (VCAM-1)[97] and the activated T cell (α4β1 & LFA-1) [97,98]. The AEII establishes specific chemokine gradients through the production of chemokines such as interferon inducible-protein 10 (IP-10) [96,99]. T cells migrate into the lung interstitium and airway lumen following these established chemokine gradients. The mass arrival of T cells to the lungs occurring between 18 and 20 days post infection is associated with the ultimate plateau of bacterial growth [64,92,94,100].

Critical chemotactic molecules: It has been demonstrated that CCL5 (RANTES) dramatically increases between day 10 and day 20 post-*M.tb* infection [101]. However, the specific role of this chemokine in T cell homing to the lungs has only recently been elucidated. Through the use of CCL5 knockout (KO) mice, Vesosky *et al* [102] have shown that CCL5 is critically required for the early recruitment of CCR5-expressing CD4 T cells to the lung in *M.tb*-infected mice. The delay in effector T cell recruitment in CCL5 KO mice caused a significant reduction in IFN-γ production and impaired granuloma formation, resulting in significantly higher bacterial burden within the lungs of these animals when compared to wild type controls [102]. Best known for their critical role in DC homing to the MLN, CCL19 (MIP-3β) and CCL21 have been recently shown to be essential in the trafficking of IFN-γ^+ T cells from the MLN to the lungs of *M.tb* infected

mice. In the study conducted by Khader [103], CCL19 was shown to increase in the lungs of infected mice between 15 and 18 days, correlating with the arrival of effector T cells and the initiation of granuloma formation. Mice deficient in CCL19 and 21 showed significantly impaired $CD4^+$ IFN-γ^+ T cell kinetics to the lungs prior to day 30 post-infection [103]. The blunted T cell recruitment in the lungs of CCL19/CCL21-deficient mice resulted in delayed IFN-γ and iNOS production, macrophage activation, and bacterial control [103]. This leads to severely impaired granuloma formation and increased bacterial loads for at least 80 days post-infection, indicating the critical role of timely T cell trafficking to the lung [103].

Adhesion molecules: In addition to chemokine expression within the lung, several studies have focused on identifying the required adhesion molecules and specific integrins which mediate the entry of effector T cells into the lung. Vascular cell adhesion molecule 1 (VCAM-1) expression is up-regulated in *M.tb* infected lungs by day 21 and is associated with the recruitment of the majority of IFN-γ-producing T cells [104]. The preferential expression of $\alpha_4\beta_1$ or $\alpha_4\beta_7$ on activated CD4 T cells makes VCAM-1 essential to efficient recruitment of T cells into lung [104]. Furthermore, depletion of either α_4 or $\alpha_4\beta_7$ results in a significant decrease in the number of lymphocytes within the lung, the consequence of which manifests in granulocyte-dominated granulomas consisting of disorganized infiltrates and heightened necrosis [104]. Similar defects in granuloma formation were seen in the lungs of mice deficient for CD11a/18, where a 3-4 fold reduction in the number of antigen-specific T cells recruited resulted in increased neutrophilia, necrotic foci, and poorly formed granulomas [105]. It is therefore apparent

that the timing of effector T cell trafficking into the lungs following *M.tb* infection is critical to the establishment of granuloma formation as well as appropriate bacterial control.

Recruitment of T cells to the different lung compartments*:* The lung can be divided into two main compartments; the interstitial tissue residing in between the alveoli, and the mucosal surface of the lung known as the airway lumen. While the timing of T cell priming in the MLN ultimately determines the kinetics of effector T cell migration to the infected lung, there is growing evidence to suggest that the coordinated up-regulation of several molecules is essential to the homing of T cells to the lung. Specifically, the most recent focus has been on the kinetics of chemokine production as well as the coordinated up-regulation of specific adhesion molecules. Both the expression of integrins on T cells, and their respective addressin molecules on the vascular endothelium and alveolar epithelium are essential to appropriate recruitment of effector T cells to lung. The differential up-regulation of these molecules dictates whether a T cell traffics to the lung interstitium or airway lumen, and can dramatically effect bacterial control. While it is believed that T cell trafficking to the airway lumen is a required process for the control of *M.tb* [17], little work has been done to understand the differential inflammatory signals required to recruit T cells into the airway lumen. Based on this, most studies have focused on the molecules required for recruiting T cells into the lung as a whole rather than the differential lung compartments.

2.3. Stages of granuloma formation

The ability of the host to 'control' *M.tb* infection encompasses a number of immunological processes designed to restrain bacterial dissemination and persistence, and reduce person-to-person transmission. The classical hallmark of anti-TB host defense is the formation of type 1 granuloma in the lung. And the granuloma is currently perceived as essential to anti-TB host defense as the host is incapable of sterile clearance and thus is forced to segregate the infected cells as a means to preserve itself.

The historical view of mycobacterial granuloma: First described in 1679, pathologists discovered unique structures in the lungs of TB patients [106] (reviewed in[107]). These structures were then termed tubercles and represent what we now know as granulomas. Commonly, it was observed that persons who had died of TB had a large number of these distinct pathological lesions, and the presence of tubercles became an associated hallmark of active TB disease. It was not until 1884 that tubercles were also characterized in individuals who had died from diseases other than TB. Upon post-mortem examination, a number of these individuals had lung lesions (granulomas) containing live tuberculosis bacilli, giving the first indication that TB latency may relate to the formation of granuloma [108]. However, upon further microbiological examination, it was revealed that live bacilli persisted not only within the granuloma itself, but also in the surrounding lung tissue, albeit to a lesser degree [109,110,111,112,113]. It was around this time that the protective view of the granuloma began to gain public acceptance and it was proposed that the recruitment of activated lymphocytes and the formation of a lymphocytic cuff served to wall-off infected macrophages as a means of limiting dissemination.

Formation of the 'innate granuloma': Shortly after aerosol exposure, *M.tb* infects the resident alveolar macrophage (AM) initiating the early inflammatory response. While amplifying the host immune response, the recruitment of innate immune cells inadvertently provides a large number of new targets for *M.tb* to infect and is thought to contribute to the early dissemination of *M.tb* [114,115]. Augmenting this problem, the infected alveolar macrophage is unable to kill internalized mycobacteria due to impaired phago-lysosome fusion, a process essential to the destruction of the phagocytosed bacteria [116]. The efficiency by which a mycobacterium species arrests phago-lysosome fusion is directly attributable to its relative virulence, with highly virulent strains such as *M.tb* almost completely inhibiting fusion [117]. Incapable of killing internalized *M.tb,* infected macrophages secrete an array of proinflammatory and chemoattractant cytokines including TNF, IL-6, and IL-8 which facilitate the recruitment of new macrophages and granulocytes to the site of infection and lead to the formation of the 'innate granuloma' [118]. A systematic diagram outlining the stages of granuloma formation is shown in diagram 6.

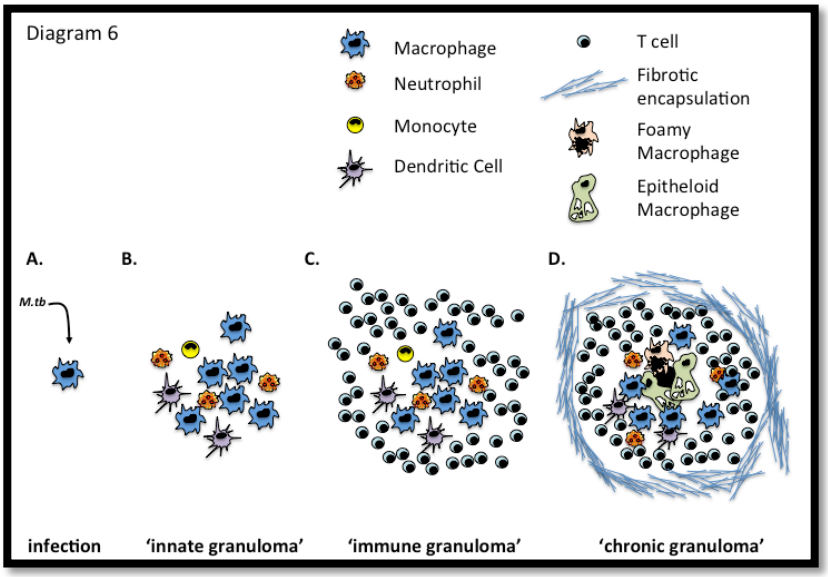

Diagram 6: **Evolution of TB granuloma formation.** Following infection sentinel macrophages cells facilitate *M.tb* uptake and the recruitment of other innate and adaptive immune cells. An *M.tb* infection begins with the inhibition of phago-lysosome fusion in alveolar macrophage populations (A), and the eventual recruitment of innate cells to form the "innate granuloma" (B). Following T cell priming and recruitment to the site of infection, T cells surround the infected macrophages and form the "adaptive immune granuloma" (C). Finally, the recruited lymphocytes are fibrotically encapsulated known as the "chronic granuloma" (D).

During the early development of mycobacterial infection, the innate granuloma dominates and is composed of a loose association of infected and uninfected macrophages. The macrophage is primarily responsible for the early production of cytokines and chemokines that activate and recruit responding innate cell populations. The early granuloma initiates some bacterial control by the recruitment and activation of effector cell populations. The recruitment of innate effectors occurs shortly after the engagement of the pattern recognition receptors on the infected macrophage. The infected macrophage produces two waves of chemokines. The first wave is composed of CCL2 (MCP-1) and CXCL10 (IP-10), which is initiated to recruit naive macrophages, dendritic

cells and innate lymphocytes [119,120]. Upon the arrival of recruited innate cells there is an enhancement in the ability of infected macrophages to produce tumor necrosis factor-α (TNF). TNF, classically defined as an alarm cytokine, functions in both an autocrine and paracrine manner to alert and rouse surrounding cells to the presence of an infection. TNF mediated signaling results in the release of a second wave of chemokines and amplification of the immune response. TNF signaling from both naïve and infected macrophages mediates the release of CCL2 (MCP-1), CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES), CXCL9 (MIG), and CXCL10 (IP-10) [120,121,122,123,124,125]. Mice deficient in TNF showed delayed chemokine production, which impairs the recruitment of both innate and adaptive mediators to the site of infection [119]. Upon the arrival of innate cells, which include neutrophils, dendritic cells and innate T lymphocytes such as γδ T cells and NKT cells, the macrophage produces a number of cytokines which function to activate or enhance the function of these newly arrived cell types [6,75,115,120,121,123,126,127,128,129]. IL-12, IL-23 and IL-15 come primarily from infected macrophages and dendritic cells [130,131,132]. IL-15, IL-23 and IL-12 are key mediators of natural killer (NK) and NK T cell survival and maturation [133]. During the early stages of infection the predominant source of IFN-γ is a population of innate lymphocytes, specifically the NK T cell and γδ T cells [81,121,122,134,135]. NK T cells are dependent on the presentation of lipid products by the CD1 family of MHC class I-like molecules which produce large amounts of IFN-γ following presentation of mycobacterial cell components, such as derivatives of LAM [135,136]. It is thought that this early activation of NK T cells provides IFN-γ prior to the initiation of the adaptive

immune response, allowing for the activation of infected macrophages to kill internalized bacteria. γδ T cells have demonstrated considerable ability to provide help by recognizing certain phosphorylated antigens from mycobacteria [134,135,137]. The ability of the γδ T cell to recognize these antigens allows them to perform numerous antigen specific responses. In addition to their capacity to produce IFN-γ, recent studies have demonstrated a strong cytotoxic perforin-dependent mechanism of protection [134].

Contribution of M.tb to the formation of the 'innate granuloma': Historically the formation of granuloma has been considered to be a host-mediated event. Using an *M. marinum* model, real-time microscopic visualization has challenged this notion, revealing that virulent mycobacterium drives the nascent formation of the early granuloma. A number of elegant studies conducted by Ramakrishnan's group have demonstrated the unique interplay between the mycobacterium and the host immune system in the early stage of granuloma formation. To this end, the early release of ESAT-6 by *M. marinum* led to the activation of the epithelium, which facilitated the recruitment of macrophages to the site of infection through inducing the production of MMP-9 [115,138]. In murine models, *M.tb* was also found to drive MMP-9 expression. Thus either broad MMP inhibition or MMP-9 specific depletion delayed granuloma formation, resulting in impaired macrophage recruitment to the site of infection and reduced granuloma size [139]. These findings indicate that *M.tb* may actually promote granuloma formation and utilizes the structure for its own benefit. Furthermore, it was demonstrated that virulent mycobacteria can utilize the innate granuloma as means of recruiting target cells allowing for the early dissemination of mycobacteria throughout the host [115]. This view is

supported further by the observation that the early granuloma is not a static environment and there is a significant movement of APC populations into and out of the early granuloma with this initial recruitment is essential to establishing the macrophage-dominated center of the 'immune' granuloma [126,140,141,142].

'Immune' granuloma formation: The induction of what is termed the 'immune' granuloma is the hallmark of immune mediated control, and is thought to represent the primary mechanism of long-term protection. The formation of the 'immune' granuloma is a very complex process, which follows the arrival of the adaptive immune cells to the lung, an event not normally seen until 2-3 weeks post infection as aforementioned [31]. The 'immune' granuloma induces a number of defined histopathological changes to the innate granuloma structure. The innate granuloma is further fortified by the arrival of effector T cells, and the ability of infected macrophages to kill internalized bacteria is enhanced by the release of IFN-γ [103]. Once in the lung, recruited T cells surround and wall-off infected macrophages, activate them for enhanced bactericidal function, and physically limit their mobility in order to restrain bacterial dissemination. Indeed, the arrival of effector T cells and the establishment of the classical 'adaptive immune granuloma' is associated with a plateau in bacterial growth [76]. The addition of effector T cells to the granuloma produces what is termed the lymphocytic cuff, where entering lymphocytes surround the infected macrophage populations forming a barrier, adjoining with the focal infection [5,75,119,120,129,143]. The formation of the lymphocytic cuff and the ensuing production of inflammatory mediators results in defined structural changes to the partitioned macrophage populations. Two major morphological changes

occur in the infected macrophage populations, within the type I granuloma: first, the induction of an epithelial-like appearance, producing epithelioid macrophages, and second, the fusion of macrophage populations to form multinucleated giant cell populations [5,75,119,120,129,143].

While the dominant subset of T cells is CD4 positive, cross-presentation also allows for the strong induction of CD8 positive T cells, collectively generating a type 1 polarized adaptive immune response [144]. Moreover, type 1 cytokines including IL-12, IFNγ and TNFα required for macrophage activation are also essential to granuloma formation [145] or the maintenance of granuloma [146]. While the prevailing immune response generated following *M.tb* infection is highly similar between the mouse and man, the structural formation of the 'adaptive immune granuloma' differs significantly (discussed below).

Granuloma as a microenvironment for mycobacterial persistence: Contrasting the classical notion of its protective role, a number of recent studies have demonstrated unaltered bacterial control even in the absence of granuloma formation, strongly arguing against the granuloma being essential to bacterial restriction [147,148,149]. Moreover, it is now known that the mycobacterium can significantly alter the immune environment of the granuloma as means to facilitate its persistence [83,150,151,152,153]. Recently many groups have begun to compartmentalize the granuloma away from the lung parenchyma and airway lumen. Mounting evidence from us and others suggests that the granuloma represents an immunologically suppressed or dampened zone that the mycobacterium preferentially resides within [142,154,155].

The 'chronic granuloma': a dynamic interplay between persisting M.tb and the host immune response: Following the establishment of the 'adaptive immune granuloma', a period of immune quiescence is established. It is during this stage that chronic immune activation leads to significant alterations in the morphology and functionality of the granuloma, many of which are not typically seen in mice. Moreover, while the granuloma has long been believed to be a protective host's response, it is now acknowledged to result from a dynamic and continuous interplay between the host's immune response and persisting *M.tb*. The continuous 'battle' between chronic immune activation and bacterial persistence causes infected macrophages to adopt an irregular epithelial-like or "epithelioid" appearance, and to fuse together forming multinucleated giant cells in the core of granuloma [156,157]. Indeed, virulent mycobacterium has been shown to induce macrophage cell death frequently throughout the course of infection, which has been proposed as a potential mechanism of gaining access to new host macrophages [115]. Newly recruited macrophages quickly phagocytose the dead bodies and become saturated with mycobacteria and lipids [158]. These lipid-rich macrophages that accumulate within the granuloma are known as foamy macrophages due to their distinct appearance, and are now recognized as a major contributing factor in the persistence of *M.tb* [158]. Recent studies have shown that foamy macrophages isolated from humans have lost key functions including their ability to phagocytose and to produce essential bactericidal agents such as nitric oxide [158]. Moreover, the polarization of macrophage populations within the granuloma is thought to shift from being a classically activated (M1) population towards that of an alternatively activated

(M2), with reduced bactericidal capacity [159]. Thus, such foamy macrophages have been proposed to function as the reservoirs of *M.tb* whereby the bacterium is able to successfully manipulate the infected macrophage into not only a safe haven but also a source of nutrients required for the synthesis of its cell wall and replication.

An infected host typically houses a highly heterogeneous mixture of granuloma types ranging from large necrotic granulomas containing large amounts of bacilli, to completely calcified structures devoid of any detectible bacteria [156,157,160]. In the later stages of granuloma evolution, fibrotic encapsulation can be seen in cases of both active and latent infection. Currently, it is unclear whether encapsulation functions to prevent bacterial escape, or to limit immune infiltration into the granuloma. Although complete mycobacterial clearance is rarely seen, latently infected humans display the evidence of healed granulomas, marked by central calcification in conjunction with fibrotic encapsulation containing no detectible bacilli [161]. Despite the observed absence of bacteria within highly calcified granulomas, it is currently unclear whether this represents immune-mediated clearance, or simply a structural artifact left behind following *M.tb* escape.

2.4. Mechanisms of immune mediated restriction and latency

Effector functions of recruited T cells in the lung: Upon the arrival of activated type I immune cells to the lung, bacterial replication plateaus and adaptive immunity contains the infection [162]. While sterile immunity is rarely achieved, the majority (>90%) of infected individuals will successfully enter into an asymptomatic latency period, accomplished through bacterial restriction and segregation, rather than clearance

[163,164,165,166,167]. Bacterial restriction is maintained through the generation of a type I granuloma formation where infected cells are spatially restricted. Any perturbation of the granuloma can dramatically impact the host's ability to contain *M.tb* [168,169]. Bacterial control is maintained through the continuous activation of infected macrophages, enhancing their ability to mediate phago-lysosome fusion, produce nitrogen and oxygen radicals, and control internalized bacteria [170,171]. As long as a sufficient degree of immune responsiveness is maintained, bacterial containment is achieved. Following an immunosuppressive event such as malnutrition, aging, chemotherapy, or HIV-AIDS, immune mediated bacterial control is lost, and clinical disease rapidly ensues [172,173].

The ability of *M.tb* to live intracellularly shields it from the host's humoral response. Thus, controlling bacterial dissemination and curtailing its replication is largely the responsibility of activated T cells subsets. Studies using MHC class II and class I deficient mice have demonstrated that while a deficiency in MHC class I has a limited impairment on bacterial control, deficiency in MHC class II results in extensive impairment, signifying the relatively greater importance of CD4 T cells [76]. Given the central role of IFN-γ in macrophage activation and the induction of nitric oxide a greater impairment is seen in iNOS deficient mice than in either MHC class I or II deficient mice, thus indicating that IFN-γ from both type I CD4 and CD8 T cells plays a critical role in protection [76]. While a type 1 immune response eventually ensues, the delayed T cell priming by early immune evasion strategies employed by *M.tb* provides a critical window for *M.tb* to grow completely unchecked in the lung.

Macrophage activation: The airway lumen is largely considered the primary site of infection. With effector T cells being recruited to 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 induction of highly toxic antimicrobial substances. 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 be removed by Fas/FasL or TNF-directed apoptosis [5,120,126,131,174]. Following IFN-γ mediated activation, the infected macrophage generates both reactive oxygen substances (ROS) and reactive nitrogen intermediates (RNI) [82,175]. 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 - NOS2 [82,175]. Resistance to RNIs is a common feature of mycobacteria, with the most virulent strains such as *M.tb* and *M. bovis* being almost completely resistant [82,175]. Although sterile clearance is never achieved, the activation of an infected macrophages is thought to be strongly bacteristatic, facilitating the persistence of *M.tb* within the host [175]. The key role of IFN-γ in this process is without question and was conclusively shown with murine IFN-γ deficiency models [176]. In the absence of IFN-γ, mice fail to up-regulate NOS2 and are unable to control bacterial dissemination, succumbing to the infection within the first few weeks [176]. Despite the unprecedented role of IFN-γ, it cannot function alone. IL-12 was shown to be essential to the optimal induction of both NO and TNF [177]. Other studies have demonstrated an essential role for TNF and it is now believed that IL-

12, IFN-γ and TNF must be present for optimal NO production [131,174]. A schematic outlining the major mechanisms of adaptive immune control is depicted in diagram 7.

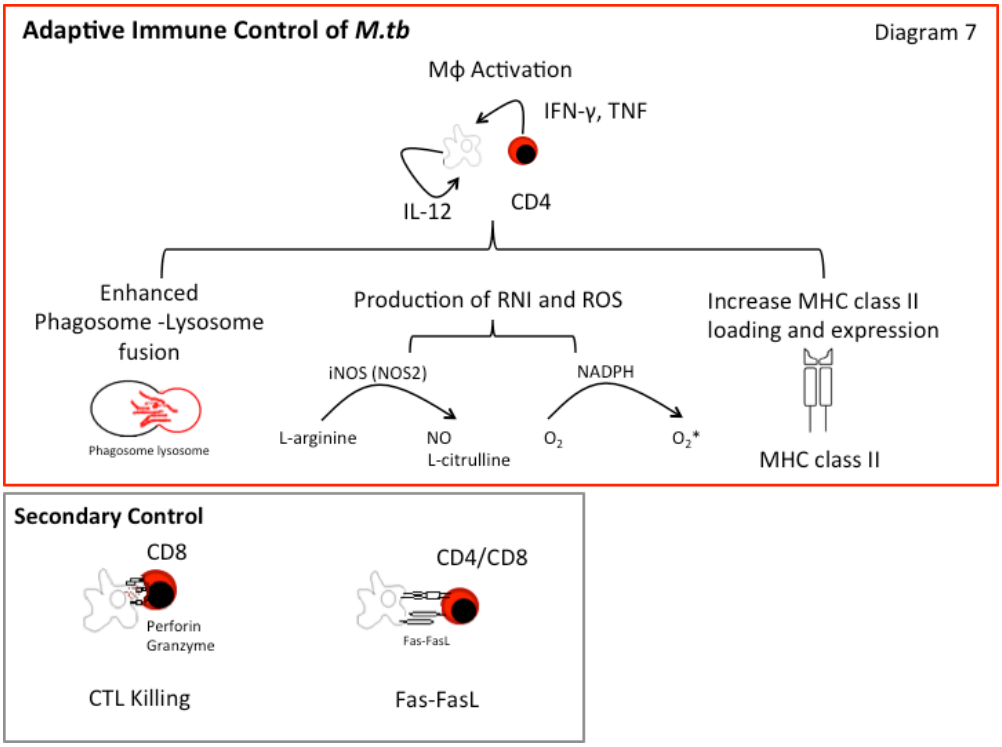

Diagram 7: A diagrammatic depiction of the major immune mechanisms responsible for *M.tb* control. The major mechanisms of macrophage activation induced following CD4+ IFN-γ production. Intracellular *M.tb* is controlled through enhanced phagolysosome fusion, the production of reactive oxygen and nitrogen species and the upregulation of MHC class II. Secondary mechanisms of control are mediated via CTL killing and contact dependent Fas-FasL.

The ability of *M.tb* to survive in the cell relies heavily on its unique ability to subvert the innate and adaptive immune systems. Its unique cell wall structure composed of lipids and glycoproteins mediate its survival in the phagosome, primarily through arresting fusion with the lysosome. One of the major components of the cell wall is mannose capped LAM which is thought to be critical to arresting phago-lysosome fusion [178,179]. The ability of LAM to arrest phagosome fusion relies on its ability to prevent

the phosphorylation of phosphatidylinositol 3-phosphate (PI3P), a required step in the conversion of an early phagosome to a late phagosome [178]. The ability of LAM to prevent the phosphorylation of PI3P is mainly attributed to its ability to prevent the cellular influx of Ca^{2+}, a required step in the activation of phosphatidylinositol kinase (PI3K) [Diagram 8] [178]. In addition to LAM, the activation of PI3P is further prevented by SapM a secreted PI3P-phosphatase, further ensuring that the phagosome is arrested at the early stage [Diagram 8][178]. In addition to preventing phagosome maturation, *M.tb* encodes a number of proteins directed at survival in an activated phago-lysosome. The ability to combat reactive nitrogen intermediates and reactive oxygen species is critical to *M.tb's* survival following the induction of adaptive immunity and correlates with strain virulence [38,179]. *M.tb* encodes two superoxide dismutases, sodA and sodC which catalyses the conversion of superoxide anions to H_2O_2, and a catalase-peroxidase katG to combat the increased levels of H_2O_2 [38]. Furthermore, *M.tb* encodes a combined NADH-dependent peroxidase and peroxynitritesreductases which is composed of four protein components; an alkyhydroperoxidereductase, a thioredoxin-related oxidoreductase, a dihydrolipoamideacyltransferase and a lipoamide dehydrogenase [38]. These four components function to detoxify both RNI and ROS and protect *M.tb* from the harsh environment of an activated phago-lysosome, limiting the availability of *M.tb* antigen [38]. The ability of *M.tb* to survive within the APC, coupled with its' slow replication rate, functions to limit the amount of antigen available to prime required T cell responses.

Interestingly, while a number of *M.tb's* lipid and protein components function to protect from or evade immune activation, some components such as trehalose 6,6'

dimycolate (TDM) or cord factor are essential to host granuloma formation [180]. It would appear at odds that some bacterial components are immunoevasive while others function to enhance host immunity. The ability of the host to form granulomas has been linked to protective immunity, as well as paradoxically to bacterial persistence. It would appear *M.tb* induces host strategies that allow for its persistence, while inhibiting the abilities that promote its clearance.

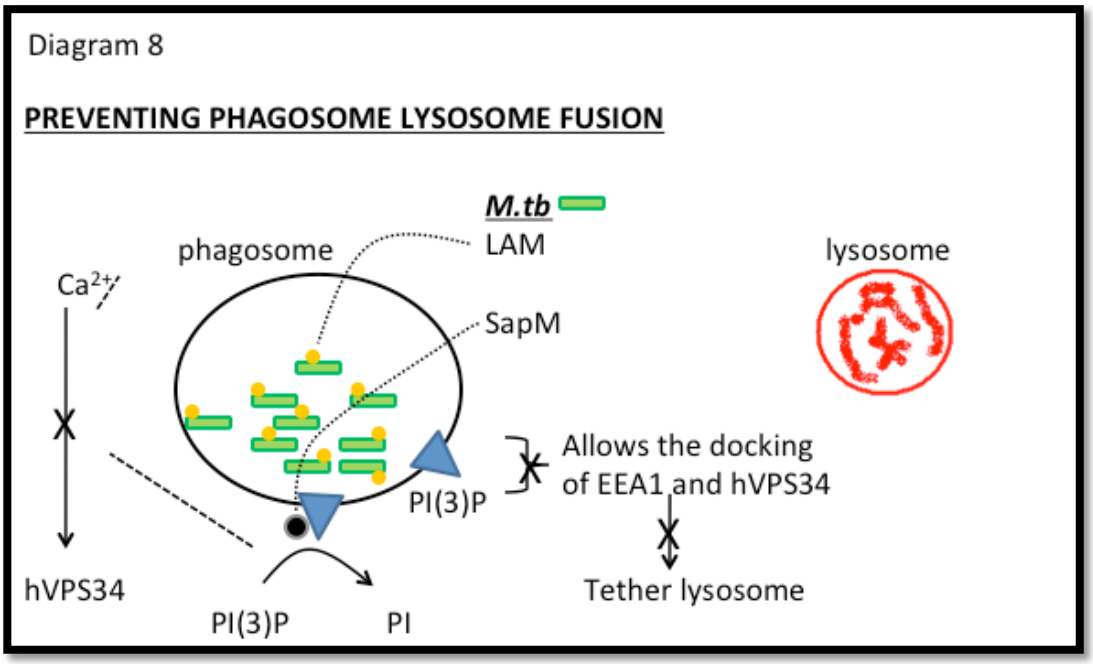

Diagram 8: Virulence factors utilized by *M.tb* to prevent phagosome lysosome fusion. A diagrammatic depiction of how LAM, and SapM target key points in the phagosome fusion pathway.

3. Risk factors for disease progression

M.tb represents a major world threat and remains one of the top three infectious diseases worldwide [181]. It is estimated that one third of the world's population is latently infected and that one in ten latently infected individuals will progress to active

tuberculosis disease in their life time [181]. Controlled latent tuberculosis causes limited pathology to infected hosts and can remain latent for many years, even decades. In immune competent hosts the bacterial load is controlled and maintained through the establishment of granuloma formation. Granuloma formation represents a delicate balance between the host immune system and bacterial control. The control of bacterial dissemination is highly dependent on a continual minimal adaptive immune response; as such any suppression of the host immune system can significantly reduce the ability to prevent active bacterial replication. Given that the reactivation of bacterial replication and dissemination can cause significant lung pathology and in many cases death, exploring the mechanisms many underlying factors that influence the delicate balance between host immune responses and bacterial control should be thoroughly investigated.

3.1. Contribution of the granuloma to bacterial pathogenesis

M.tb facilitates person-to-person transmission through alterations to the granuloma: Despite the attempts of the host to contain *M.tb* within the granuloma, as the infection progresses, the majority of individuals will develop granulomas with a necrotic focus formed due to the caesation of the macrophage infused center [182] (Diagram 9). This eventual necrosis of the granuloma is now accepted as a necessary event in facilitating the transmission of *M.tb* by disrupting the lung structure and allowing *M.tb* to gain access to the major airways. It should be noted that, in addition to person-to-person transmission, the active granuloma leaking bacteria into the airways might also allow for intrapulmonary dissemination [183,184,185]. It is therefore likely that the heterogeneity in granuloma structure seen in different lung regions of the same host represents different

evolutionary timelines. Although much remains to be understood, it is clear that the evolution of granuloma is the result of the dynamic interplay between persisting mycobacteria and the host immune response, continuously evolving throughout the course of *M.tb* infection. Interestingly, *M.tb* may actually utilize the host immune response to facilitate the structural changes required to facilitate person-to-person transmission. In line with this, while essential to preventing bacterial dissemination, paradoxically IFN-γ-producing Th1 cells may also play a role in facilitating bacterial transmission [186]. The ability of *M.tb* to manipulate the host immune response as means to facilitate central granuloma necrosis and facilitate its transmission while deterring immune-mediated bacterial clearance is a remarkable but poorly understood feature of *M.tb***.** It is important to note that the processes of bacterial dissemination within a host, and transmission between hosts may be independently regulated. For instance, clinically it has been observed that despite exaggerated bacterial burdens and extensive dissemination, HIV-AIDS individuals co-infected with *M.tb* transmit *M.tb* person-to-person far less efficiently [114,187,188,189]. The inability of the HIV-infected host to spread *M.tb* has been attributed to a failure of *M.tb* to drive central granuloma necrosis and cavitation, and the transport of bacilli to the airway. These observations argue that *M.tb* utilizes the necrotic granuloma as a portal for person-to-person transmission.

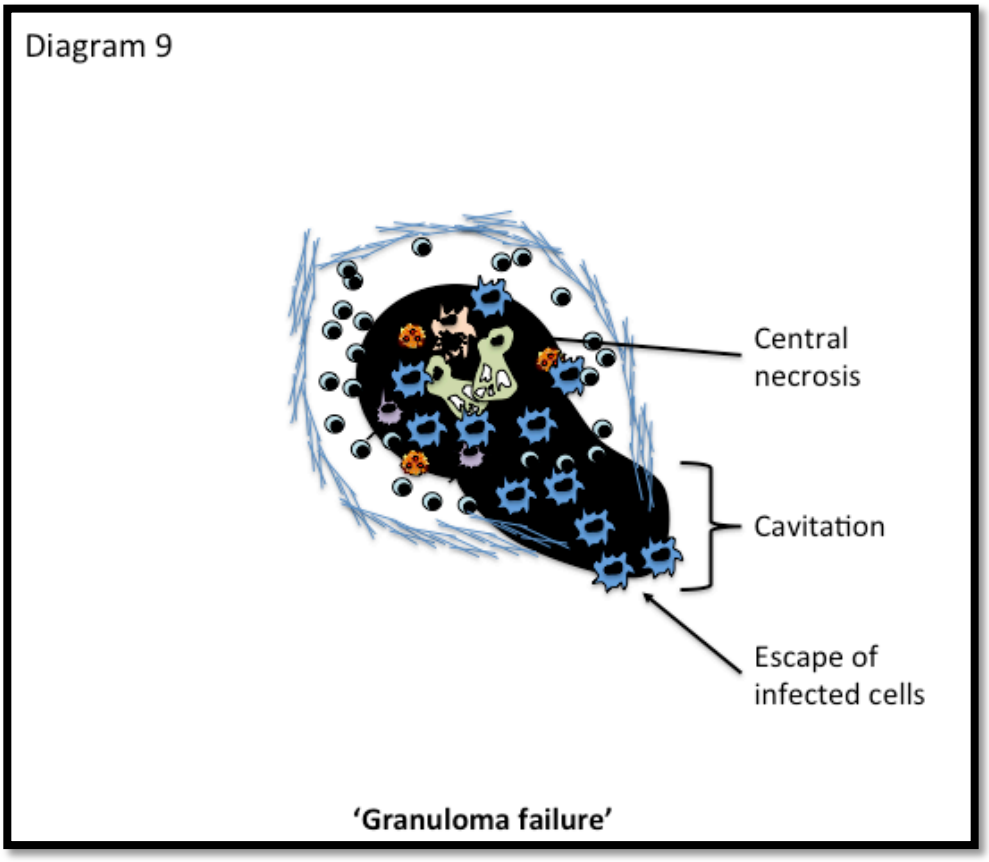

Diagram 9: The failure of the TB granuloma. The characteristic changes associated with the disintegration of the TB granuloma required for dissemination. The center of the granuloma forms a necrotic foci, and initiates the process of cavitation. This results in the disintegration of immune control and the escape of infected cells into the surrounding tissue.

While it is well known that changes to the granuloma structure are required for bacterial transmission, it is currently unknown whether the host or bacteria are responsible for these changes. Recently, studies have examined the granuloma at early and chronic stages of disease revealing a dramatic shift in the genes expressed by both *M.tb* and the host immune response. Notably, within the granuloma, the host immune response shifts from predominately pro-inflammatory during the early phases of infection, to immunosuppressive during the chronic stages [153,190,191]. Coincidentally,

M.tb expresses a defined set of genes that function to facilitate immune activation, while simultaneously expressing enzymes to combat immune mediated clearance [192]. This is later followed by a shift in gene expression thought to facilitate immune senescence within the granuloma, allowing for *M.tb's* persistence [192]. While traditionally *M.tb* is thought to lie dormant, it has recently been demonstrated that throughout the course infection *M.tb* will periodically 'awaken', up-regulating a number of genes and sample the immune environment [190]. This sampling allows *M.tb* to identify the optimal conditions for facilitating person-to-person transmission. Moreover, during the caseous stage of granuloma formation there is a further shift in the genes expressed by *M.tb* with a significant up-regulation of genes associated with lipid metabolism [182]. Notably, most mycobacterial species are rich in immunomodulatory lipids, which play a central role in immune evasion. Intriguingly, the distribution and release of certain lipids by *M.tb* varies significantly over the course of infection, providing a means by which *M.tb* directs the host immune response. To this end, *M.tb* can release toxic lipids and generate targeted tissue damage. The generation of central necrosis is essential to facilitating cavitation and promoting *M.tb* transmission. Trehalose 6, 6'dimycolate (cord factor) has potent cytotoxic effects and has been implicated in the generation of central necrosis of the granuloma and the transmission of *M.tb* [182,193]. Recently, it has been shown that neutrophils and alveolar macrophages recognize mycobacterial cord factor through their surface c-type lectin receptor, mincle [194,195]. The engagement of mincle leads to a pro-inflammatory cytokine pathway that aids in the early cellular recruitment and control of mycobacteria [194,195]. Interestingly however, the proportion of cord factor varies

greatly throughout the course of infection with its synthesis heavily up-regulated by *M.tb* during the development of central necrosis and cavitation [182,193]. Indeed, studies have linked the amount of cord factor released by *M.tb* to the extent of necrosis and cavity formation [182,193]. Moreover, previous studies have documented mincle as a key receptor in the detection of necrosis and the development of an inflammatory response upon tissue damage [196]. Given that cord factor is known for its cytotoxic effects, one may speculate that engaging mincle may be central to the development of a pro-inflammatory response capable of aiding the formation of cavitation within the granuloma. Utilizing the host immune machinery *M.tb* facilitates the necessary structural changes to ensure its own transmission, which occurs at a time when the immune system is most vulnerable.

While the true nature of the granuloma still remains to be defined, it is now clearly evident that the granuloma is not just a host-mediated entity of segregation and rather, it is a dynamic battlefield bearing the scars left both by the pathogen and the host immune response. While it may have been originally destined to restrain bacterial dissemination, *M.tb* efficiently hijacks the granuloma to provoke the generation of an immunologically sheltered niche to reside within and persist until the situation is favorable to bacterial transmission. Further investigation is required to unravel the mechanisms that allow *M.tb* to mediate its persistence within the granuloma.

3.2. TB and anti – TNF therapy

Much controversy surrounds the role of TNF in host immune responses. In many situations TNF plays a critical role acting as an early released alarm type cytokine and is

required for proper immune activation. However, in the disease progression of inflammatory diseases, such as rheumatoid arthritis, TNF is thought to play a negative or potentiating role. Evidence has been shown that neutralization of TNF in some inflammatory diseases may reduce the severity and slow the progression [197,198]. In other disease types, TNF neutralization accelerates and worsens the disease as has been seen with tuberculosis [197,198,199]. The differential efficacy of such treatment may be attributed to the differential roles of TNF. During an inflammatory process it may increase or worsen the condition, while in a cellular-immunity mediated disease it may be acting as a negative regulator. Irregardless, there has been an increase in the use of anti-TNF alpha therapy as a means of controlling the inflammatory mediated diseases. Given the increase in diagnosis and treatment of inflammatory diseases in the general population, there is an increased risk of negative side effects arising in individuals who also have underlying type I controlled disease, such as TB. Indeed, it has been demonstrated that the use of anti-TNF alpha may significantly increase the risk of latent TB reactivation in humans and if left untreated, becomes rapidly fatal [74,200,201]. This raises the question as to how anti-TNF alpha treatment mediates decreased morbidity in the treatment of inflammatory diseases, but results in potentially fatal immune driven pathology in individuals latently infected with TB.

In addition to classical regulatory cytokines, a number of pro-inflammatory cytokines are being shown to have strong regulatory properties. Following mycobacterial infection, mice deficient in either IFN-γ or TNF fail to regulate the type I immune responses and succumb to immune induced respiratory failure rather than bacterial

dissemination [202,203]. Not surprisingly, following mycobacterial infection IFN-γ deficient mice fail to control *M.tb* which rapidly disseminates. However, interestingly, in addition to bacterial dissemination there is substantial accumulation of activated lymphocytes in the lungs of infected IFN-γ deficient mice. It was demonstrated that the accumulation of antigen specific T cells, and not the bacterial dissemination, is what leads to the massive immunopathology and the rapid death of infected mice [203]. The elevated bacterial levels were demonstrated to be consistently lower than required to be the cause of this degree of immunopathology and swiftness of death [203]. These findings led to the hypothesis that IFN-γ may play a biphasic role as both a pro-inflammatory as well as an immune regulatory cytokine [203]. The mechanism through which IFN-γ influences T cell regulation was demonstrated to be indirect [203]. The absence of IFN-γ was shown to cause a profound failure of NOS2 induction and the production of nitric oxide [203]. Investigation in the potential role of nitric oxide on T cell regulation showed that in the absence of nitric oxide there was a significant reduction in the apoptosis of infiltrating T cells [203]. Further studies were carried out using iNOS deficient mice and the similar conclusions reached [82]. These results highlight the importance of nitric oxide in the regulation of T cells during infection, and a recent paper indicated that the ability of virulent *M.tb* to resist nitric oxide may allow the bacteria to exploit the immunosuppressive nature of nitric oxide as a mechanism of immune evasion [204].

Similar to IFN- γ, a loss or polymorphism in TNF can increase the susceptibility of the host to mycobacterial infection, as well as enhance reactivation of a latent infection. When TNF is absent we see a highly similar phenomenon to that observed in

the lungs of IFN-γ deficient mice. Determining the underlying mechanism of action has been complicated by the multifaceted role that TNF plays during mycobacterial host defense. Previously, our lab has documented that in the complete absence of TNF there is a loss of type I immune regulation following mycobacterial BCG challenge [202]. This loss of type I immune regulation is not restricted to the lung but is also demonstrated in the spleen and mediastinal lymph nodes of infected TNF deficient animals [202]. Following infection there is a rapid accumulation of IFN-γ producing lymphocytes in the lung parenchyma [202]. The massive accumulation of type I cells in the lung was thought to have profound effects on the establishment of granuloma formation [202]. Although detectible, the granulomatous response in TNF deficient mice is significantly altered compared to wild type animals [202]. It was estimated that at least one third of the lung area of TNF deficient mice was undergoing massive necrosis, with enhanced fragmentation of macrophages contained within the granuloma [202]. The granuloma had poorly defined edges and was less compacted [202]. There was evidence of massive perivascular infiltrates consistent with the accumulation of type I lymphocytes [202]. While 100% of mice deficient in TNF succumb to respiratory failure following pulmonary mycobacterial infection the mechanisms governing this mortality are not currently known.

3.3. TB and cigarette smoke exposure

Despite the prevalence of individuals infected with *M.tb*, the causative bacterium of TB rarely (5-10% of cases) results in active disease [31]. While the majority of TB cases are seen in the developing world, North America is 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 [205,206]. While nutrition, population density, and access to advanced health care are significant factors in the development of active disease, a number of preventable elements exist that can substantially increase an individual's risk [207,208]. Typically, the development of active TB is associated with the acquisition of an immune compromising disease that significantly impairs CD4 T cell responses, such as HIV-AIDS [42,209,210]. While the influence of HIV-AIDS on TB has long been acknowledged and commonly perceived as a leading risk factor, it is only responsible for one quarter of all deaths [42]. Shockingly, the single leading cause of TB mortality is the exposure to smoked tobacco products [211]. Regrettably, this highly preventable social activity accounts for approximately 60% of all TB related deaths, and is the leading risk factor associated with acquisition, active disease and mortality. Further augmenting the problem, primary as well as secondhand cigarette exposure significantly enhances these risks, especially in children [212,213]. Given the link between cigarette exposure and increased TB mortality, as well as countless other health concerns, it is particularly disturbing that 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 [214]. The collision of these two epidemics makes unraveling how cigarette exposure impacts TB immunity a particularly relevant issue. With an estimated 60-80% of adult males in China regularly consuming tobacco products, and similar percentages in India, the overwhelming prevalence of cigarette in

these regions places not only those actively smoking, but also their non-smoking friends, colleagues, and household contacts at increased risk for TB [214]. Given the shear number of latently infected individuals and the vast consumption of tobacco products in these afflicted countries, understanding the immunological bases for cigarette mediated suppression of anti-tuberculosis immunity remains a question of paramount importance.

Protective immunity to *M.tb* largely relies on the generation of a robust type 1 immune response, specifically the generation of Th1 polarized, CD4+ IFN-γ producing T cells [31,176]. A failure to generate, or localize these cells to the lung significantly impairs bacterial control, and active disease rapidly ensues [102,145,215]. The generation of Th1 CD4+ IFN-γ+ T cells is dependent on the ability of the host to detect *M.tb's* presence in the lung, uptake *M.tb* antigen and transport it to the local draining lymph node (dLN) [31]. Once in the dLN, specialized antigen presenting cells present antigen to naïve T cells. The presentation of antigen must be conducted in the correct immunological context to ensure protective immunity is generated. The production of Th1 polarizing cytokines, specifically IL-12, are essential to the generation of protective Th1 CD4+ IFN-γ+ T cells and in the context of an *M.tb* infection a failure to produce IL-12 will abrogate the generation of Th1 responses, significantly impairing host immunity [145].

Given that cigarette smoke exposure has long been associated with alterations to the local lung APC populations, and that the alveolar macrophage represents the primary host for *M.tb*, determining if cigarette smoke exposure affects the activation status of infected alveolar macrophages is a highly relevant question. Traditionally, cigarette smoke influences the alveolar macrophage to adopt an alternative activation phenotype,

preferentially facilitating Th2 responses and suppressing the generation of Th1 [216,217,218,219]. Furthermore, it has been repeatedly demonstrated that cigarette smoke exposure significantly impairs the generation of type 1 immunity leaving infected hosts highly susceptible to certain viral and bacterial pathogens [220,221,222]. Despite these observations, little is known about how cigarette smoke exposure influences the generation of Th1 immunity following a pulmonary *M.tb* infection.

Despite the immense epidemiological data, firmly establishing a link between cigarette smoke exposure and the development of active TB, the role of cigarette smoke exposure on TB disease progression has been largely ignored both clinically and scientifically. Likely owing to the misconceived significance of cigarette smoke exposure on TB, little experimental data has surfaced demonstrating the underlying mechanisms of how cigarette smoke influences TB immunity. Recently however, two groups have begun to address this question, firmly establishing that prior cigarette smoke exposure suppresses the development of type 1 immunity in the lung, resulting in impaired bacterial control [223,224]. While these studies have conclusively demonstrated a link between cigarette smoke exposure and impaired type 1 immunity in the lung, they have only assessed the impact of prior cigarette smoke exposure on anti-TB immunity, crippling any real world extrapolation. Owing to technical limitations, no group to date has evaluated the effect of continuous cigarette smoke exposure on the course of an *M.tb* infection, leaving a significant gap in our understanding. Clinically, cigarette smoke cessation rapidly decreases the risk of TB mortality by more than 60% and within a short period is indistinguishable from similarly infected individuals with no history of cigarette

smoke exposure [225,226]. Regardless of experimental limitations, the question of how continuous cigarette smoke exposure influences mycobacterial immunity after infection is of significant importance and must be addressed. Reverberated by clinical observations, cessation models can provide only limited insight into the impact of cigarette smoke on *M.tb* immunity, providing a very short, and largely undefined experimental window in which to study the effects of cigarette smoke exposure. The global smoking statistics and rapid recovery following cigarette smoke cessation emphasizes the urgency for an in depth analysis of both continuous and discontinuous smoke exposure and the ensuing impacts on *M.tb* immunity to be conducted.

4. Human vs murine TB models

The use of animal models affords the ability to perform assays and analysis for mechanistic studies that would not otherwise be possible. Regardless, the role of granuloma in tuberculosis has remained enigmatic largely because of the unavailability of reliable animal models and appropriate techniques to observe the dynamic process of granuloma evolution. Although different experimental models (mice, guinea pig, rabbit, cattle and macaque) have been developed, only cattle and macaque monkeys form the type of granuloma that closely resembles those seen in humans [157,227,228,229,230]. The use of a murine model of mycobacterial infection has provided us with significant insight into the initiation and maintenance of mycobacterial disease. Despite its usefulness, the murine model of mycobacterial granuloma does differ structurally and progresses differently from that of those seen in humans. Being extensively used as a model of TB, the murine granuloma lacks many of the unique characteristic features of

the human granuloma including centralized necrosis, giant multinuclear cells, and a defined 'lymphocytic cuff' [231]. The structural development of multinucleated giant cell or Langhans cells is not commonly seen in the murine model of tuberculosis and is considered a defining difference [120]. Further, central necrosis, or caseation is generally not seen in wild type models of murine tuberculosis, and thus the ability to model chronic granuloma formation is significantly impaired [120]. Moreover, the essential role of CD4 T cells in the control of mycobacterial growth has largely been attributed to their potent IFN-γ production and subsequent macrophage activation. While macrophage activation is essential to the control of *M.tb* in mouse and man, the role of nitric oxide has been an issue of some debate. Historically, studies have shown that human macrophages do not produce nitric oxide to the same degree *in vitro* as those isolated from mice [232]. Recently however, several groups have demonstrated that human macrophages from TB infected patients, as well as human macrophage cell lines are capable of inducing iNOS and producing nitric oxide in response to *M.tb* antigen [233,234,235]. Indeed, it appears that while the timing of nitric oxide and its role in TB control may differ somewhat between mouse and man, nitric oxide remains important in both species. Despite their differences, the insights gained from murine models have allowed for a better understanding of the progression and functionality of the granuloma following mycobacterial infection.

5. Central hypothesis and objectives of study

Hypothesis: The integrity of the granuloma is fundamental to the host ability to control *M.tb* infection. The granuloma is a dynamic battlefield in which there is a continuous struggle between the host immune system and the persisting bacteria. Any perturbation to this balance will be detrimental to the host.

Objectives:

[1] To address the question: How does the immune environment within the granuloma compare to that outside the granuloma following mycobacterial infection?

[2] To address the question: How does TNF regulate the integrity of the granuloma and limit the induction of lethal immunopathology?

[3] To address the question: How does cigarette smoke alter the host immune response to mycobacterial infection, and how does this impair bacterial control?

CHAPTER 2: How does the immune environment within the granuloma compare to that outside the granuloma following mycobacterial infection?

Christopher R. Shaler, Kapilan Kugathasan, Sarah McCormick, Daniela Damjanovic, Carly Horvath, Cherrie-Lee Small, Mangalakumari Jeyanathan, Xiao Chen, Ping-Chang Yang & Zhou Xing.

Pulmonary mycobacterial granuloma: Increased IL-10 production contributes to establishing a symbiotic host-microbe microenvironment.

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How does the immune environment within the granuloma compare to that outside the granuloma following mycobacterial infection?

In this study we set out to evaluate the immune microenvironment within the granuloma compared to the airway luminal space. The airway lumen represents the first site where invading *M.tb* meets the immune response. Conventional knowledge considers the pulmonary granuloma a central hallmark of TB disease. Given the incomplete ability of the host immune response to clear *M.tb,* the granuloma is thought to serve as the primary mechanism of bacterial control. Given the central role of the granuloma in mycobacterial control, it is imperative to understand the immune microenvironment within the granuloma structure, and how this functions to limit bacterial dissemination.

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

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Immunopathology and Infectious Diseases

Pulmonary Mycobacterial Granuloma

Increased IL-10 Production Contributes to Establishing a Symbiotic Host–Microbe Microenvironment

Christopher R. Shaler, Kapilan Kugathasan, Sarah McCormick, Daniela Damjanovic, Carly Horvath, Cherrie-Lee Small, Mangalakumari Jeyanathan, Xiao Chen, Ping-Chang Yang, and Zhou Xing

From the Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, and M.G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario, Canada

The granuloma, a hallmark of host defense against pulmonary mycobacterial infection, has long been believed to be an active type 1 immune environment. However, the mechanisms regarding why granuloma fails to eliminate mycobacteria even in immune-competent hosts, have remained largely unclear. By using a model of pulmonary *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) infection, we have addressed this issue by comparing the immune responses within the airway luminal and granuloma compartments. We found that despite having a similar immune cellular profile to that in the airway lumen, the granuloma displayed severely suppressed type 1 immune cytokine but enhanced chemokine responses. Both antigen-presenting cells (APCs) and T cells in granuloma produced fewer type 1 immune molecules including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and nitric oxide. As a result, the granuloma APCs developed a reduced capacity to phagocytose mycobacteria and to induce T-cell proliferation. To examine the molecular mechanisms, we compared the levels of immune suppressive cytokine IL-10 in the airway lumen and granuloma and found that both granuloma APCs and T cells produced much more IL-10. Thus, IL-10 deficiency restored type 1 immune activation within the granuloma while having a minimal effect within the airway lumen. Hence, our study provides the first experimental evidence that, contrary to the conventional belief, the BCG-induced lung granuloma represents a symbiotic host-microbe microenvironment characterized by suppressed type 1 immune activation. ***(Am J Pathol 2011, 178:1622–1634; DOI: 10.1016/j.ajpath.2010.12.022)***

Secondary only to HIV, pulmonary tuberculosis remains a leading cause of death by a single infectious pathogen.[1–3] Pulmonary infections caused by other mycobacterial species also often pose a serious problem to immune-compromised hosts.[4–6] After mycobacterial infection in the lung, the host attempts to control the infection by locally segregating the bacteria in a granuloma formed under type 1 immune conditions.[7–10] Granuloma formation is thus the pathological hallmark of pulmonary mycobacterial infection readily detectible on a radiographical image. The granuloma is an organized collection of inflammatory and immune cells primarily composed of infected macrophages, recruited dendritic cells, and activated lymphocytes. The evolution of the granuloma is divided into two stages. First, the initial formation of the "innate" granuloma comprises early recruited innate immune cells including macrophages, dendritic cells, and neutrophils, which function to contain early mycobacterial infection and to recruit adaptive immune components. Second, the formation of the "immune" granuloma is hallmarked by the arrival of antigen-specific T lymphocytes and the activation of infected macrophages to further control internalized mycobacteria.[9,10] The "immune" granuloma formation is not fully developed until 21 to 25 days, the time when maximal antigen-specific T cells reach the lung.[3] On arrival in the lung, some antigen-specific T cells remain in the interstitium, presumably assisting in the formation of the "im-

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Address reprint requests to Zhou Xing, M.D., Ph.D., Room 4012-MDCL, Department of Pathology & Molecular Medicine, McMaster University, 1200 Main St W, Hamilton, ON L8N 3Z5, Canada. E-mail: xingz@mcmaster.ca.

mune" granuloma, whereas others enter the airway lumen. It has long been believed that the granuloma represents purely a host response mechanism, creating an immune-active microenvironment to concentrate antimycobacterial immune responses, contain infection, and limit systemic dissemination.[2,3] This belief is based mainly on the findings from us and others that the inability of the host to form or maintain granuloma structures as a result of either lacking or having dysregulated type 1 immune responses will inevitably lead to uncontrolled mycobacterial infection in the lung and severe disseminated disease.[2,3,10–15]

However, the notion that the granuloma is an immune-active microenvironment formed solely as a mechanism for the host to control infection and limit systemic dissemination has recently been challenged. There is the evidence that by expressing a set of "persistence" genes, the mycobacterium, together with the host, contributes to granuloma formation as a mechanism to facilitate bacterial dissemination and persistence.[16,17] This suggests that the granuloma microenvironment is subject to immune subversive influences from mycobacteria throughout the course of infection. Furthermore, recent studies demonstrate that mycobacteria would rather make the granuloma their home than the extragranuloma environment,[18,19] suggesting that the granuloma is permissive to mycobacterial survival and persistence while also being immune-protective. Indeed, even infected immune-competent human beings or animals have hardly ever been observed to eliminate the mycobacteria, resulting in a high incidence of latent infection and the reactivation of disease when the immune system is weakened.[20,21] Together, the emerging evidence supports the contemporary view that the granuloma represents a symbiotic tissue microenvironment for the mutual benefit of both the host and the mycobacterium. However, whether the mycobacterial granuloma is indeed an immunologically suppressed microenvironment remains largely to be established, and the underlying mechanisms are still unclear. Investigation of such questions entails the understanding and comparison of the phenotype and functional characteristics of the immune cells both inside and outside of the granuloma.

In the present study, we have addressed this issue by using a murine model of pulmonary mycobacterial infection elicited by *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG). We found that, compared with the antigen-presenting cells (APCs) and T cells residing within the airway luminal (bronchoalveolar) space outside of the granuloma, the cells from the granuloma were functionally immune suppressed. Such an immune-suppressed phenotype of the granuloma cells was characterized by heightened production of the immune-regulatory cytokine IL-10. Thus, the absence of IL-10 restored the type 1 immune responses and antimycobacterial activities in the granuloma. Together, our study findings strongly suggest that, during pulmonary mycobacterial infection, the airway lumen and the granuloma represent two immunologically different lung microenvironments, with the latter being immune suppressed.

Materials and Methods

Mice

Female C57Bl/6 mice 6 to 10 weeks old were purchased from Harlan Laboratories, (Indianapolis, IN). IL-10 knockout mice of C57Bl/6 background (B6.129P2-Il10tm1Cgn/J) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a specific pathogen-free level B facility. All experiments were conducted in accordance with the McMaster University Animal Research Ethics Board.

Mycobacterial Preparation and Model of Pulmonary Mycobacterial Granuloma Formation

M. bovis BCG (Connaught strain) was prepared as previously described.[12,22] Briefly, BCG was grown in Middlebrook 7H9 broth (Difco, Lawrence, KS) 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 10^6 colony forming units (CFU) per mouse for elicitation of vigorous granuloma formation.

Isolation of Granuloma and Airway Luminal Cells from Mycobacterium-Infected Lungs

We have previously observed that pulmonary "immune" granulomatous responses peak at approximately day 25 postinfection.[11,12] Thus, mice were sacrificed 25 days after mycobacterial infection to allow for sufficient yields of total and fractionated airway lumen and granuloma immune cells. Mice were first bled via the abdominal vessels and their lungs were exhaustively lavaged with PBS to remove bronchoalveolar lavage cells following our previously described protocol.[23] Lung granulomas were then isolated by using a well-established protocol.[24–26] Briefly, the lavaged lungs were homogenized in a tissue blender (Waring Commercial, Torrington, CT) using the low speed setting for 10 to 15 seconds, which leaves the granulomas intact. Lung granuloma-containing homogenates were then filtered through a 40-μm cell strainer. Granulomas collected on top of the filter were then digested with collagenase type 1 (Sigma-Aldrich, Oakville, ON, Canada) (2000 units/mL in a volume of 5 mL/lung) for 20 minutes under agitation at 37°C and washed three times with 5% FBS in PBS. Red blood cells in granuloma cell preparations were lysed using ACK lysis buffer and the cells were then washed with 5% FBS in PBS and filtered through a 40-μm cell strainer. Cells were spun

down and resuspended in cRPMI media (RPMI 1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin) and counted. Cell viability was determined by Trypan Blue exclusion.

Purification of CD11c+ and CD11b+ APC Populations from Total Airway Luminal and Granuloma Cells

Single cell suspensions of total airway lumen and granuloma cells isolated above from multiple mice were pooled and then first incubated with CD11c microbeads (Miltenyi Biotec, Auburn, CA) for purification of CD11c+CD11b+/− APCs as we recently described.[27] CD11c-labeled cells were then passed through a mass spectrometry (MS) column on the OctoMACS separator (Miltenyi Biotec, Auburn, CA). Samples were run through magnetically activated cell sorting (MACS) separation columns twice to achieve high purity. Collected CD11c-negative fractions were then further labeled with CD11b microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions and passed through the MACS column twice for isolation and enhanced purity of CD11c-CD11b+ APCs. Cells were counted and their viability was verified by Trypan blue exclusion. Purity of recovered CD11c+CD11b+/− and CD11c-CD11b+ APC populations was determined by fluorescence-activated cell sorting (FACS) and was consistently >90%.

Cell Surface Immunostaining and Intracellular Cytokine Staining

All monoclonal antibodies (mAbs) used were purchased from BD Pharmingen. Immunostaining and FACS were carried out as previously described.[12,22,28] Briefly, cells were blocked for nonspecific binding of their Fc receptors with anti-CD16/CD32 Abs for 15 minutes and then stained for 30 minutes on ice with the appropriate combinations of fluorochrome-conjugated mAbs. Fluorochrome-conjugated mAbs to CD11b, CD11c, Gr.1, CD19, NK1.1, CD3, CD4, and CD8 were used. Appropriate isotype controls were used for each antibody. For intracellular cytokine staining, single cell suspensions from airway lumen and granuloma were cultured and stained as previously described.[29] 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, ON, Canada) was added 18 hours after stimulation. After culture, cells were washed and blocked with CD16/CD32 for 15 minutes on ice and stained with cell surface Abs. In some experiments, cells were then washed, permeabilized and stained with interferon-γ (IFN-γ), and IL-10 Abs according to the manufacturer's instructions included in the intracellular cytokine staining kit (BD Pharmingen). Stained cells were run on the LSRII (BD Biosciences, Mississauga, ON, Canada) flow cytometer using FACSDiva software and data were 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.

Cell Culture and Cytokine Measurement

Total airway lumen and granuloma cells (0.25×10^6/well) or purified CD11c+ and CD11b+ APC fractions from the airway lumen and granuloma (0.1×10^6/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. The antigens used for stimulation were *M. tuberculosis* culture filtrate proteins (*M.tb*-CF) (2 μg/well) or live *M. bovis* BCG (5 CFU/cell or 2 CFU/cell for total and purified cell cultures, respectively). 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, and IL-10 concentrations were measured by using duoset ELISA kits (R&D Systems) and IL-17, keratinocyte chemoattractant, macrophage inflammatory protein-1 β, and monocyte chemoattractive protein-1 were measured by Luminex multianalyte technology (Luminex Corporation, Austin, TX).

Nitric Oxide Production Measurement

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.[30] Briefly, diluted supernatants were added at a 1:1 ratio with Griess reagent buffer (Sigma-Aldrich, St Louis, MO). 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 μmol/L of sodium nitrite concentrations.

Mycobacterium *Phagocytosis Assay by Flow Cytometry and Confocal Microscopy*

To fluorescently detect bacterial uptake by APCs, *M. bovis* BCG was labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) for 30 minutes at room temperature to ensure a high level of binding. After CFSE labeling, BCG was opsonized with naive C57BL/6 serum at 37°C for 30 minutes. Subsequently, 0.25×10^6 purified CD11c+ cells were combined with fluorescence-labeled BCG-CFSE (5 CFU/cell) in a 96-well plate and co-cultured at 37°C overnight. After overnight co-culture, cells were blocked for nonspecific binding of their Fc receptors with anti-CD16/CD32 for 15 minutes on ice and then stained with mAb against CD11c+ and CD11b+ (BD Biosciences) for 30 minutes. To evaluate the uptake of mycobacteria by APC populations, stained cells were run on the LSRII (BD Biosciences, Mississauga, ON, Canada) flow cytometer using FACSDiva software, and data were analyzed with Flowjo software (Tree Star, Ashland, OR) to determine the frequency of bacterial uptake by the percentage of cells that stained CD11b+CFSE+ and CD11c+CFSE+. Depending on the number of cells available, 100,000 to 250,000 events per sample were ana-

lyzed. In an independent setup, CD11c+ cells were purified by MACS double column purification from infected airway luminal and granuloma cells. Purified CD11c+ cells (0.25×10^6) were cultured overnight with opsonized, fluorescence-labeled BCG prepared as described above. After overnight culture, cells were fixed for 30 minutes using 2% paraformaldhyde, washed three times with PBS, followed by 30-minute staining with PI, a DNA intercalating agent with fluorescent properties, allowing the visualization of the nucleus. After PI staining, cells were imaged for PI and CFSE localization using a Zeiss LSM10 laser capture confocal microscope.

T-Cell Proliferation Assay

CD11c+ cells were purified by MACS purification from infected airway luminal and granuloma cells. CD3+ T cells were purified from the spleen of BCG-infected mice and labeled with CFSE as described above. APCs and T cells were combined at a ratio of 1 CD11c+ cell to 10 CFSE-labeled T cells for a total of 2×10^6 cells per well cultured for 72 hours at 37°C and 5% CO_2 in complete RPMI. After culture, cells were blocked for nonspecific binding of their Fc receptors with anti-CD16/CD32 for 15 minutes on ice, and then stained with mAb against CD3 and CD4. Stained cells were run on the LSRII (BD Biosciences) flow cytometer using FACSDiva software and data were analyzed with Flowjo software (Tree Star, Ashland, OR). The dilution of CFSE on CD3+$CD4^+$ cells was assessed as an indication of T-cell proliferation.

Immunohistochemical and Immunofluorescence Staining for Localization of CD11c+ and CD11b+ APCs

For immunohistochemical staining, lungs were removed and placed in OCT compound, at which time they were stored at −70°C until sectioning. Frozen tissue sections were mounted on slides and fixed with cold acetone for 15 minutes. After fixation, sections were washed with Tris-buffered saline and covered with 2% BSA for 20 minutes at room temperature to block non-specific binding. Sections were then blocked with H_20_2 and avidin/biotin block (Vector Laboratories, Burlington, ON, Canada). Tissues were covered with hamster anti-mouse CD11c and on a separate occasion with rat anti-mouse CD11b mAb (BD Pharmingen, Mississauga, ON, Canada) overnight at room temperature. Sections were then incubated for 1 hour with a secondary biotinylated anti-hamster or anti-rat Ab, after which sections were incubated with streptavidin HRP for 45 minutes. Slides were then incubated with 3-amino, 9 ethyl-carbozole (AEC) chromogen solution for 30 minutes, rinsed, and counterstained with hematoxylin.

For immunofluorescence staining, lungs were removed and placed in OCT compound, at which time they were stored at −70°C until sectioning. Cold acetone-fixed, 5 μmol/L sections were washed and treated with 3% goat serum in PBS for 30 minutes to block nospecific binding. Sections were then washed and stained with primary antibodies for CD11c (hamster anti-mouse, overnight) and CD11b (rat anti-mouse, 1 hour). Sections were subsequently washed and incubated with secondary antibodies toward the primary CD11c hamster anti-mouse (Alexfluor 488 conjugated goat anti-hamster) and CD11b rat anti-mouse (Alexafluor 633 conjugated goat anti-rat). (Molecular Probes, Invitrogen, Burlington, ON, Canada) for 1 hour at room temperature. Slides were mounted with Vectashield mounting media (Vector Laboratories) with SYTO 83 (Molecular Probes) and visualized using the Carl Ziess LSM 510 confocal microscope and images were analyzed using LSM510 image software.

Statistical Analysis

Statistical analysis was performed using unpaired, one-tailed or two-tailed Student's *t*-test with Excel spreadsheet software (Microsoft Corporation, Redmond, WA). Values of $P < 0.05$ were considered to be statistically significant. The one-tailed Student's *t*-test was used to analyze the information of colony forming units, whereas the two tailed Student's *t*-test was used to analyze all other values.

Results

Similar Immune Cellular Composition Observed in Airway Lumen and Granuloma in Mycobacterial Infection

After mycobacterial infection, two major APC populations are primarily infected: the alveolar macrophage and the alveolar dendritic cell. These two populations share similar cell surface makers and are broadly defined by their expression of complement receptors: CR3(CD11b/CD18) and CR4(CD11c/CD18).[31] Thus we first examined the tissue localization and accumulation of CD11b+ and CD11c+ cells in mycobacterium-infected lung by immunohistochemistry. By day 21, there was a large accumulation of both CD11b+ and CD11c+ cells in the lung, with the CD11b+ cells being more abundant in the granuloma (Figure 1A). As one of the pulmonary interstitial dendritic cell subsets expresses both CD11b and CD11c,[28,32] we next examined the double-positive APCs in the granuloma by confocal microscopy. Confocal microscopic examination reveals mostly singly CD11c+ or CD11b+ cells, and again there was a greater number of the latter in the granuloma (Figure 1B), reaffirming the results by immunohistochemistry. The frequency of CD11c+CD11b+ double positive cells was found to be low (Figure 1B). Both CD11c+ and CD11b+ APC populations increased to a maximum by day 35 and slowly declined, returning to levels similar to those in naïve mice by day 75 (data not shown).

To gain further insight into different specific immune cell types recruited to the lung after mycobacterial infection, cells contained within the airway lumen were isolated by bronchoalveolar lavage, and the granuloma cells were isolated from the lung paranchyma.[23,24,26]

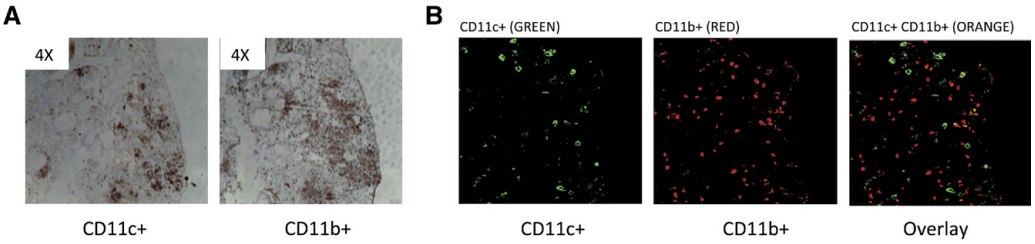

Figure 1. Localization and accumulation of CD11c+ and CD11b+ APCs in the lung after mycobacterial infection. Infected mice were sacrificed at day 21. **A:** Immunohistochemical staining of mycobacterial infected lungs for CD11c- and CD11b-positive cells. **B:** Immunofluorescent confocal microscopy of murine mycobacterial infected lungs and demonstration of CD11c (green), CD11b (red), and overlay (orange) positive cells. Images are representative of three sets of stained sections.

Purified airway luminal and granuloma cells were stained with antibodies against CD3, CD4, CD8, CD19, CD11c, Gr.1, CD11b, or NK1.1 and analyzed by FACS. The FACS analysis revealed the presence of CD3+CD4$^+$ (CD4 T cells), CD3+CD8+ (CD8 T cells), CD3-CD19+ (B cells), CD3-NK1.1+ [natural killer (NK) cells], CD11c+-CD11b+/− (CD11c+ APCs), CD11c-CD11b+ (CD11b+ APCs), and CD11c-Gr.1+ (neutrophils) cell populations in both the airway lumen and granuloma (Table 1). T and B lymphocytes and CD11c+ and CD11b+ APCs (macrophages and dendritic cells) were found to represent the majority of inflammatory cells recruited to both the airway lumen and the granuloma. The overall frequencies of inflammatory cells were highly comparable between the airway lumen and granuloma (Table 1). The greater ratio of CD11c-CD11b+ APCs versus CD11c+ APCs is in agreement with the data by immunohistochemistry and confocal microscopy (Figure 1, A and B). These two APC populations most likely represent macrophages and myeloid dendritic cells, respectively. There were also many more CD4$^+$ T cells than CD8+ T cells in both the airway lumen and granuloma compartments, supporting the notion that the former is the major effector T-cell subset involved in host defense during mycobacterial infection.[12,33,34] Together, these data reveal a similar immune cellular profile in the airway lumen and the granuloma after pulmonary mycobacterial infection, and indicate that macrophages, dendritic cells, and T cells are the predominant antimycobacterial cellular components.

Table 1. Immune Cellular Profile of the Airway Lumen and Granuloma of Mycobacterium-Infected Mice

Cell surface marker	Airway lumen (% ± SE)	Granuloma (% ± SE)
CD3+	31.97 ± 3.71	29.87 ± 3.3
CD3+CD4$^+$	17.46 ± 5.16	17.51 ± 3.7
CD3+CD8+	9.65 ± 2.99	7.09 ± 1.87
CD3-CD19+	12.51 ± 3.22	15.51 ± 4.17
CD3-NK1.1+	1.77 ± 0.11	4.05 ± 0.7
CD3-CD11c+CD11b+/−	14.87 ± 2.38	14.82 ± 2.73
CD3-CD11c-CD11b+	25.59 ± 3.48	30.08 ± 3.99
GR1+CD11b+/−	18.13 ± 2.13	19.33 ± 2.94

Inflammatory cells were isolated from the airway lumen and granuloma of mycobacterium-infected lungs and stained with various leukocyte surface cell markers. Results are expressed as the cumulative average percent of total cells SEM from five independent experiments.

Differential Pro-Inflammatory and Anti-Inflammatory Molecules Are Produced by Total Airway Lumen and Granuloma Inflammatory Cells during Mycobacterial Infection

Having observed a similar overall cellular immune profile within the airway lumen and granuloma, we next began to examine the functionality of the immune cells from these two compartments. We first compared cytokine production by total inflammatory cells isolated from these two compartments. In this regard, we focused on the two type 1 cytokines, TNF-α and IFN-γ, that are known to play key roles in host defense against mycobacterial infection.[11,12,14] On stimulation with live mycobacteria, total airway luminal cells produced significantly greater amounts of TNF-α compared with total granuloma cells after 12-, 24-, or 48-hour culture (Figure 2A). Similarly, airway luminal cells produced significantly higher levels of IFN-γ than granuloma cells on stimulation with granuloma cells, producing nearly undetectable levels of this cytokine after 12-, 24-, or 48-hour culture (Figure 2B). In light of decreased pro-inflammatory cytokines TNF-α and IFN-γ by granuloma cells (Figure 2, A and B), we evaluated the level of NO, a critical mycobactericidal and phagosome maturation-stimulating biochemical product produced by infected alveolar macrophages and dendritic cells.[32,35,36] On stimulation with mycobacteria, airway luminal cells produced markedly increasing levels of NO over 12-, 24-, and 48-hour culture (Figure 2C). In sharp contrast, stimulated granuloma cells produced much lower levels of NO, which were hardly inducible over the 12 hours (Figure 2C).

Given the depressed type 1 immune phenotype seen in the granuloma, we examined the potential immune mechanism. Evidence in the literature suggests that mycobacterial infection can induce the production of IL-10,[37–39] which is a critical immune regulatory cytokine. We thus examined whether granuloma cells produced the immune regulatory cytokine IL-10 and compared its level with that by airway luminal cells. In contrast to their much-heightened production of pro-inflammatory cytokines, airway luminal cells produced little IL-10 in response to mycobacterial stimulation, whereas granuloma

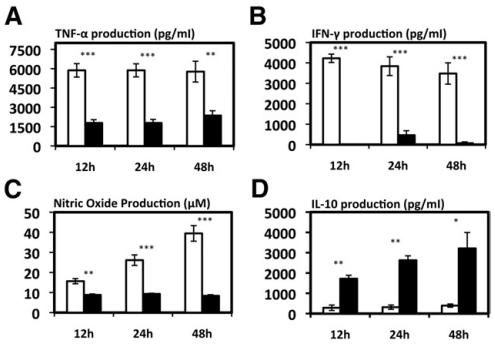

Figure 2. Production of pro-inflammatory and anti-inflammatory molecules by total airway luminal and granuloma cells. Cytokines and NO were measured by ELISA or a chemical assay in supernatants collected from cell cultures stimulated with live mycobacteria. **A:** TNF-α production by granuloma (**filled bar**) and airway luminal (**open bar**) cells. **B:** IFN-γ production by granuloma and airway luminal cells. **C:** Nitric oxide production by granuloma and airway luminal cells. **D:** IL-10 production by granuloma and airway luminal cells. Data are expressed as the average values ± SEM of three sets of cell preparations (three mice/set) and four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the corresponding granuloma or airway luminal cells. h, hours; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

cells produced significantly higher levels of IL-10 over the entire course of culture (Figure 2D). As it was shown that IL-10 could suppress Th17 cells and the production of IL-17,[40,41] we examined the levels of IL-17 in the culture supernatant of airway luminal and granuloma cell cultures. Indeed, airway luminal cells produced larger amounts of IL-17 than granuloma-derived cells (Table 2).

To address whether the granuloma represents a generalized immune suppressed environment, we compared the production of chemokines by airway luminal and granuloma cells. To this end, we focused on measuring the chemokines involved in neutrophil, APC, and lymphocyte chemotaxis: keratinocyte chemoattractant , monocyte chemoattractive protein-1, and macrophage inflammatory protein-1 β. Of interest, compared with airway luminal cells, granuloma cells produced similar or even increased levels of chemokines (Table 2), in contrast to their much-reduced production of type 1 cytokines (Figure 2, A and B).

Together, the above data indicate that, first, compared with the airway lumen, the granuloma represents a differentially regulated immune environment with greatly reduced type 1 cytokines but yet similar or increased chemokine responses; and second, the reduced type 1 immune activation in the granuloma is associated with markedly enhanced production of immune suppressive cytokine IL-10.

Differential Pro-Inflammatory and Anti-Inflammatory Molecules Are Produced by CD11b+ and CD11c+ APC Populations of Airway Lumen and Granuloma during Mycobacterial Infection

Thus, despite their similar cellular composition, total granuloma cells demonstrate a suppressed type 1 immune phenotype with reduced cytokine and NO responses. To address the major cellular sources displaying such type 1 immune suppression, we first analyzed the major APC populations by using purified CD11c+ and CD11b+ cells, which are most likely dendritic cells and macrophages, respectively.[28,42] Our data show that these CD11c+CD11b+/− and CD11c-/CD11b+ APCs are the most prominent APC populations in both the airway lumen and granuloma (Table 1). These cells were shown to be a significant cellular source of TNF-α, IFN-γ, IL-10, and NO in various models of pulmonary mycobacterial infection.[36,43,44] Thus, we purified CD11c+CD11b+/− (CD11c+) and CD11c-/CD11b+ (CD11b+) APC populations from total airway lumen and granuloma cells by double column-based MACS purification and assessed their cytokine and NO responses. Both airway luminal CD11c+ and CD11b+ APCs produced more TNF-α than their corresponding granuloma counterparts on stimulation with soluble mycobacterial antigens (*m.tb* CF) (Figure 3, A and E). Similarly, mycobacterium BCG-infected airway luminal CD11c+ APCs also produced more TNF-α than the granuloma-derived CD11c+ APCs. In comparison, airway luminal CD11b+ cells produced similar amounts of TNF-α as the granuloma-derived CD11b+ APCs (Figure 3, A and E). Both airway luminal CD11c+ and CD11b+ APCs produced more IFN-γ than the corresponding granuloma counterparts on stimulation by soluble mycobacterial antigens or mycobacterial BCG (Figure 3, B and F). Of note, CD11b+ APC populations, whether airway lumen or granuloma derived, produced less TNF-α and IFN-γ than CD11c+ APCs (Figure 3, B and F). Associated with reduced type 1 cytokine production by granuloma APC populations is markedly decreased NO production by both granuloma CD11c+ and CD11b+ APCs (Figure 3, C and G).

Similar to the unfractionated granuloma cells (Figure 2), the production of immune suppressive or anti-inflammatory cytokine IL-10 by both granuloma CD11c+ and CD11b+ APC populations was significantly upregulated compared with their airway luminal counterparts (Figure 3, D and H). Together the above results suggest that, relative to extragranuloma bronchoalveolar macrophages and dendritic cells, the APC populations present in mycobacterial granuloma demonstrate reduced type 1 immune activation that is associated with enhanced production of immune-suppressive IL-10.

Table 2. Cytokine and Chemokine Production Profile by Airway Lumen and Granuloma Cells

Cytokine	Airway lumen (pg/mL ± SE)	Granuloma (pg/mL ± SE)
IL-17	7807.99 ± 682.72	959.94 ± 46.16
KC	18890.96 ± 399.56	14319.42 ± 213.52
MCP-1	167.35 ± 6.85	1224.89 ± 36.09
MIP-1β	2325.22 ± 160.53	7982.31 ± 203.30

Isolated total airway luminal and granuloma cells were cultured with live mycobacteria. IL-17 and chemokines in culture supernatants were assessed by luminex assay. The data are expressed as the average values ± SEM of triplicate samples.

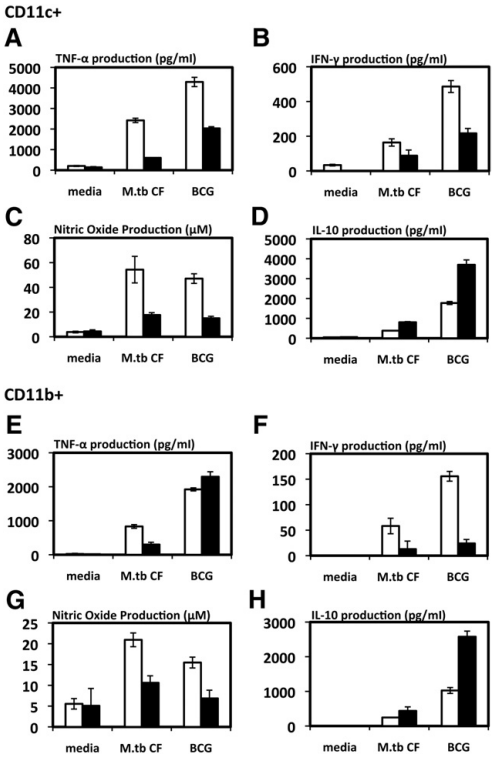

Figure 3. Production of pro-inflammatory and anti-inflammatory molecules by purified CD11c+CD11b+/− and CD11c-CD11b+ APCs from airway lumen and granuloma. APC populations were purified from total airway luminal and granuloma cells. Cytokines and NO were measured by ELISA or a chemical assay in the supernatants of cell cultures with or without stimulation by soluble mycobacterial antigens *m.tb*-CF or live mycobacteria. **A** and **E:** TNF-α production by airway luminal (**open bar**) or granuloma cells (**filled bar**). **B** and **F:** IFN-γ production by airway luminal or granuloma cells. **C** and **G**. Nitric oxide production by airway luminal or granuloma cells. **D** and **H:** IL-10 production by airway luminal or granuloma cells. Data are expressed as the average values ± SEM of triplicate samples obtained from nine pooled mice, representative of two independent experiments. h, hours; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; *M.tb* CF, mycobacterial antigens culture filtrate; BCG, Bacillus Calmette-Guerin.

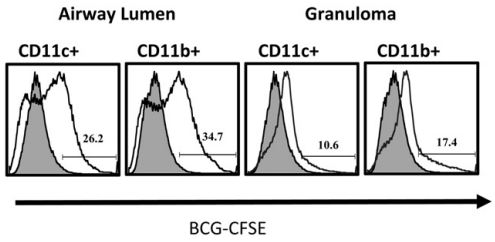

Figure 4. Differential phagocytosis rate of mycobacteria by airway luminal and granuloma derived APC populations assessed by FACS. Purified APCs from the airway lumen and granuloma were incubated with CFSE fluorescence-labeled mycobacteria, and phagocytosis rates were assessed by gating on CFSE+ CD11c+ and CD11b+ APCs. Histograms are representative of duplicate cultures obtained from nine pooled mice and two independent experiments. BCG, Bacillus Calmette-Guerin; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester.

Granuloma-Derived APCs Have Reduced Capacity to Phagocytose Mycobaceria

Given the suppressed type 1 immune profile displayed by mycobacterial granuloma-derived APC populations, we investigated whether this suppressed immune profile might lead to altered antimycobacterial activities of APCs. To this end, we assessed the ability of granuloma-derived CD11c+ and CD11b+ APCs to phagocytose fluorescence-labeled mycobacterium BCG as analyzed by FACS. Compared with airway luminal CD11c+ and CD11b+ APCs, granuloma-derived APCs showed a reduced capacity to phagocytose fluorescence-labeled mycobacteria (airway lumen CD11c + 26.2%, CD11b + 34.7% vs granuloma CD11c + 10.6%, and CD11b + 17.4%, respectively) (Figure 4). This observation was supported by using confocal microscopy, which showed a lower intensity of fluorescence-labeled mycobacteria uptake by granuloma-derived CD11c+ APCs when compared with airway luminal CD11c+ APCs (Figure 5). These data suggest that reduced type 1 immune activation within granuloma can lead to diminished antimycobacterial function of granuloma APCs.

Given the impaired ability of granuloma APCs to uptake mycobacteria (Figures 4 and 5) and their diminished type 1 cytokine responses (Figure 3), we questioned whether these APCs might also have reduced ability to drive T-cell proliferation. To this end, airway luminal and granuloma APCs were paired with splenic CFSE-labeled CD4 T cells, and the level of T-cell proliferation was evaluated by the extent of CFSE dilution. We found that granuloma-derived APCs showed an approximately 40% reduced capacity to drive *ex vivo* $CD4^+$ T-cell proliferation compared with airway luminal APCs (Figure 6).

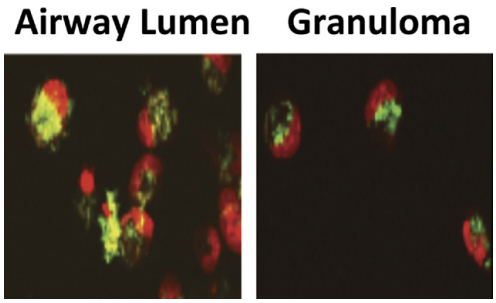

Figure 5. Differential phagocytosis of fluorescence-labeled mycobacteria by airway luminal and granuloma derived APCs visualized by confocal microscopy. Purified APCs from the airway lumen and granuloma of mycobacterium-infected mice were incubated with opsonized fluorescence-labeled mycobacteria. After incubation, cells were fixed and stained for the localization of the nucleus (red) and the uptake of fluorescence-labeled mycobacteria (green). Uptake of fluorescence-labeled mycobacteria was assessed by laser scanning confocal microscopy. Cells were isolated from nine pooled mice.

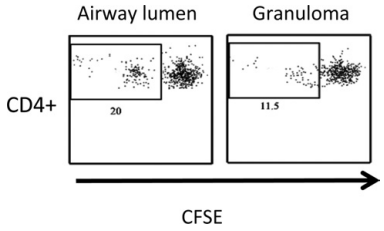

Figure 6. Differential $CD4^+$ T-cell proliferation stimulated by airway luminal and granuloma APCs. Airway luminal or granuloma derived APCs purified from mycobacterium-infected mice were paired with 5,6-carboxyfluorescein-diacetate-succinimidyl-ester–labeled (CFSE) splenic T cells and cultured for proliferation analysis. Proliferation was assessed by the CFSE dilution of $CD3+CD4^+$ T cells assessed by flow cytometry using $CD3+CD4^+$ gating. Dot plots are representative of quadruplicate samples per condition obtained from nine pooled mice.

Differential Pro-Inflammatory and Anti-Inflammatory Cytokines Are Produced by T Cells of Airway Lumen and Granuloma during Mycobacterial Infection

It is known that, after mycobacterial infection, activated type 1 T cells, particularly those of the $CD4^+$ subset, are the most abundant cellular source of IFN-γ, which is a signature type 1 cytokine.[33,44,45] It is believed that infected APCs and T cells interact and reciprocally affect each other's activation. Having observed a suppressed type 1 immune phenotype of APCs in the granuloma, we next examined its impact on T-cell activation in the granuloma compared with the T cells present in the airway lumen by using intracellular cytokine staining. To this end, total cells isolated from the airway lumen and granuloma were stimulated with mycobacterial antigens, stained for T-cell surface markers and intracellular IFN-γ and IL-10, and analyzed by flow cytometry. There was clearly a much greater portion of total airway luminal CD4 T cells responding to mycobacterial antigen stimulation by producing IFN-γ (6.5% of CD4 T cells) compared with granuloma T cells (3.27% of CD4 T cells) (Figure 7). In addition to CD4 T cells, granuloma CD8 T cells also showed a lower level of IFN-γ positivity, indicating a global suppression of T-cell responses (data not shown). In contrast to their markedly reduced IFN-γ responses, granuloma CD4 T cells displayed markedly increased IL-10 production (4.61% in granuloma vs 0.41% in the airway lumen) (Figure 7). Together with the data on APC populations (Figure 3), these results reveal that, first, both APCs and CD4 T cells are significant cellular sources of type 1 cytokines in mycobacterium-infected lung; and second, in keeping with the suppressed type 1 activation of granuloma APC populations, the type 1 mycobacterial antigen-specific T cells present within granuloma are also phenotypically altered with reduced type 1 cytokine production and increased immune-suppressive IL-10 production, relative to those present in the extragranuloma, bronchoalveolar environment of the same mycobacterium-infected host.

Increased IL-10 Contributes to Suppressed Type 1 Immune Activation in Granuloma Microenvironment

Thus far we have established an inverse functional relationship between type 1 immune activation and IL-10 production in the airway lumen and granuloma of mycobacterium-infected lung. This suggests the involvement of heightened IL-10 expression in immune suppression within granuloma. To investigate the role of IL-10 in immune regulation in the airway luminal and granuloma environments, we infected both wild-type (wt) and IL-10 knockout (IL-10KO) mice with mycobacteria and purified airway luminal and granuloma cells. First, we compared the level of activation of type 1 CD4 T cells derived from the airway lumen and granuloma of wt and IL-10KO mice. Consistent with the data presented in Figure 7, there was a much smaller frequency of IFN-γ-producing CD4 T cells (2.86%) in the granu-

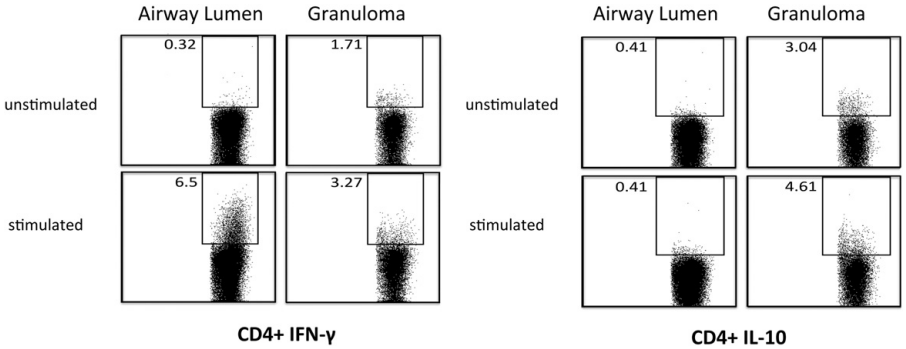

Figure 7. Suppressed antigen-specific type 1 immune activation and enhanced IL-10 responses in granuloma CD4 T cells. Total cells isolated from the airway lumen and granuloma of mycobacterium-infected mice were cultured with or without stimulation by mycobacterial antigens and subject to cell surface and intracellular cytokine staining and flow-cytometric analysis. Dot plots are representative of three sets of cell preparations (each set of cell preparation was pooled from three mice) and three independent experiments. IFN-γ, interferon-γ.

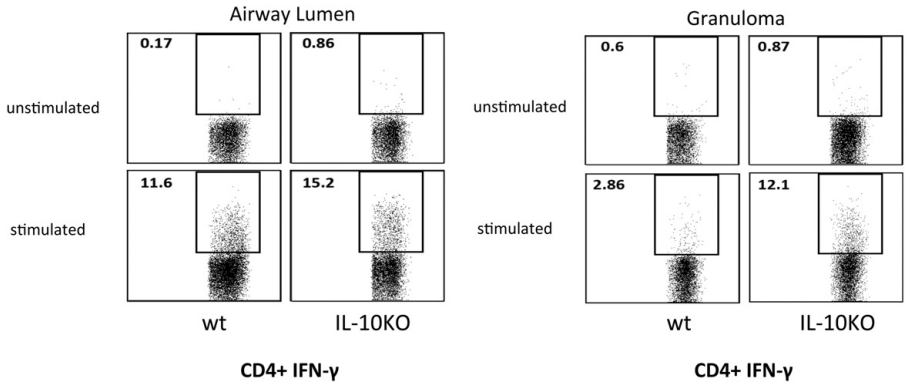

Figure 8. Restored type 1 immune activation of granuloma CD4 T cells by removal of IL-10. Total cells isolated from the airway lumen and granuloma of mycobacterium-infected wild-type (wt) and IL-10 knock-out (KO) mice were cultured with or without stimulation by mycobacterial antigens and subject to cell surface and intracellular cytokine staining and flow cytometry. Dot plots are representative of three sets of cell preparations (each set of cell preparation was pooled from three mice). IFN-γ, interferon-γ.

loma of wt hosts than in the airway lumen (11.6%) of wt hosts (Figure 8). However, lack of IL-10 remarkably restored the level of IFN-γ responses in granuloma CD4 T cells (from 2.86% to 12.1%), which is comparable to 11.6% positivity found in wt airway luminal CD4 T cells (Figure 8). In comparison, the lack of IL-10 had a much smaller impact on IFN-γ-producing CD4 T cells in the airway lumen (from wt 11.6% to 15.2%) (Figure 8), in keeping with relatively little IL-10 protein produced by airway luminal APCs and T cells (Figures 2, 3, and 7).

Furthermore, we examined the production of IFN-γ by total airway luminal and granuloma cells. Again, in keeping with the data in Figure 2, wt granuloma cells produced much less IFN-γ than wt airway luminal cells (Figure 9A). However, lack of IL-10 markedly restored IFN-γ production by granuloma cells from approximately 500 pg up to 8000 pg/mL, which is comparable to that produced by wt airway luminal cells (9000 pg/mL) (Figure 9A). In comparison, lack of IL-10 only modestly further enhanced IL-10 production by airway luminal cells (from 9000 to 13,000 pg/mL) (Figure 9A). The above results indicate that enhanced IL-10 responses within the granuloma account significantly for the markedly suppressed type 1 immune activation.

To evaluate the impact on antimycobacterial activities of enhanced type 1 immune activation in the mycobacterial graunloma of IL-10KO hosts, we assessed the total mycobacterial burden in the lungs of WT and IL-10KO mice. Indeed, in keeping with markedly improved type 1 immune activation in granuloma of IL-10KO hosts, the total bacterial counts were found to be significantly lower in the lungs of these mice when compared to wt counterparts (Figure 9B). Together the above results indicate that enhanced IL-10 production accounts not only for suppressed type 1 immune activation but also for decreased antimycobacterial activities.

Discussion

The formation of the granuloma is considered a crucial event in host defense against pulmonary mycobacterial

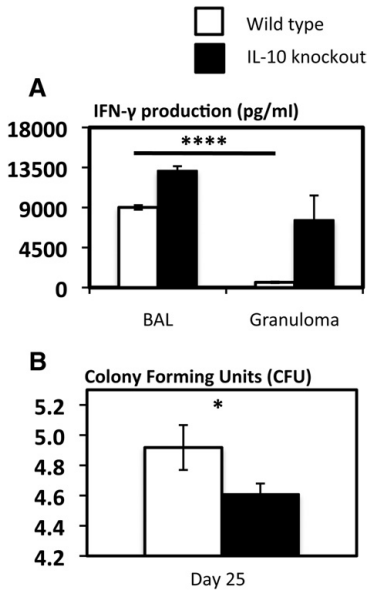

Figure 9. Restored type 1 immune activation of granuloma cells and enhanced antimycobacterial host defense by removal of IL-10. **A:** Total cells were isolated from the airway lumen and granuloma of mycobacterium-infected wild-type (wt) and IL-10 knockout mice and cultured with stimulation by live mycobacteria. Culture supernatants were measured for interferon-γ (IFN-γ) protein content by ELISA. BAL, brochoalveolar lavage cells. **B:** Lung homogenates from mycobacterium-infected wt or IL-10 knockout mice were subject to a mycobacterial CFU assay. Values displayed in **A** are average values ± SEM of three sets of cell preparations. Values in **B** are expressed as average values ± SEM of nine mice/group. $^{*}P < 0.05$, as compared with IL-10KO group; $^{****}P < 0.0001$ as compared with wt granuloma.

infection. Despite the importance of granuloma formation and integrity to antimycobacterial host defense,[10,46] little is understood about why the mycobacterium is able to paradoxically survive within the intact granuloma for a long time. Although recently emerging evidence has begun to suggest a symbiotic nature of mycobacterial granuloma, it remains unclear whether the granuloma environment is immune suppressed relative to the tissue environment outside of granuloma and, if so, what are the underlying immune mechanisms.

We and others have documented that the successful control of mycobacteria is mediated largely through the interaction of activated T lymphocytes with infected APCs.[6,36,44] The ability of the host to control mycobacteria is dependent on the ability of recruited T cells, specifically type 1 polarized CD4 T cells, to secrete IFN-γ and activate the intracellular killing mechanisms of the infected APCs.[3,36] Although the two main APC populations, macrophages and dendritic cells, can be readily infected by mycobacteria, macrophages are better equipped to control internalized mycobacteria, primarily through the production of NO.[47] On the other hand, infected dendritic cells are better able to activate and polarize CD4 T cells through the production of type 1 immune cytokines such as IL-12.[47] Both macrophage and dendritic cell populations provide an essential link between the innate and the adaptive immune systems that may be subject to the exploitation by mycobacteria after infection.

Mounting evidence indicates the importance of examining immune responses in different lung compartments, particularly the airway luminal space and the parenchyma.[1,48,49] Thus, in relation to pulmonary mycobacterial infection, it would be important to examine the immune responses both in the airway lumen, ie, the site of initial infection, and the granuloma, ie, the focal point of bacterial control and a site to which the majority of mycobacteria home.[12,50] The granuloma is composed of a nucleus of infected macrophages along with recruited monocytes, T cells, neutrophils, and NK cells, and it is formed within the lung parenchymal tissue around the vessel and bronchi. Despite the ample information about the pivotal role of both APC and activated T-cell populations in antimycobacterial host defense, to date no study has examined and compared the phenotype and functional properties of these important immune cell populations located in the airway lumen and granuloma during pulmonary mycobacterial infection. This line of study will help to address the question whether the granuloma environment is immune suppressed relative to the tissue environment outside of the granuloma.

In the present study, we have examined and compared the phenotypic and functional characteristics of the two major immune cell populations, APCs and T cells, within the granuloma against those located in the airway luminal space outside of the granuloma. Our study reveals that, despite the largely comparable immune cellular profile in the airway lumen and granuloma compartments, the granuloma cells have adopted a suppressed type 1 immune phenotype. This is typified by lower levels of type 1 immune cytokines and mycobactericidal NO and higher levels of immune suppressive IL-10, compared with airway luminal cells. In addition, this is the case for both granuloma APCs (CD11c+ dendritic cells and CD11b+ macrophages) and mycobacterial antigen-specific T cells. As a result, the antimycobacterial and T-cell-activating activities of granuloma APCs are compromised. Of note, the host immune responses are only selectively suppressed in the granuloma, as we found the chemokine responses within the granuloma to be largely intact or even more intense than the extragranuloma environment, in keeping with the high demand for continual recruitment of inflammatory cells into the granuloma.[34] Although chemokine responses were similar or elevated in the granuloma, IL-17 responses were decreased, paradoxical to the current belief that IL-17 is involved in the induction of chemokines in some experimental models.[40,51] This provides further support that the granuloma is a differentially regulated immune environment because of the heavy presence of mycobacteria, a phenomenon that likely evolves over the course of the infection. Of importance, we further demonstrated a critical role of enhanced IL-10 in mediating suppressed type 1 immune activation within the granuloma, as the lack of IL-10 restored type 1 immune responses in the granuloma, whereas it had a minimal effect on airway luminal immune responses. Based on these findings, we believe that the high concentration of mycobacteria and the intimate nature, ie, the close proximinity of infected APCs and adaptive cellullar immune components, account for the immune-suppressive environment in the granuloma relative to the extragranuloma environment. Thus our study has revealed, for the first time, an immune suppressive nature of mycobacterial granuloma. The study findings suggest that, in contrast to the traditional belief, the granuloma environment represents an immunological niche that may paradoxically favor the persistence of mycobacteria. Given that many mycobacterial species are notoriously difficult to eliminate, our current findings provide a plausible mechanism to explain why mycobacterial infections such as *M.tb* can remain latent for a lifetime in the lung of otherwise healthy, immune-competent human beings.[7–9]

It is noteworthy that we used an attenuated strain of mycobacteria (*M. bovis* BCG), but not virulent *M.tb*, in our current study. The current physical and technical constrains in our level III biohazard containment facility does not allow us to carry out the elaborate procedures needed for isolating bronchoalveolar and granuloma cells from *M.tb*-infected lungs. However, after pulmonary BCG or *M.tb* infection in mice, the overall kinetics and cellular makeup of the granuloma are comparable between models of BCG and *M.tb* infection.[11,12,42,52] Furthermore, the critical role of type 1 T-cell immunity, including the role of CD4 T cells and type 1 cytokines in antimycobacterial host defense, has shown to be true for pulmonary infection elicited by exposure to either BCG or *M.tb.*[11, 12, 53–57] *M. bovis BCG* also uss similar immune evasion strategies as *M.tb* to dampen host immune activation mechanisms including intracellular persistence,[58–60] inhibiting MHC molecule expression,[42,61] and inducing IL-10 production.[37–39,62] Despite these described similarities, we must caution that, compared with

BCG, *M.tb* is a significantly more virulent and persistent pathogen. For instance, in the absence of TNF-α, infection with either BCG or *M.tb* will result in mortality, but the onset of mortality is seen earlier after *M.tb* infection.[11,55] Similarly, whereas infection with either BCG or *M.tb* in the absence of TLR2 or TLR4 results in enhanced bacterial burden, it occurs to a higher magnitude after *M.tb* infection.[63,64] Thus, given the difference in virulence between *M. bovis* BCG and *M.tb*, the findings from our current study will need to be independently verified in a model of pulmonary *M.tb* infection when it becomes technically possible in the future.

Although our current study carries a focus on the host mechanisms, based on some recent evidence,[16] it is plausible to speculate that mycobacteria have adopted the mechanisms to fully parasitize the granuloma in an attempt to evade host-mediated clearance, and that some of these mechanisms may involve the works of the mycobacterial "persistence" gene products.[16,17] Many parallels exist between the granuloma and other immune-evasive microenvironments, such as those within tumors. The ability of the tumor to shield itself from immune infiltration is mediated by using many mechanisms similar to those seen after the formation of mycobacterial granuloma. Specifically, many tumors as well rely on the induction of IL-10 and other immune suppressive cytokines to dampen type 1 immune responses and to remain immune evasive.[65] In a fashion similar to that of the tumor, although it is believed that mycobacteria may simultaneously use multiple mechanisms of immune evasion including mechanisms of down-regulating antigen presentation,[42,61] in the current study we have shown that IL-10 is one of the essential mechanisms through which the immune suppressive environment of mycobacterial granuloma is maintained. Despite the overwhelming evidence to support a direct role of mycobacterial infection in inducing IL-10 production by APCs,[37–39,62] it is plausible for us to consider the possibility that some level of IL-10 may be actively induced as part of host immune regulatory mechanisms as a means to limit immunopathology, a concession that could be exploited by mycobacteria and has recently been suggested by others.[66] Although our current findings and a large body of literature information support the immune suppressive role of IL-10 in mycobacterial infection, one study shows the addition of exogenous IL-10 to enhance macrophage activation.[67]

In conclusion, our study, for the first time, has examined the profile and characteristics of granuloma APCs and T cells in a model of pulmonary mycobacterial infection and compared them with those located in the extragranulomatous bronchoalveolar space of the same host. Contrary to the previous belief, we found that, within the granuloma, both APC and T-cell populations differed from their extragranuloma counterparts in that they assumed a suppressed and functionally impaired immune phenotype. Our study thus provides an important clue as to in which way granuloma may offer the mycobacterium a unique biological niche for its stand-off with the immune system.

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CHAPTER 3: How does TNF regulate the integrity of the granuloma and limit the induction of lethal immunopathology?

Christopher R. Shaler+ Carly N. Horvath+, Rocky Lai, Sarah McCormick, Anna Zganiacz and Zhou Xing. **+ Equal Contribution**

The TNF - Nitric oxide - Tim-3 axis is critically required to maintain anti-tuberculosis granuloma integrity via restricting Th1 cell over-activation –

Prepared for submission

How does TNF regulate the integrity of the granuloma and limit the induction of lethal immunopathology?

Previous studies have demonstrated an essential role for TNF in the control of mycobacterial infection. Having been shown to play a central regulatory role in limiting pathogenic activation of recruited type 1 T cells, the mechanisms by which TNF controls this balance are key to our understanding the delicate immune response generated following *M.tb* infection. Our previous work showed that TNF was central to limiting the pathogenic activation of type 1 T cells. Indeed, depletion of T cells effectively rescues mycobacterial-infected mice from immunopathology and mortality. Despite the observation that T cells are responsible for the pathology, little is known about the underlying mechanisms by which the absence of TNF leads to this population of pathogenic T cells. In this study we evaluated the critical role of TNF in the regulation of immunity and immunopathology following pulmonary *M.tb* infection.

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

The TNF - Nitric oxide - Tim-3 axis is critically required to maintain anti-tuberculosis granuloma integrity via restricting Th1 cell over-activation

Christopher R. Shaler[1,2], Carly N. Horvath[1,2], Rocky Lai[1], Sarah McCormick[1], Anna Zganiacz[1] and Zhou Xing[1*]

[1]McMaster Immunology Research Centre, Department of Pathology & Molecular Medicine, and M.G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario CANADA

[2]Equal contribution

***Corresponding Author:**
Dr. Zhou Xing, MDCL-Rm. 4012, Department of Pathology and Molecular Medicine, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada
xingz@mcmaster.ca

Abstract

With an estimated one third of the world's population latently infected with the bacterium *Mycobacterium tuberculosis* (*M.tb*), the causative agent of pulmonary tuberculosis (TB), TB is a significant global health concern. Despite the high prevalence of infection, the rates of active disease are low, with the majority of infected individuals achieving clinical latency. Central to the control of *M.tb* is a robust type 1 immune response, where the mobilization of CD4+IFN-γ+ T cells into the infected lung results in bacterial containment within granulomas, but is rarely cleared. Currently, little is known about the mechanisms by which the host maintains long-term bacterial control, and even less about the mechanisms responsible for limiting immune mediated pathology. In our current study, we demonstrate the essential role of TNF for the production of nitric oxide following a pulmonary *M.tb* infection. Moreover, we establish a novel regulatory mechanism whereby nitric oxide induces the expression of Tim-3 on lung CD4+ effector T cells, an essential feedback mechanism for limiting T cell activation and preventing the development of lethal lung pathology during *M.tb* infection.

Introduction

Pulmonary tuberculosis (TB) caused by *Mycobacterium tuberculosis (M.tb)* is responsible for approximately 9 million new cases and 1.5-2 million deaths each year [1]. However, despite the high global prevalence of TB the majority of infected individuals never progress to active disease. Such latency is achieved by immune restriction mediated by the formation of Th1 granuloma and the maintenance of its structural integrity [2,3,4]. Although *M.tb* may adapt to the granuloma microenvironment for its persistence, granuloma forms to contain infection and limit tissue immunopathology [5,6]. The maintenance of granuloma integrity is critical to anti-mycobacterial host defense and the disintegration/liquefying necrosis of granuloma leads to loss of infection control both in experimental animals [7,8,9,10,11,12,13] and humans [14,15], which is an important cause of person-to-person *M.tb* transmission and intrapulmonary dissemination [6]. However, the immune mechanisms for maintaining granuloma integrity are largely unclear.

Effective control of mycobacterial replication within Th1 granuloma requires the action of Th1 effector cells and molecules such as IFN-γ and TNF-α (TNF) [16,17,18]. Compared to the relatively well-known mechanisms of action by IFN-γ [17,19], the role of TNF in anti-mycobacterial Th1 immunity has been recognized to be complex and incompletely understood [20,21,22,23]. On one hand, it is well-established both in experiment animals and humans that TNF is absolutely required for host anti-TB defense. Indeed, genetic TNF deficient mice or those depleted of TNF succumb to infection by *M.tb* or mycobacterial BCG due to granuloma disintegration and necrosis [8,9,10,11]. On

the other hand, anti-TNF immunotherapy for humans with autoimmune/inflammatory conditions was found to disrupt the granuloma integrity and thus increase the risk of re-activation of latent *M.tb* infection and TB progression [14,15]. For long, it has been commonly believed that TNF contributes to host anti-TB immunity via its effects on granuloma formation. However, compelling emerging evidence indicates that TNF is not required for the initial granuloma formation but rather, it is required for the maintenance of granuloma integrity [8,9,11,23,24]. Although one of the plausible mechanisms by which TNF maintains the integrity of granuloma is via its inductive effects on nitric oxide (NO) production and inhibition of mycobacterial replication and macrophage necrosis within the granuloma [21,22,23,24,25,26,27], there is the evidence that fatal granuloma necrosis proceeded without heightened mycobacterial replication in TNF-deficient animals [10]. Thus, the immune cellular mechanisms for TNF-mediated maintenance of anti-mycobacterial granuloma still remain poorly understood [21,22,23].

Mounting evidence suggests that TNF is a double-edged sword in host defense against intracellular infection including mycobacterial and viral infections [21,22,28]. Experimental studies in models of pulmonary *M. avium* [11] and mycobacterial BCG infection [9] have implicated the dys-regulated Th1 activation in fatal granuloma disintegration and necrosis in TNF-deficient hosts. These findings are in line with the observations that TNF deficiency or depletion paradoxically caused heightened T cell activation and immunopathology in experimental models [29,30] or human studies [31] of autoimmune/inflammatory conditions. These lines of evidence support a newly emerged notion that it is to the advantage of *M.tb* to induce a robust Th1 response [32]. Indeed, the

immune dominant CD4 T cell epitopes were found to be highly conserved in *M.tb* strains [33]. Strong Th1 responses are closely associated with transmissible necrotic and cavitary pulmonary TB in humans [32]. Thus, HIV-AIDS and *M.tb* co-infected individuals, although suffering worsened TB infection, have much reduced rates of *M.tb* transmission, probably due to suppressed Th1 immunity and subsequently sustained granuloma integrity [6,34,35]. The potential contribution of aberrant Th1 responses to the break-down of mycobacterial granuloma has also been seen in the hosts with other immune deficiency conditions [13,19,36]. Nevertheless, whether TNF maintains granuloma integrity and prevents necrotic immunopathology in pulmonary *M.tb* infection via negatively regulating Th1 immune responses, has remained to be an important outstanding question. The immune molecular pathway underlying this TNF-mediated process is also currently unknown. Given the irreplaceable role of TNF in anti-TB host defense, the enhanced knowledge in this regard will help us not only in the fight against pulmonary TB but also in further improving anti-TNF therapies that have entered the clinical practice for treating a number of autoimmune/inflammatory disorders.

In our current study, by using murine models of pulmonary *M.tb* infection we have identified TNF to be a critical negative regulator of Th1 cell differentiation and activation. Thus, loss of such TNF-imposed regulation leads to the development of immunopathogenic Th1 cells which cause the lethal disintegration and necrosis of mycobacterial granuloma and lung injury, and impaired bacterial clearance. We further identify that TNF regulates Th1 activation via its indispensable role in induction of NO in *M.tb*-infected macrophages, which is critically required to control Th1 cell proliferation

and activation via its inducing effects on T cell immunoglobulin-3 (Tim-3), a negative T cell regulatory molecule expressed on T cells.

Results

TNF is critically required to limit the development of fatal granuloma disintegration and control tuberculous infection in the lung

We first established the overall role of TNF in host defense against pulmonary *M.tb* infection by using wild type (wt) and TNFKO mice (Figure 1A). All of the TNFKO mice succumbed to *M.tb* infection by day 34, contrasting with 100% survival rate of wt mice and TNFKO mice (Figure 1B). Compared to discrete mycobacterial granulomas seen in the lungs of wt hosts, increased susceptibility of TNFKO mice was associated with granuloma disintegration and necrosis, extensive immunopathology, and intense lymphocytic infiltration (Figure 1C). Accompanied with rapid mortality of TNFKO hosts was the loss of control of *M.tb* infection in the lung (>10 log) (Figure 1D). Remarkably increased susceptibility of TNFKO mice is not unique to virulent *M.tb* infection or relative levels of mycobacterial infection in the lung as TNFKO hosts also succumbed to pulmonary infection with attenuated *M.tb*H_{37}Ra (data not shown) or BCG, demonstrating similar survival kinetics and granuloma disintegration and necrosis as with virulent *M.tb* infection, despite 3-4 log less bacterial burden in the lung (Supplementary Figure 1A/B/C/D-BCG). These findings re-affirm that TNF is critically required for host defense against pulmonary *M.tb* infection and suggest that the high mycobacterial burden is unlikely the major driving force to cause the break-down of granuloma and loss of its function in the lung of TNFKO mice following mycobacterial infection.

To further examine the overall role of TNF in host defense against pulmonary *M.tb* infection, we determined whether it was in the early or later stage of infection that TNF was required to avert a fatal pathological consequence. To this end, we utilized an adenoviral vector (Ad)-mediated transgene expression approach which allows a physiologic level of TNF expression locally in the lung for about 10 days [9]. Thus, AdTNF or a control vector (Ad Control) was intranasally given to TNFKO mice either at the time of mycobacterial infection (data not shown) or at day 21 post-infection, the time when significant T cell recruitment to the lung was seen. We found that reconstitution of TNF around the time of infection was unable to significantly alter the fatal pathologic sequela (data not shown). In contrast, reconstitution of TNF from day 21 onward markedly improved the survival of infected TNFKO mice (Figure 1B) and prevented granuloma disintegration and severe immunopathology (Figure 1C), despite only modestly reduced mycobacterial burden in the lung which was still 3 log above the wt control (Figure 1D). These results suggest that fatal granuloma disintegration may be regulated independent of the overall mycobacterial burden and that the development of granuloma disintegration and necrosis seen in TNFKO hosts relates to the timing of arrival of T cells en masse in the lung.

Lack of TNF leads to Th1 cell over-activation following pulmonary *M.tb* infection

We next determined the magnitude of Th1 responses in the lung of *M.tb* infected TNFKO hosts. We found that the lung of TNFKO mice at the endpoint time with intense lymphocytic infiltration (Figure 1C) had highly increased levels of IFN-γ protein ($p<0.0001$) (Figure 2A). Compared to the T cells in wt mouse lungs, there were markedly

increased numbers of T cells, particularly the CD4 T cells, in the lung of TNFKO mice that expressed Th1 markers, T-bet and IFN-γ (Figure 2B). By using *M.tb* antigen-specific MHC class II tetramers, we further found much greater numbers of Ag85b- and ESAT-6-specific CD4 T cells in the lung of TNFKO mice (Figure 2B). In light of much heightened Th1 responses in the lung of infected TNFKO mice, we evaluated the frequency of CD4 T cells expressing phosphorylated (p) STAT-1 and STAT-4, the transcription molecules critical to Th1 cell differentiation and activation [37]. Indeed, the frequencies of CD4+ T cells expressing pSTAT 1 and 4 were much higher in the lung of infected TNFKO mice (Figure 2C). Thus, lack of TNF did not impair Th1 differentiation and activation following pulmonary *M.tb* infection but rather, it led to uncontrolled aberrant Th1 activation. However, such much heightened Th1 responses failed to control pulmonary *M.tb* infection and its systemic dissemination.

Over-activated Th1 cells lead to granuloma disintegration and necrosis following pulmonary *M.tb* infection in TNFKO hosts

To investigate whether it was the over-activated T cells that caused the fatal granuloma disintegration and immunopathology in the lung of infected TNFKO hosts, we depleted T cells in TNFKO mice from day 21 post-*M.tb* infection on until the time of sacrifice (Figure 2D). As expected, all of the infected TNFKO with intact T cell populations succumbed with granuloma disintegration and necrosis, and much heightened infection by day 34 (Figure 2E/F/G). In stark contrast, T cell depletion led to much improved survival of infected TNFKO mice (Figure 2E), which was associated with retained granuloma integrity and much reduced immunopathology in the lung (Figure

2F). Of note, depletion of over-activated Th1 cells in these hosts did not significantly alter mycobacterial burdens in the lung (Figure 2G). These data suggest the over-activated Th1 cells, but not heightened mycobacterial burden, resulting from TNF deficiency to be the direct cause for fatal granuloma disintegration and necrosis and mortality in *M.tb* infected TNFKO hosts.

TNF is required for induction of nitric oxide production following pulmonary *M.tb* infection

We have thus far identified that TNF maintains the granuloma integrity and averts a fatal immunopathologic outcome via critically restricting the over-activation of Th1 responses. To begin dissecting the molecular mechanisms by which TNF regulates Th1 cell differentiation and activation, we first examined the level of expression of iNOS and nitric oxide (NO) in infected TNFKO animals. NO is well-known to be required for control of pulmonary *M.tb* or mycobacterial infection and both IFN-γ and TNF are important to iNOS induction and NO production [19,21,22,25,26,38,39]. We found that compared to its high levels (>400 fold increase) in the lung of infected wt mouse lungs, the gene expression of iNOS was severely blunted in the lung of infected TNFKO mouse lungs (Figure 3A). In accordance with blunted iNOS gene expression, lung mononuclear cells (MNC) isolated from infected TNFKO mice produced much reduced NO compared to their wt counterparts (Figure 3B). To determine whether impaired NO production seen in the lung of infected TNFKO mice was due to an intrinsic inability of TNFKO antigen-presenting cells (APCs; including macrophages and dendritic cells) to produce NO independent of mycobacterial infection, wt and TNFKO CD11c+ APCs isolated from

naive lungs were exposed to varying doses of recombinant IFN-γ. Indeed, in contrast to wt APCs, TNFKO APCs had an impaired ability to respond to IFN-γ in producing increasing levels of NO production or iNOS gene expression unless recombinant TNF protein was supplemented (Suppl. Figure 2A/B). These data indicate that TNF is critically required for IFN-γ-mediated NO production by APCs. They further suggest severely blunted NO production in APCs to be the cause for impaired control of *M.tb* replication in infected TNFKO mouse lungs (Figure 1D and Figure 2G) regardless of markedly heightened Th1 cell activation and IFN-γ production (Figure 2A/B/C).

TNF regulates the level of Th1 cell activation via nitric oxide induction following pulmonary *M.tb* infection

Given the blunted NO production observed in infected TNFKO hosts, we sought to investigate whether TNF restricted Th1 cell over-activation via the action of NO during pulmonary *M.tb* infection. In addition to its mycobactericidal activities, recent emerging evidence has suggested an immune- or T cell-regulatory role by NO [40,41,42,43,44]. Since we have demonstrated that TNF reconstitution by transient transgenic expression initiated at day 21 post-*M.tb* infection, was able to markedly improve the survival and prevent fatal granuloma disintegration in the lung of infected TNFKO mice (Figure 1A/B/C/D), we first investigated the relationship of TNF-mediated NO production to the regulation of Th1 cell activation in this model. To this end, TNFKO mice were infected with *M.tb* and at day 21, received TNF gene transfer vector (AdTNF) for TNF reconstitution, and were subjected to the inhibition of NO production through the use of an iNOS inhibitor L-NAME (Figure 3C). While TNF reconstitution restored the ability of

the MNC in TNFKO mice to produce NO, in vivo administration of L-NAME in these mice effectively inhibited NO production by the MNC (Figure 3D). Inhibition of NO production in these hosts resulted in enhanced Th1 responses including IFN-γ production (Figure 3E) and IFN-γ-producing Th1 cells in the lung (Figure 3F) which were otherwise brought back down by TNF-restored NO production near the wild type control levels (Figure 3E/F). Associated with dys-regulated Th1 responses by NO inhibition in TNF-reconstituted TNFKO mice (TNFKO+TNF+L-NAME) were a decreased survival rate (Figure 3G), severe granuloma disintegration and necrosis similar to immunpathology seen in the lung of infected TNFKO control animals (Figure 3H), and increased mycobacterial burden (Figure 3I).

Next, we further examined the relationship of NO production to the regulation of Th1 cell activation in infected wild type hosts. Indeed, blockade of NO production by the use of L-NAME in wt mice (once each 2 d x5; i.p-describe it briefly in figure legend) also resulted in markedly increased numbers of CD4+IFN-γ+ T cells (Figure 3J) and *M.tb* antigen Ag85b+ specific CD4+ T cells (Figure 3K). In accord with dys-regulated Th1 cell responses due to NO inhibition in wt animals, the lungs of these animals had intensified lymphocytic infiltration (Figure 3L). The above data together indicate that TNF is critically required to negatively regulate the level of Th1 cell responses and maintain mycobacterial granuloma integrity via NO induction.

APC-derived nitric oxide is critical to restraining Th1 cell over-activation following *M.tb* infection

Having demonstrated the importance of TNF-induced NO in restricting Th1 cell over-activation and maintaining anti-TB granuloma integrity in the lung, we set out to specifically address the issue regarding the interaction of antigen-presenting cells (APCs) and CD4 T cells, and the requirement of APC-derived NO for the regulation of Th1 cell activation. To investigate this, we first isolated both CD11c+ APCs and CD4 T cells from mycobacterial (figure legend-consistency) infected wt and TNFKO mice. We have demonstrated the intrinsically impaired ability of TNFKO CD11c+ APCs to produce NO (Suppl. Figure 2A). Thus, we paired APCs isolated from infected wt or TNFKO mice ex vivo with in vivo-primed Th1 cells (from wt or TNFKO mice) at 1:2 ratio and analyzed the extent of Th1 cell activation and proliferation. No matter whether wt or TNFKO Th1 cells, co-incubation with TNFKO APCs resulted in increased frequencies of IFN-γ-producing Th1 cells (Suppl. Figure 3A) and increased rates of their proliferation (Suppl. Figure 3B). On the other hand, TNFKO Th1 cells, when co-incubated with wt APCs, displayed decreased activation and proliferation (Suppl. Figure 3A/B). We next determined whether APC-mediated regulation of Th1 activation was NO-competent APC dose-dependent. The *M.tb*-infected wild type NO-competent APCs inhibited IFN-γ-producing (Figure 4A) and T-bet-expressing (Figure 4B) Th1 cells in an APC-concentration-dependent manner. As expected, TNFKO APCs even when plated at high APC:T cell ratios failed to suppress Th1 activation (data now shown). We further found that the regulation of Th1 cell activation required the interaction of T cells with NO-competent APCs as exogenously introduced NO was unable to robustly regulate Th1 cells in the absence of APCs (data not shown). These results together suggest that it is the

altered immune property in TNFKO APCs that fails to negatively regulate the level of Th1 activation.

To investigate whether it was APC-derived NO that mediated the negative regulation of the level of Th1 activation, we first examined the level of Th1 cell activation in *M.tb*-infected wt and iNOSKO mice. iNOSKO mice were previously found to suffer mycobacterial granuloma disintegration and necrosis [19] similar to the phenotype of *M.tb*-infected TNFKO mice seen in our current study. Indeed, infected iNOSKO animals had much greater frequencies of IFN-γ-producing and *M.tb* antigen Ag85b-specific Th1 cells in the lung (Figure 4C/D). We next examined the interaction of iNOSKO APCs with T cells and its effect on CD4 T cell activation. Instead of using in vivo primed CD4 T cells which would be present in differential frequencies between wt mice and the mice defective of NO production, we used an identical number of CFSE-labeled *M.tb* Ag85b transgenic CD4 T cells (Tg-P25 CD4+ T cells) to pair up with APCs isolated from infected wt and iNOSKO mice. Upon incubation, there was a much greater frequency (>10%) of IFN-γ-producing Tg-P25 CD4+ cells paired with iNOSKO APCs than those (2.5%) paired with wt APCs (Figure 4E). Increased frequencies of activated Tg-P25 CD4+ T cells paired with iNOSKO APCs resulted from much increased rates of proliferation (Figure 4F). The level of Tg-P25 CD+ T cell activation was inversely correlated with the capability of APCs to produce NO in the co-culture (Figure 4G).

To further understand the relationship of APC-derived NO to the negative regulation of Th1 activation, we examined whether exogenous NO reconstitution could restore the T cell-regulating capability of TNFKO APCs which were otherwise incapable

of negative regulation of Th1 activation (Suppl. Figure 3A/B). Thus, lung MNC isolated from infected TNFKO mice were cultured in the presence or absence of various doses of a nitric oxide donor molecule SIN-1 or SNAP. The wt MNC were included as a comparison. Indeed, NO reduced the frequencies of IFN-γ-producing (Figure 4H), STAT1-expressing (Figure 4I/K), and STAT-4-expressing (Figure 4J/K) Th1 cells. At the highest doses of NO donor, the level of Th1 cell activation in TNFKO MNC cultures was brought down to wt controls (Figure 4H/I/J/K).

TNF regulates Th1 cell activation via inducing T cell expression of Tim-3 following pulmonary *M.tb* infection

Our observation that NO-mediated Th1 cell regulation required the presence of APCs suggests the involvement of T cell surface molecules capable of interacting with the cognate ligands expressed by APCs. Thus, we next evaluated the potential involvement of a recently identified Th1 regulatory molecule Tim-3 that is expressed on activated T cells with its cognate ligand Gal-9 expressed by APCs [45]. Tim-3 has recently been implicated in anti-TB immunity [45,46,47,48]. In this regard, we first examined Tim-3 expression on Th1 cells in the lung of *M.tb*-infected wt and TNFKO animals, and found markedly reduced frequencies of Tim-3-expressing CD4 T cells in the lung of infected TNFKO mice compared to wt counterparts (Figure 5A). This was in sharp contrast to uncompromised expression of another Th1 regulatory molecule PD-1 on the CD4 T cells from infected TNFKO mice (Figure 5B). These data suggest Tim-3 on Th1 cells to be a mechanistic point downstream of NO and TNF, controlling Th1 activation. To examine this issue, we first used our TNF-reconstituted TNFKO models.

Thus, infected TNFKO mice that received AdTNF gene transfer vector were injected either with Tim-3 blocking mAb or the control IgG (Figure 5C). Wt mice and TNFKO receiving control Ad vector and IgG were used as controls. Upon examination of Tim-3-expressing CD4 T cells, we found that as seen earlier (Figure 5A), infected TNFKO lungs had decreased frequencies of Tim-3-expressing CD4 T cells (TNFKO+Ad control; Figure 5D). However, TNF reconstitution in TNFKO mice (TNFKO+AdTNF) restored Tim-3-expressing T cells to the wt levels (Figure 5D). On the other hand, Tim-3 blocking treatment (TNFKO+AdTNF+αTim-3) led to markedly diminished Tim-3-expressing T cells (Figure 5D). Thus, TNF-restored Tim-3 expression on CD4 T cells (TNFKO+AdTNF) markedly enhanced the survival rate and prevented granuloma disintegration and necrosis of infected TNFKO mice (Figure 5E/F). In contrast, Tim-3 blockade in these mice (TNFKO-AdTNF-αTim-3) worsened survival resulting in granuloma disintegration and necrotic immunopathology similar to TNFKO control (TNFKO Ad control) group (Figure 5E/F). The fatal immunopathology observed in TNFKO-AdTNF-αTim-3 animals was linked to dys-regulated Th1 cell activation (Figure 5G) and proliferation (Figure 5H).

To further examine the role of Tim-3 in Th1 cell regulation following *M.tb* infection, the Tim-3-blocking antibody was administered to *M.tb*-infected wild type mice. Similar to heightened Th1 cell activation seen in TNF-deficient (Figure 2B) or NO-deficient/depleted hosts (Figure 4C/D), Tim-3 blockade led to heightened Th1 cell activation in the lung (Figure 5I) and significantly increased lung T cell infiltration and immunopathology (Figure 5J). Together, these results indicate that Tim-3 expression is

up-regulated by TNF following pulmonary *M.tb* infection and is essential to controlling the level of Th1 cell activation, therefore preventing fatal granuloma disintegration and immunopathology caused by Th1 over-activation.

TNF-induced nitric oxide regulates Th1 cell activation via inducing T cell expression of Tim-3 following pulmonary *M.tb* infection

Thus far we have causally linked the TNF-nitric oxide pathway or the TNF-Tim-3 pathway to the negative regulation of Th1 cell activation and Th1 fatal immunopathology, and demonstrated that TNF induces T cell expression of Tim-3 following pulmonary *M.tb* infection (Figure 5A/D). This prompted us to investigate whether TNF-induced NO regulates Th1 cell regulation through the induction of Tim-3 expression on CD4 T cells. To address this question, we first examined whether NO exposure could directly induce Tim-3-expressing CD4 T cells in the absence of TNF. We isolated lung MNC from *M.tb*-infected TNFKO mice and these cells were then cultured without or with increasing doses of NO donor SNAP. We found that NO enhanced Tim-3 expression on CD4 T cells in an NO dose-dependent manner (Figure 6A). Having established a direct relationship between nitric oxide and T cell Tim-3 expression, we next determined whether NO restricts Th1 cell activation through Tim-3. Indeed, we found that NO-induced Tim-3 expression on Th1 cells from infected TNFKO animals led to reduced Th1 cell over-activation as measured by T cell proliferation (40% reduction) (Figure 6B), which was associated with a 50% reduction in the frequency of *M.tb* antigen Ag85b-specific CD4 T cells similar to the level of wt control T cells (Figure 6C). On the other hand, when the same cells were exposed to a Tim-3-Fc chimeric molecule (Tim-3-fc), a competitive

inhibitor for the Tim-3 ligand expressed on APCs (Tim-3 pathway activation inhibitor) [49], the Tim-3-imposed suppressive effect on CD4 T cell activation was completely abolished (Figure 6B/C). We further confirmed that the observed reversal of NO-mediated T cell suppression by disabling Tim-3 signaling pathway was not due to altered NO concentration since the levels of NO remained unchanged in the presence (+SNAP/Tim-3-fc) and absence (+SNAP) of Tim-3-Fc (Figure 6D).

As the suppressor of cytokine signaling (SOCS) proteins such as SOCS-3 are important intracellular negative regulators of cytokine-mediated STAT signaling pathways [50], we also examined the relationship of the TNF-nitric oxide- axis with SOCS-3 gene expression in CD4 T cells. Indeed, we found the mRNA expression of SOCS-3 was severely blunted in the lung tissue of infected TNFKO animals, compared to the level of expression in wt controls (Figure 7A), which was mirrored in the CD4+ T cells isolated from TNFKO lungs (Figure 7B). Of importance, while TNF reconstitution in TNFKO hosts completely restored SOCS-3 expression to wt levels, while impairing NO production in these animals led to the loss of restored SOCS-3 expression (Figure 7C). On the other hand, the introduction of NO donor SNAP increased SOCS-3 expression in TNFKO lung MNCs in a dose-dependent manner (Figure 7D). The above results together suggest that APC-derived nitric oxide is critical to the negative regulation of Th1 cell activation and such regulation is APC-T cell contact-dependent.

Since we observed the inducing role of NO in T cell SOCS-3 expression, we examined the relationship of Tim-3 to SOCS-3 expression. We found that blockade of Tim-3 resulted in the inability of TNF reconstituted in TNFKO hosts to up-regulate

SOCS-3 expression (Figure 7E), suggesting that the TNF-nitric oxide-Tim-3 axis operates to negatively regulate Th1 cell activation via its activating effects on SOCS-3 within T cells. Taken together the above data indicate that following *M.tb* infection, TNF restricts Th1 cell over-activation by triggering an immune regulatory cascade which involves the initial induction of nitric oxide in APCs and subsequent induction of Tim-3 on Th1 cells.

Discussion

While the critical bactericidal role of TNF has long been reported, the mystery as to why humans latently infected with *M.tb* undergoing anti-TNF rapidly progress to active TB, doing so more rapidly than even HIV infected immune compromised hosts [35,51,52,53]. Indeed, the speed at which TNF deficiency causes mortality in both murine models and humans cannot solely be attributed to the enhanced bacterial burden seen following *M.tb* infection. Previously we have published that TNFKO mice rapidly succumb to avirulent mycobacteria, displaying profound T cell mediated lung pathology. Despite these observations the common perception remains; TNF deficiency leads to bacterial dissemination, which is responsible for the death of TNFKO mice following *M.tb* infection. In our current study we challenge this preconceived notion, demonstrating that while undeniably TNF is essential to bacterial control, the cause of death in TNFKO mice is T cell mediated pathology. Employing a murine model of pulmonary TB, we investigated the contribution of TNF to the regulation of mycobacterial immunity demonstrating that, in line with previous studies, TNFKO mice uniformly succumb to *M.tb* infection, in less than 6 weeks. Further, despite mounting a strong Th1 T cell response, TNFKO mice are profoundly impaired in their ability to control bacterial

replication, a defect attributed to their reduced capacity to produce nitric oxide. In our current study, we demonstrated that TNF maintains granuloma integrity carefully tuning T cell activation through its contribution to the production of nitric oxide. Further, we show that nitric oxide attenuates T cell activation by up-regulating Tim-3, an effect that down-regulates the Th1 response, while up-regulating suppressive molecule SOCS-3. Overall our study begins to unravel the mystery clouding the role of TNF in mycobacterial immunity.

While we demonstrate that following *M.tb* infection TNFKO mice develop exaggerated T cell responses, previous studies have demonstrated TNFKO mice to develop similar T cell responses, despite their enhanced bacterial burdens [54]. Indeed, we have noted that at time points earlier than day 30 TNFKO mice have highly similar T cell numbers to wt (data not shown), and it is not until between day 30 and 36 that TNFKO develop higher T cell numbers, the time in which TNFKO mice invariably die due to lung pathology. We attributed the differences in findings to the precise time point in which T cell responses were analyzed.

While it is not surprising that anti-TNF therapy results in active disease, the speed at which latently infected individuals progress to severe disease is astounding, typically within 3 months of starting therapy. In contrast, HIV-AIDS has only a 10% risk of developing TB per year, and typically takes years to cause active TB disease in latently infected individuals [35,53,55]. Indeed, even CD4+ T cell knockout mice do not succumb as rapidly as TNFKO mice to *M.tb* infection [56,57]. Given that T cell depletion significantly enhances the survival of TNFKO mice while further compromising bacterial

control, we attribute the mortality of TNFKO mice to a loss of granuloma integrity and the development of pathogenic T cells. Supporting this notion, despite having 3-4 logs less CFU in their lungs, TNFKO mice develop identical pathology and rapidly succumb to avirulent BCG infection. Indeed, TNFKO mice are extraordinarily susceptible to mycobacterial infection, which cannot be explained solely by the heightened bacterial burdens.

Despite being one of the most successful human pathogens, *M.tb* rarely causes disease (<10%), emphasizing the importance of striking a balance between immunity and immunopathology [1]. Recently this has been highlighted when examining the granuloma formation. Well known for its role in preventing dissemination, the granuloma has recently been demonstrated to be an immune suppressed environment, suggesting a parasitic relationship between *M.tb* and the infected host [4,58,59,60,61,62]. Undeniably a unique relationship has evolved between *M.tb* and humans; it remains plausible that sterile clearance may be sacrificed in order to limit potentially lethal tissue pathology. Indeed, previous literature has documented that following an *M.tb* infection PD-1KO mice rapidly succumb due to T cell mediated pathology rather than bacterial dissemination. Likewise, our current study has demonstrated the essential role of TNF-nitric oxide mediated up-regulation of Tim-3 on the prevention of lethal pathology and the integrity of the granuloma. Indeed, in the absence of TNF mice rapid develop disorganized and highly necrotic granulomas. There is a plethora of studies demonstrating that the granuloma core is immunological suppressed. Moreover, it has been observed that impairing key regulatory molecules, Tim-3, PD-1, SOCS-3, or IL-10, results in a

profound loss of granuloma integrity and bacterial control. Taken together, the immune regulatory environment within the granuloma is highly relevant to the overall control of *M.tb* and the mechanisms required for maintaining must be carefully examined.

Recently, Carow *et al* demonstrated the essential role of SOCS-3 in CD4 T cells following *M.tb* infection. In line with our current study, they demonstrate that a deficiency in T cell SOCS-3 expression results in a loss of granuloma integrity, bacterial control and is unilaterally fatal by 6 weeks [63]. Moreover, in the absence of SOCS-3 mice demonstrate enhanced IFN-γ responses, but highly impaired bacterial control furthering the notion that the integrity of granuloma can only be maintained by a carefully balanced immune response [63].

In addition to its bactericidal function, nitric oxide has been shown to selectively regulate Th1 responses, while having little effect on Th2 T cells [64,65,66]. Given that nitric oxide is the most downstream product of macrophage activation, we propose that nitric oxide functions as an immune rheostat, maintaining bacterial control while fine-tuning T cell activation to prevent pathology. Indeed, we have demonstrated that nitric oxide is capable of regulating Th1 immunity, in dose dependent manner. Further, we have shown that nitric oxide regulates Th1 immunity by up-regulating the expression of SOCS-3 and Tim-3 on Th1 CD4+ T cells, providing a highly sensitive negative feedback signal.

While Tim-3 has been identified as key Th1 regulatory molecule, little is known about what regulates its expression on activated T cells, or how T cells become aware that the pathogen has been cleared/controlled. While it has been demonstrated that T-bet plays a role in the regulation of Tim-3 expression, these observations fail to explain why an

active Th1 polarized cell expressing high levels of T-bet, displays very low levels of Tim-3 until the point of contraction [67]. Indeed, the infected cells require a means to signal the adjacent T cell confirming that they have been appropriately activated; nitric oxide provides just such a signal, linking the infected macrophage, and recruited T cell through Tim-3. The induction of nitric oxide is highly complex requiring coordinated signals from both the infected cells (TNF) and the recruited CD4 T cells (IFN-γ). While TNF is required to turn on the iNOS system, IFN-γ is the master regulator of nitric oxide production levels, which in turn feedback and regulate CD4 T cell mediated IFN-γ production. This complexity may serve a dual purpose, both preventing unnecessary activation and tissue damage, as well ensuring that the adaptive immune response is adequate to control the infection prior to its shutdown. Certainly, the balance between immunity and immunopathology must be carefully regulated. Indeed, premature exhaustion through the up-regulation of Tim-3 has recently been linked to the impaired control of Hepatitis B [68], an effect that could be reversed by blocking Tim-3.

Furthermore, recently literature suggests that Tim-3 may play a dual role during intracellular bacterial infections. Indeed, Jeyearaman et al (2010) has recently demonstrated the critical requirement for Tim-3 to the control of bacterial replication following *M.tb* infection [46]. Following this, recent studies have demonstrated that TNF, IFN-γ and nitric oxide are not absolutely required for host immunity to *M.tb*, while paradoxically deficiency is universally fatal [12,57,72].

While bacterial control is essential to host survival, this control comes at a cost, one that must be carefully weighed to ensure the best possible outcome for the infected

host. This raises the notion that while it is commonly believed that latency is a failure of the host to eliminate the pathogen, this may not be the case. It remains plausible that the host may be capable of eliminating the pathogen, however the price for this clearance may be too great, leading the host to compromise, allowing for the establishment of latency. Indeed, the cliché expression, 'the immune system is a double edged sword' is particularly fitting.

In summary, we demonstrate that the extreme susceptibility of TNFKO mice to *M.tb* is due to the development of pathogenic T cells, rather than bacterial dissemination where TNF is essential to the production of nitric oxide; critical to both bacterial control and T cell regulation. Further, we demonstrate a novel mechanism whereby the infected macrophage signals the recruited T cell to 'stand down' through the production of nitric oxide, an effect mediated by the selective up-regulation of Tim-3, and production of SOCS-3. Overall, our study highlights the importance of balance between bacterial control and immunopathology, demonstrating the detrimental outcome that occurs when both are lost.

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 Jackson laboratories (Bar Harbor, Maine, USA) and housed in a specific pathogen-free, level B facility. Tg-P25 mice were purchased from Jackson laboratories, and an in house colony of TNFKO mice (originally purchased from Jackson Laboratories- B6.129S-Tnftm1Gkl/J- #005540) were maintained at specific pathogen-free level A until 6-8 weeks of age. Mice were either infected with *M. bovis* Bacille Calmette Guerien (BCG) 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.

Mycobacterial preparation and infectious dose

Mycobacterium bovis BCG (Connaught strain) was prepared as previously described [75,76]. 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 $0.5x10^6$ cfu/mouse for elicitation of strong Th1 immune responses and granuloma formation. A

higher inoculum of BCG was used to compensate for its avirulent nature, allowing generation of Th1 immune responses highly similar to those seen following *M.tb* challenge.

Mycobacterium tuberculosis $H_{37}Rv$ was prepared and processed the same as BCG, described above. Infection with *M.tb* was delivered intranasally at our previously established dose of 10 000 CFU/mouse. At this dose we have demonstrated significant Th1 immunity and the formation of robust granulomas formation throughout the lung.

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, lungs were 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 (5x, 10x, 20x), looking for structural and morphological changes associated with mycobacterial infection and overall pathological changes within the lung.

Cell isolation from the lung interstitium

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.

Cell culture and cytokine measurement

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 timepoints depending on experimental readout. 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). In some experiments, cells were cultured in the presence of nitric oxide donor or inhibitor molecules, or with a TIM-3 FC chimera (R&D Systems) as a means of competitively inhibiting TIM-3. Cells were cultured in a total volume of 250μl 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).

Cell surface immunostaining, tetramer, and intracellular cytokine staining (ICCS)

All monoclonal antibodies (mAbs) used were purchased from BD Pharmingen or ebiosciences. Immunostaining and FACS were carried out as previously described [75,76,77]. 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 various surface markers were used. Appropriate isotype or fluorescence minus one (FMO) controls were used for each antibody.

In order to enumerate antigen-specific T cells, we used class II tetramers against *M.tb* Ag85b and ESAT-6 (NIH core tetramer facility). Cells were stained with tetramers for 1 hour at 37°C prior to extracellular surface staining and ICCS. For intracellular cytokine staining (ICCS), single cell suspensions from airway lumen, lung, spleen and MLN were cultured and stained as previously described [78]. Briefly, cells were cultured for 24 hours with or without mycobacterial antigens (*M.tb*-Culture Filtrate-CF and crude BCG), Golgi Plug (5μg/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 Abs according to the manufacturer's instructions included in the ICCS kit (BD Pharmingen). Further, in some experiments, cells stained for transcription factors pSTAT-1 and 4 as well as T-bet, using ebioscience's transcription factor staining buffer according to the manufacturer's protocol. 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 a minimum of 100,000 to 250,000 events per sample were analyzed.

Nitric oxide production measurement

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[79]. 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.

Nitric oxide donor and inhibitor molecules

Throughout the study two nitric oxide donor molecules were used interchangeably. The inhibitor molecule L-NAME was used to inhibit nitric oxide production *in vivo* and *in vitro*. L-NAME, SIN-1 and SNAP were purchased from Sigma Aldrich (Oakville, Ontario, Canada). The donor molecules were used at concentrations determined empirically to represent a spectrum of physiologically relevant nitric oxide concentrations. L-NAME was used at a concentration 2.5mg/kg delivered in 200μL intraperitoneally *in vivo* and 1μg/mL *in vitro*.

T cell proliferation Assay

T cell proliferation assays were conducted as previously described [62], briefly CD11c+ APCs, and CD4+ 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 CD4 microbeads for the purification od CD4+ T cells. Labelled cells were then 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 CD4+ T cell populations was determined by FACS and was consistently >90%. Isolated T cells were stained with CFSE, and paired with isolated CD11c+ APCs at a ratio of 1:5. Cells were cultured in the presence of mycobacterial antigen, and various concentrations of nitric oxide donor molecules. Cells were cultured for 72hrs, at which time cells were collected and stained for ICCS as described above. Cells were run on LSRII flow cytometry, and the extent of proliferation determined by means of CFSE dilution of isolated T cells.

mRNA extraction and quantitative PCR analysis

Following cell culture, RNA was extracted using RNeasy (Qiagen) spin columns as per manufacture's protocol. Extracted RNA was reverse transcribed into cDNA using a quanta qPCR supermix kit. Converted cDNA was then used for qPCR using quanta PerfeCTa® SYBR® Green SuperMix with ROX and relevant fwd and rev primers. Primer sequences for iNOS, SOCS-1 and SOCS-3 were constructed using the NCIB primer blast tool and are summarized in the table below.

Primer	Forward	Reverse
iNOS		
SOCS-1	GCT GTG CCG CAG CAT TAA G	CCA GAA GTG GGA GGC ATC TC
SOCS-3	TAC TGA GCC GACC TCT C	CCG TTG ACA GTC TTC CGA CAA

Adenovirus vector delivery

In some experimental setups, TNFKO mice were reconstituted for TNF via the intranasal delivery of a gene transfer vector, administering 1 x 10^8 PFU/mouse in 40μL saline of an Adenovirus previously constructed to express soluble murine TNF [9]. In all experiments, empty Adenoviral backbones (Addl70) were delivered to control groups at a dose of 1 x 10^8 PFU/mouse in 40μL saline to account for any effects caused by the viral delivery.

Delivery of Depleting Antibodies

In certain experiments, mice received anti-CD4/CD8 (made in house) or anti-TIM-3 (BioXcell) blocking antibody commencing at day 21 post infection at a dose of 100μg/injection 3 times weekly. Antibodies were delivered via the intraperitoneal route in 200μL. Appropriate isotype matched controls were delivered to control groups.

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.

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Figures and Legends

Figure 1.

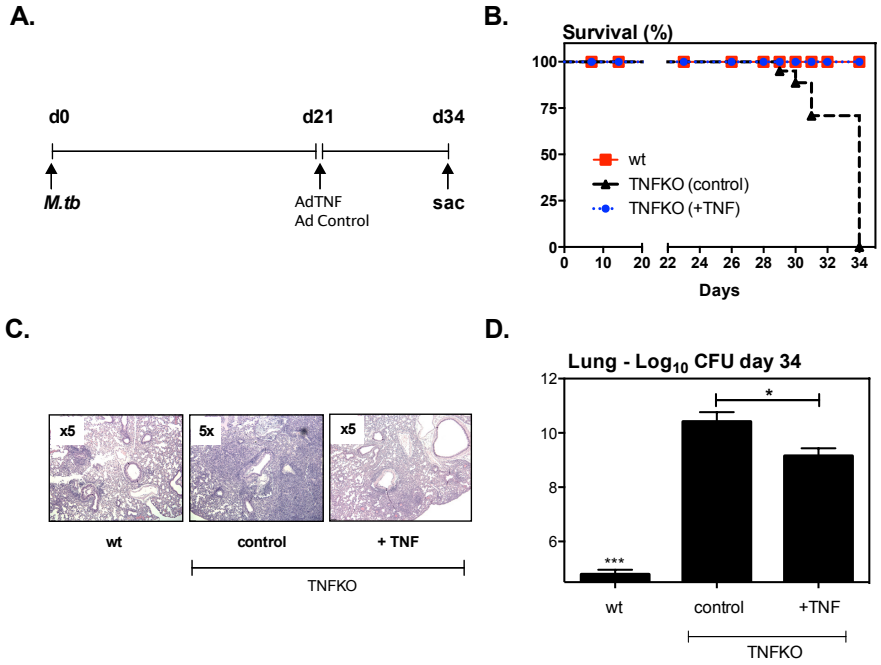


Figure 1. TNF is critically required to limit the development of lethal pathology and prevent bacterial dissemination following virulent *M.tb* infection. Wild type and TNFKO mice were infected with M.tb-H_{37}Rv. Three weeks following infection TNFKO mice were reconstituted for TNF using viral vector transfer (AdTNF) or given control vector (A). Mice were monitored for endpoint and mice were sacrificed at the time point when the majority of TNFKO reached endpoint (B). At the time in which TNFKO mice reached endpoint, lung histology was collected and stained for H&E to assess gross lung pathology (C), and bacterial burden assessed by colony formation assay (D). Representative of at least 3 independent experiments with 5 mice per group. * $p \leq 0.05$.

Figure 2.

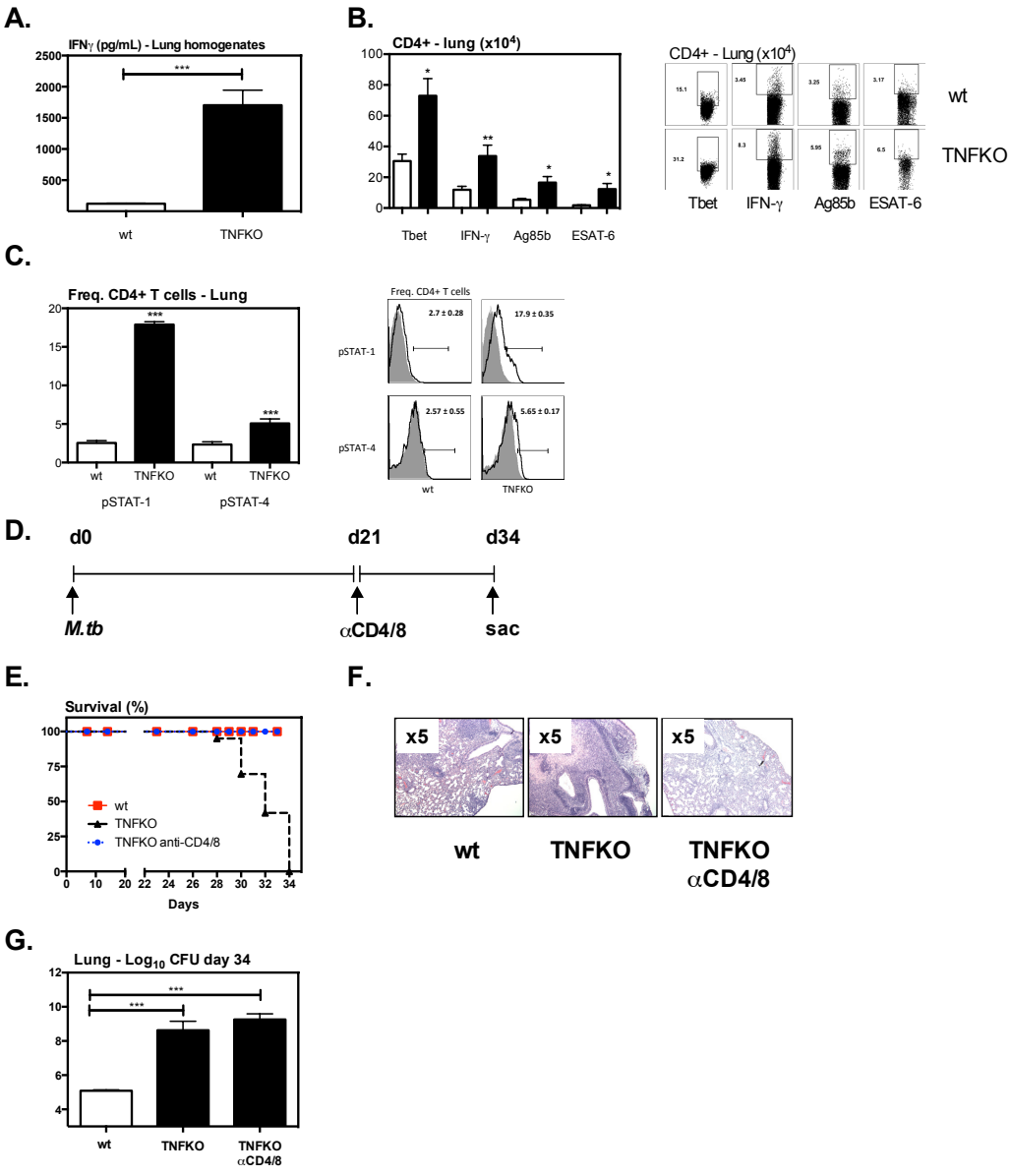

A.
IFNγ (pg/mL) - Lung homogenates
wt
TNFKO
B.
CD4+ - lung (x10^4)
Tbet
IFN-γ
Ag85b
ESAT-6
CD4+ - Lung (x10^4)
wt
TNFKO
C.
Freq. CD4+ T cells - Lung
pSTAT-1
pSTAT-4
Freq. CD4+ T cells
2.7 ± 0.28
17.9 ± 0.35
2.57 ± 0.55
5.65 ± 0.17
D.
d0
d21
d34
M.tb
αCD4/8
sac
E.
Survival (%)
wt
TNFKO
TNFKO anti-CD4/8
Days
F.
x5
wt
TNFKO
TNFKO αCD4/8
G.
Lung - Log10 CFU day 34
wt
TNFKO
TNFKO αCD4/8

Figure 2. The absence of TNF following *M.tb* infection leads to exaggerated T cell activation. At the time in which TNFKO mice reached endpoint, lung mononuclear cells were collected and the levels of IFN-γ were assessed (A). Further, cells were stained for markers of T cell activation and TB antigen specificity (B), and STAT1 and STAT4 phosphorylation (C). Three weeks following infection TNFKO mice were depleted of CD4 and CD8 T cells (D). Survival was monitored until un-depleted TNFKO mice reached endpoint (E) and lung histology was collected and stained for H&E to assess gross lung pathology at the time of endpoint (F), and bacterial burden assessed by colony formation assay (G). Representative of at least 3 independent experiments with 5 mice per group. *** < 0.001.

Figure 3.

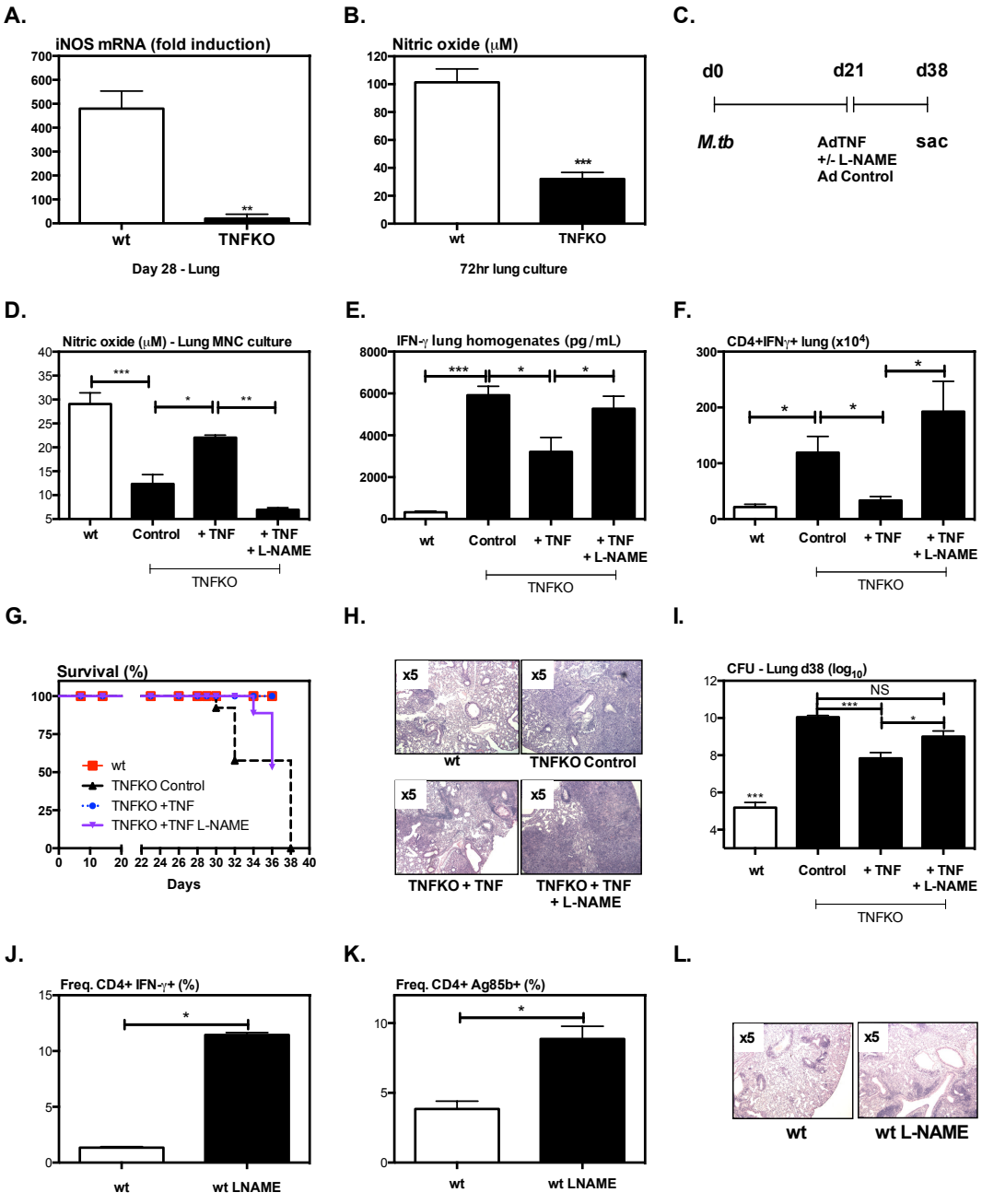

A.
iNOS mRNA (fold induction)
wt
TNFKO
Day 28 - Lung
B.
Nitric oxide (μM)
wt
TNFKO
72hr lung culture
C.
d0
d21
d38
M.tb
AdTNF
+/- L-NAME
Ad Control
sac
D.
Nitric oxide (μM) - Lung MNC culture
wt
Control
+ TNF
+ TNF
+ L-NAME
TNFKO
E.
IFN-γ lung homogenates (pg/mL)
F.
CD4+IFNγ+ lung (x10⁴)
G.
Survival (%)
wt
TNFKO Control
TNFKO +TNF
TNFKO +TNF L-NAME
Days
H.
x5
wt
TNFKO Control
TNFKO + TNF
TNFKO + TNF
+ L-NAME
I.
CFU - Lung d38 (log10)
NS
J.
Freq. CD4+ IFN-γ+ (%)
wt
wt LNAME
K.
Freq. CD4+ Ag85b+ (%)
L.
wt
wt L-NAME

Figure 3. The absence of TNF leads to the development of pathogenic T cells following *M.tb* infection. Following *M.tb* infection, mRNA was extracted from lung homogenates and the transcripts of iNOS were assessed in wild type and TNFKO mice (A) and the ability of lung mononuclear cells to produce nitric oxide assessed (B). Three weeks following infection TNFKO mice were reconstituted for TNF using viral vector transfer (AdTNF) or given control vector (C). In the mice reconstituted for TNF one group received L-NAME to inhibit nitric oxide production. At the time in which TNFKO mice reached endpoint, lung mononuclear cells were collected and the ability of reconstituted mice to produce nitric oxide was assessed at the time of sacrifice (D) and the levels of IFN-γ were assessed in the lung homogenates. Further, the numbers of CD4+ IFN-γ T cells were assessed at endpoint (F) Mice were monitored for endpoint and mice were sacrificed at the time point when the majority of TNFKO reached endpoint (G). At the time in which TNFKO mice reached endpoint, lung histology was collected and stained for H&E to assess gross lung pathology (H) and bacterial burden assessed by colony formation assay (I). In a similar setup, nitric oxide production was blocked in wild type through the administration of L-NAME. Five weeks following infection, wild type and wild type L-NAME mice were sacrificed, and the frequency of CD4+IFN-γ T cells (J), and TB antigen Ag85b specific T cells assessed (K). Further, lung histology was collected and stained for H&E to assess gross lung pathology (L). Representative of 2 independent experiments with 5 mice per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 4.

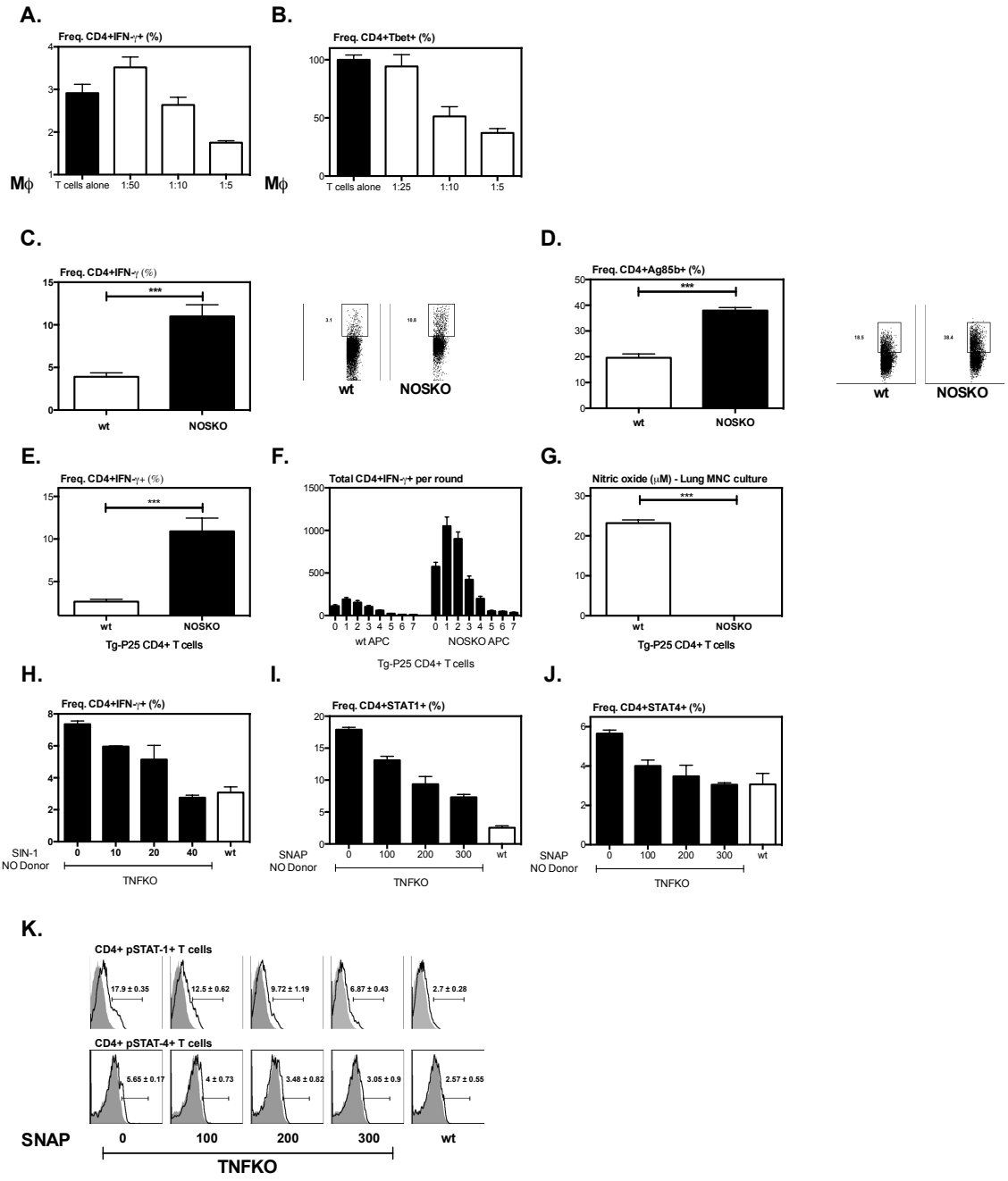

A.
Freq. CD4+IFN-γ+ (%)
Mϕ
T cells alone
1:50
1:10
1:5
B.
Freq. CD4+Tbet+ (%)
Mϕ
T cells alone
1:25
1:10
1:5
C.
Freq. CD4+IFN-γ (%)
***
wt
NOSKO
wt
NOSKO
D.
Freq. CD4+Ag85b+ (%)
***
wt
NOSKO
wt
NOSKO
E.
Freq. CD4+IFN-γ+ (%)
***
wt
NOSKO
Tg-P25 CD4+ T cells
F.
Total CD4+IFN-γ+ per round
wt APC
NOSKO APC
Tg-P25 CD4+ T cells
G.
Nitric oxide (μM) - Lung MNC culture
***
wt
NOSKO
Tg-P25 CD4+ T cells
H.
Freq. CD4+IFN-γ+ (%)
SIN-1
NO Donor
TNFKO
I.
Freq. CD4+STAT1+ (%)
SNAP
NO Donor
TNFKO
J.
Freq. CD4+STAT4+ (%)
SNAP
NO Donor
TNFKO
K.
CD4+ pSTAT-1+ T cells
17.9 ± 0.35
12.5 ± 0.62
9.72 ± 1.19
6.87 ± 0.43
2.7 ± 0.28
CD4+ pSTAT-4+ T cells
5.65 ± 0.17
4 ± 0.73
3.48 ± 0.82
3.05 ± 0.9
2.57 ± 0.55
SNAP
TNFKO

Figure 4. Reduced production of nitric oxide results in exaggerated T cell activation following *M.tb* infection. T cells were non-specifically activated overnight and then paired with M.tb infected macrophages at various ratios. Following 72hrs co-culture, cells were stained for CD4+IFN-γ (A) and CD4+ T bet (B). Five weeks following infection, wild type and NOSKO mice were sacrificed and lung mononuclear cells isolated. C ells were stained for IFN-γ (C) and TB Ag85b antigen specificity (D). At the time of sacrifice CD11c+ APCs were isolated and paired with isolated Tg-P25 CD4+ T cells and cultured for 72hrs. Following 72hr culture cells were stained for CD4 and IFN-γ the frequency of CD4+IFN- γ + cells (E) and proliferation of isolated T cells was assessed by CFSE dilution for total CD4+ T cells (F) and nitric oxide was measured in the supernatant (G). Following infection, at the time of endpoint, TNFKO lung mononuclear cells were isolated and exposure for 24hrs to a nitric oxide donor. Following exposure, cells were stained for CD4, IFN-γ (H), pSTAT-1 (I&K) and pSTAT4 (J&K) and the effect of the nitric oxide determined. Representative of 2 independent experiments with 5 mice per group.

Figure 5.

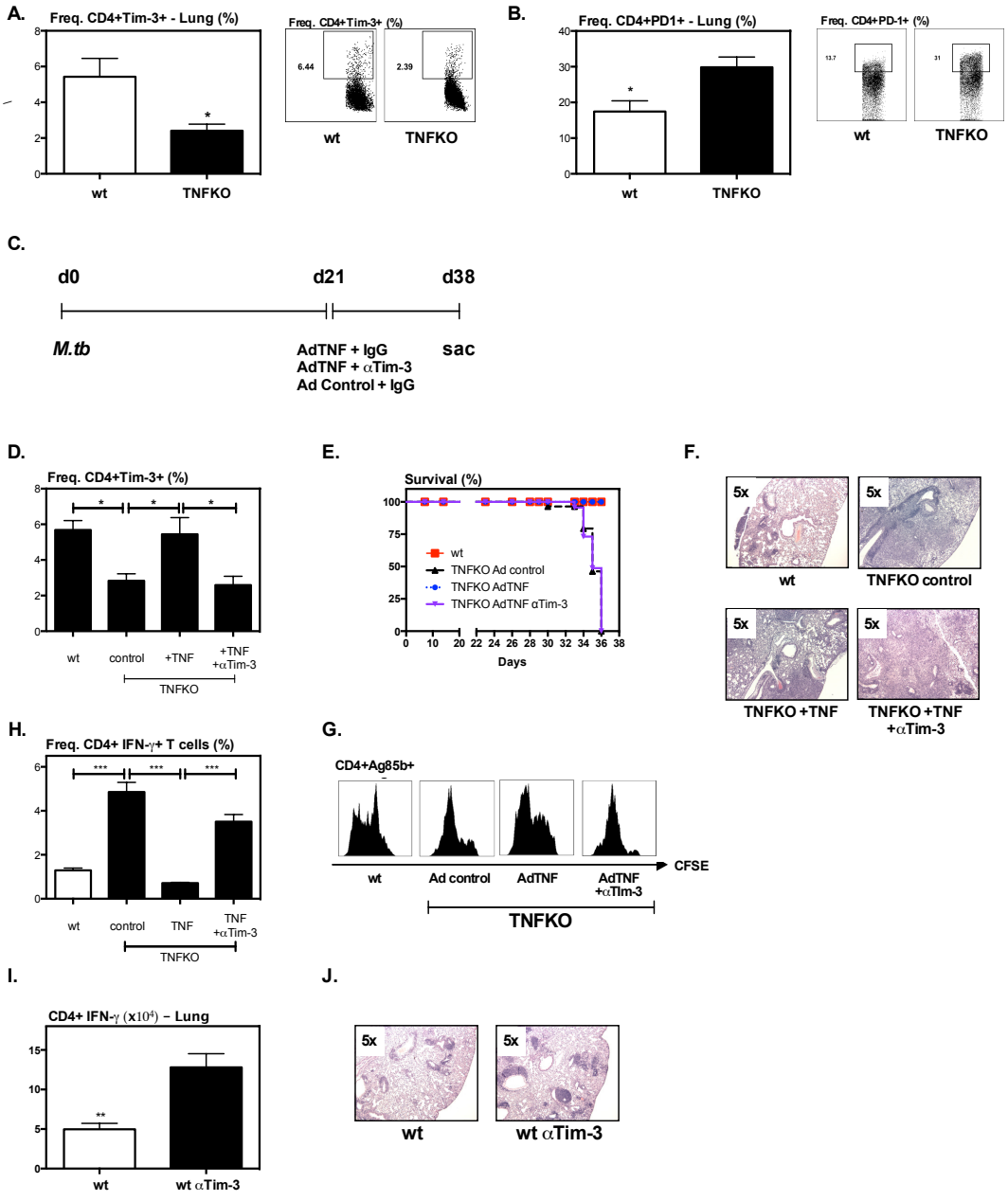

A.
Freq. CD4+Tim-3+ - Lung (%)
wt
TNFKO
Freq. CD4+Tim-3+ (%)
6.44
2.39
wt
TNFKO
B.
Freq. CD4+PD1+ - Lung (%)
wt
TNFKO
Freq. CD4+PD-1+ (%)
wt
TNFKO
C.
d0
d21
d38
M.tb
AdTNF + IgG
AdTNF + αTim-3
Ad Control + IgG
sac
D.
Freq. CD4+Tim-3+ (%)
wt
control
+TNF
+TNF +αTim-3
TNFKO
E.
Survival (%)
wt
TNFKO Ad control
TNFKO AdTNF
TNFKO AdTNF αTim-3
Days
F.
5x
wt
TNFKO control
TNFKO +TNF
TNFKO +TNF +αTim-3
H.
Freq. CD4+ IFN-γ+ T cells (%)
wt
control
TNF
TNF +αTim-3
TNFKO
G.
CD4+Ag85b+
wt
Ad control
AdTNF
AdTNF +αTim-3
CFSE
TNFKO
I.
CD4+ IFN-γ (x10⁴) – Lung
wt
wt αTim-3
J.
5x
wt
wt αTim-3

Figure 5. TNF regulates T cell activation following *M.tb* infection via up-regulation of Tim-3. Five weeks following infection, wild type and TNFKO mice were sacrificed and lung mononuclear cells isolated. Cells were stained for CD4, Tim-3 (A) and PD-1 (B). Three weeks following infection TNFKO mice were reconstituted for TNF using viral vector transfer (AdTNF) or given control vector (C). In the mice reconstituted for TNF one group received αTim-3. Mice were monitored for endpoint and mice were sacrificed at the time point when the majority of TNFKO reached endpoint. At the time in which TNFKO mice reached endpoint, lung mononuclear cells were collected and the frequency of CD4+ Tim-3+ (D) and survival were determined (E). Further, lung histology was collected and stained for H&E to assess gross lung pathology (F) and the number of IFN-γ+ T cells were assessed (H) and the level of antigen specific T cell proliferation was assessed by CFSE dilution (G). Similarly, cells were isolated from wild type mice in which Tim-3 was blocked, and CD4+IFN-γ+ T cells (I) and lung histology (J) were assessed. Representative of 2 independent experiments with 5 mice per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 6.

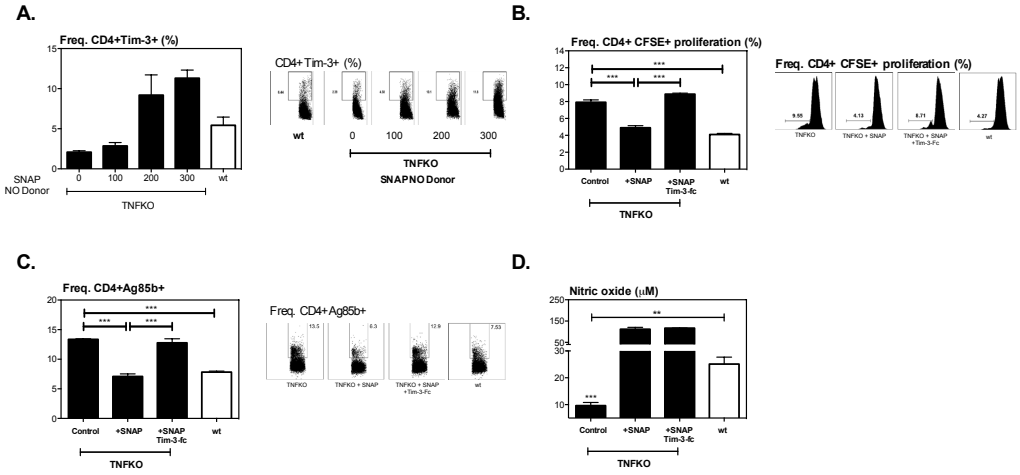


Figure 6: Nitric Oxide regulates CD4 T cell activation via the induction of Tim-3 surface expression. Following *M.tb* infection, mice were sacrificed when TNFKO reached clinical endpoint and isolated lung MNC were stimulated and subjected to increasing doses of the NO donor SNAP and expression levels of TIM-3 on CD4+ were assessed via flow cytometry (A). In subsequent experiments where TIM-3 was inhibited, following *M.tb* infection, mice were sacrificed when TNFKO reached clinical endpoint and isolated lung MNC were stimulated and assessed via flow cytometry for proliferation via CFSE dilution (B) and the frequency of antigen-specific T cells (C). In the same experiments, the level of nitric oxide production was also assessed via greiss assay (D). All assays were conducted in triplicate with a minimum of 5 mice per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 7.

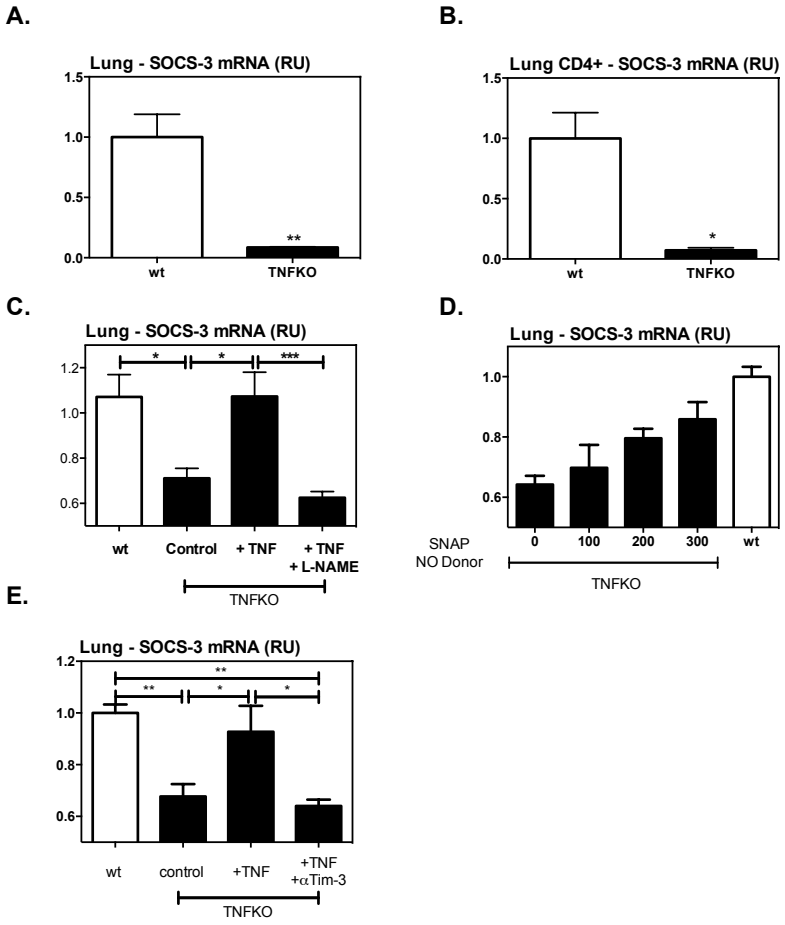
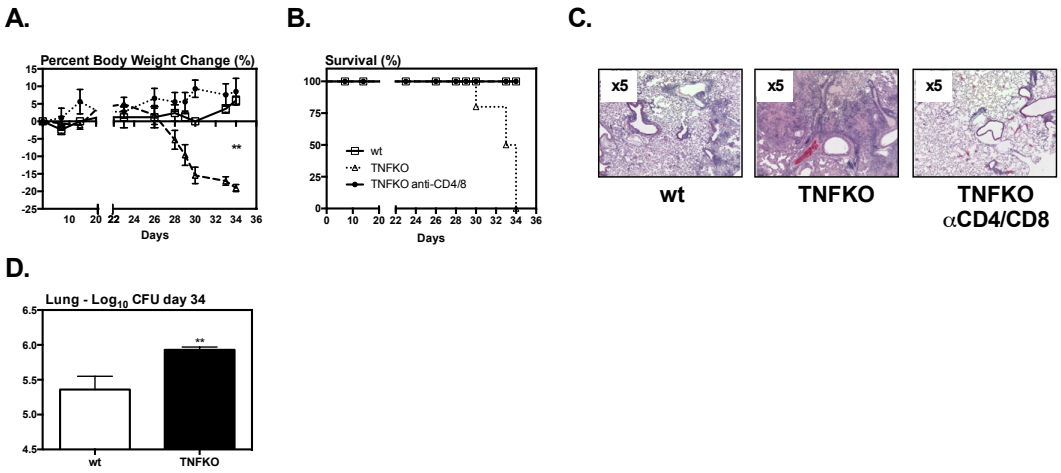

A.
Lung - SOCS-3 mRNA (RU)
1.5
1.0
0.5
0.0
**
wt
TNFKO
B.
Lung CD4+ - SOCS-3 mRNA (RU)
1.5
1.0
0.5
0.0
*
wt
TNFKO
C.
Lung - SOCS-3 mRNA (RU)
1.2
1.0
0.8
0.6
*
*
***
wt
Control
+ TNF
+ TNF
+ L-NAME
TNFKO
D.
Lung - SOCS-3 mRNA (RU)
1.0
0.8
0.6
SNAP
NO Donor
0
100
200
300
wt
TNFKO
E.
Lung - SOCS-3 mRNA (RU)
1.2
1.0
0.8
0.6
**
**
*
*
wt
control
+TNF
+TNF
+αTim-3
TNFKO

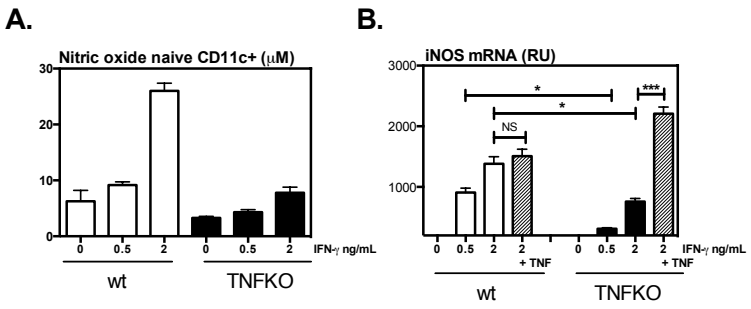
Figure 7: Nitric oxide regulates the development of pathogenic T cells through the induction of Tim-3 mediated SOCS-3 expression. Following *M.tb* infection, mice were sacrificed when TNFKO reached clinical endpoint and isolated lung MNC (A) as well as purified CD4 T cells (B) were analyzed for level of SOCS-3 mRNA expression. In subsequent experiments, bulk lung mononuclear cells were isolated at the time of sacrifice and analyzed for level of SOCS-3 expression by mRNA relative to wildtype (C). In the same experiment, isolated lung MNC from TNFKO mice were subjected to increasing doses of the NO donor SNAP and the level of SOCS-3 mRNA expression was assessed and compared to isolated MNC cells from wildtype controls (D). Finally, in experiments where TIM-3 was inhibited *in vivo*, isolated lung MNC were analyzed for SOCS-3 mRNA expression at the time of sacrifice (E). Representative schematic of results indicating that TNF is required for the production of nitric oxide which achieves T cell regulation via the up-regulation of Tim-3 on active Th1 T cells and suppresses T cell activation via SOCS-3 induction (F). All experiments were conducted in triplicate with a minimum of 5 mice per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Supplementary figure 1.



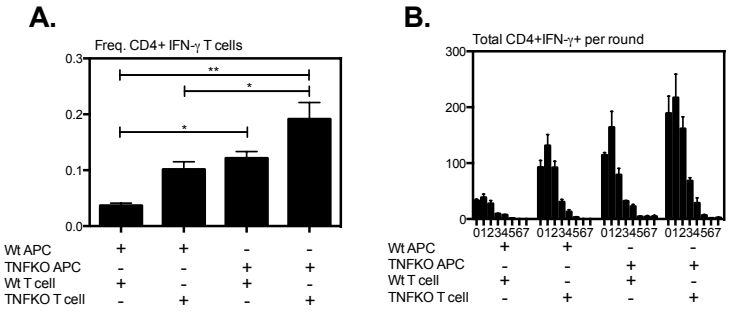
Supplementary figure 1. TNF is critically required to limit the development of lethal pathology and prevent bacterial dissemination following avirulent mycobacterial infection infection. Wild type and TNFKO mice were infected with M.tb. Three weeks following infection one group of TNFKO mice were depleted for CD4+ and CD8+ T cells. Mice were monitored for weight loss (A) and endpoint survival (B). Mice were sacrificed at the time point when the majority of TNFKO reached endpoint and lung histology was collected and stained for H&E to assess gross lung pathology (C), and bacterial burden assessed by colony formation assay in the lung (D). Representative of at least 3 independent experiments with 5 mice per group. * $p \leq 0.05$.

Supplementary figure 2.



Supplementary figure 2. Naïve TNFKO APCs do not up-regulate iNOS gene expression and produce nitric oxide in response to IFN-γ. Naïve CD11c+ APCs were isolated from the lungs of wild type and TNFKO mice. Cells were subjected to various doses of IFN-γ for 72hrs after which nitric oxide production (A) and iNOS mRNA transcription were assessed (B). Representative of at least 2 independent experiments with 5 mice per group. * $p \leq 0.05$.

Supplementary figure 3.



Supplementary figure 3. Mycobacterial infected TNFKO APCs are impaired in their ability to restrain T cell activation. Two weeks following BCG infection, CD11c+ APCs and CD4+ T cells were isolated from wild type and TNFKO mice. APCs and T cell were paired in various combinations and cultured for 72hrs. Following culture, activation of CD4+ IFN-γ+ T cells (A) and proliferation by CFSE dilution (B) were assessed. All assays were conducted in triplicate. * $p \leq 0.05$, ** $p \leq 0.01$.

CHAPTER 4: How does cigarette smoke alter the host immune response to mycobacterial infection, and how does this impair bacterial control?

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.

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How does cigarette smoke alter the host immune response to mycobacterial infection, and how does this impair bacterial control?

There exists much epidemiological data demonstrating a link between cigarette smoke exposure and TB acquisition and disease progression. Despite these observations little experimental evidence exists demonstrating the underlying mechanisms by which cigarette smoke influences the host immune response to mycobacterial infection. In our current study we evaluated the impact of pre and post cigarette smoke exposure on the hosts ability to control a mycobacterial infection. The ability to continuously expose mice to cigarette smoke has allowed us to evaluate how on going smoke expose alters many of key hallmarks of mycobacterial immunity. In addition to evaluating continuous smoke exposure, we also investigated the impact of smoke cessation on anti-mycobacterial host immunity in comparison to never smoke-exposed controls.

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

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PLOS ONE

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

Christopher R. Shaler, Carly N. Horvath, Sarah McCormick, Mangalakumari Jeyanathan, Amandeep Khera, Anna Zganiacz, Joanna Kasinska, Martin R. Stampfli*, Zhou Xing*

McMaster Immunology Research Centre, and Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

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

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* E-mail: stampfli@mcmaster.ca (MRS); xingz@mcmaster.ca (ZX)

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

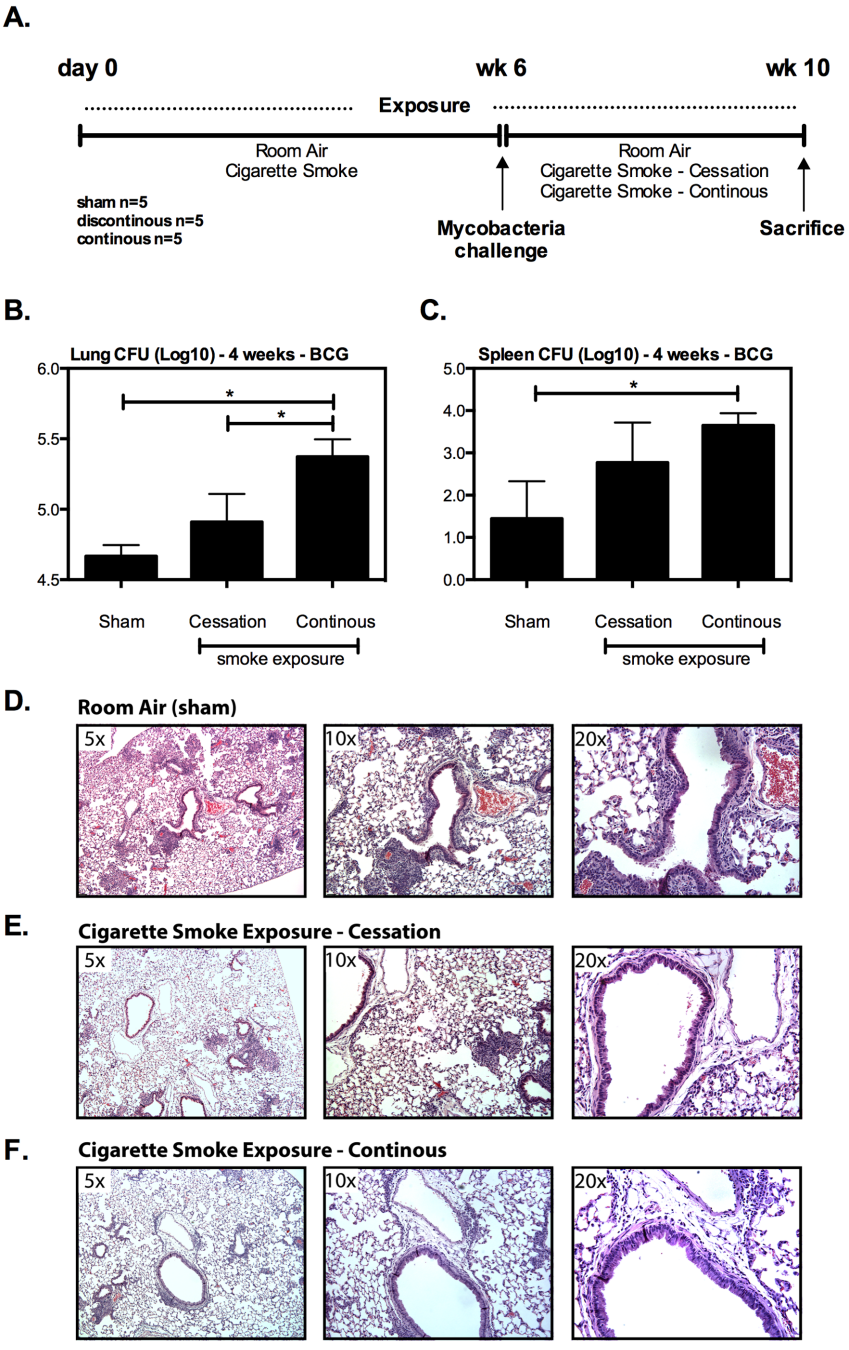
A.
day 0
wk 6
wk 10
Exposure
Room Air
Cigarette Smoke
Room Air
Cigarette Smoke - Cessation
Cigarette Smoke - Continous
sham n=5
discontinous n=5
continous n=5
Mycobacteria challenge
Sacrifice
B.
Lung CFU (Log10) - 4 weeks - BCG
6.0
5.5
5.0
4.5
Sham
Cessation
Continous
smoke exposure
C.
Spleen CFU (Log10)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

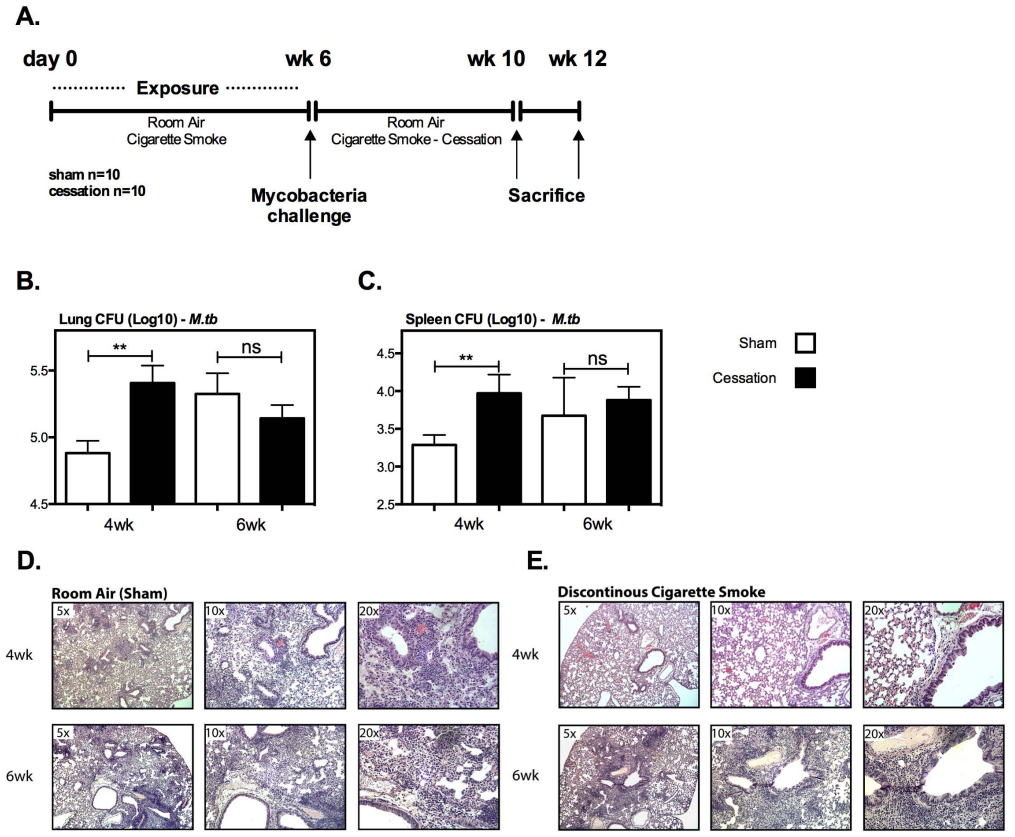

Figure 2. Prolonged cigarette smoke cessation enhances cellular infiltration, granuloma formation and bacterial control following mycobacterial challenge. Mice where either exposed to cigarette smoke or room air for a period of 6 wks, at which time both groups were subjected to challenge with *M.tb* $H_{37}Rv$ (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

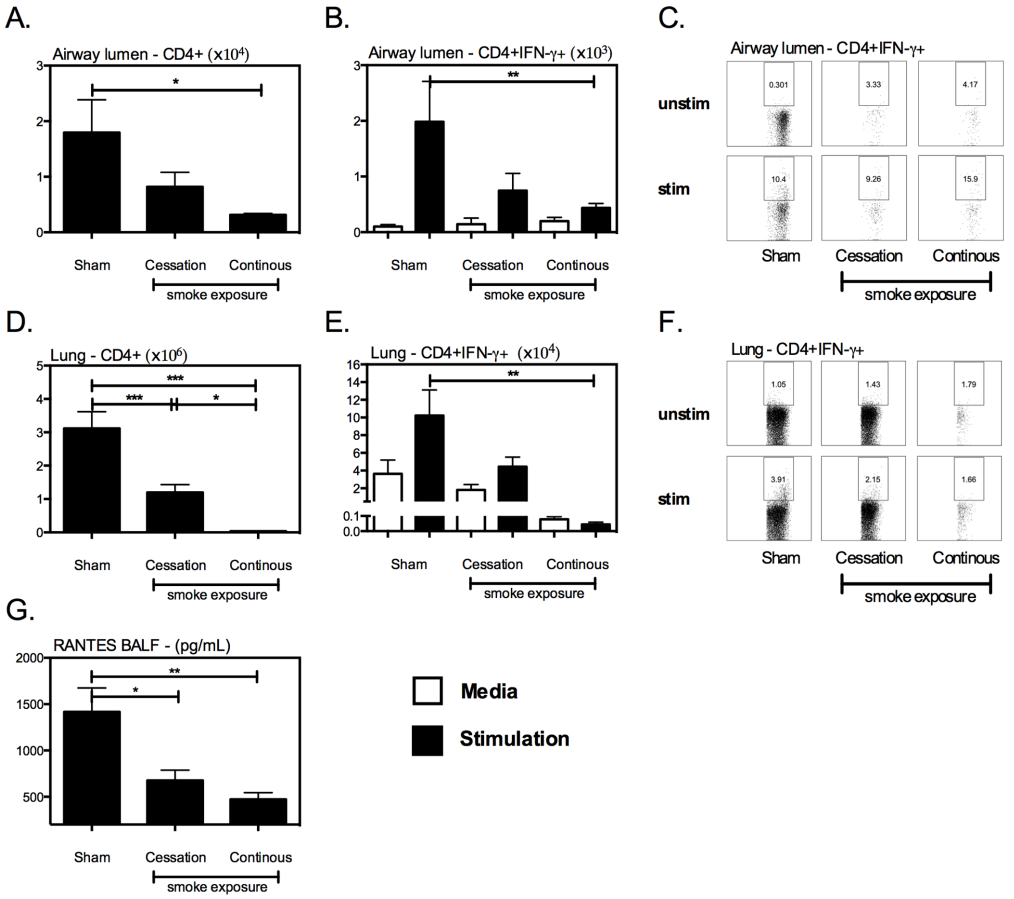

Figure 3. Continuous cigarette smoke exposure impairs the establishment of type 1 immunity in the lung of mycobacteria infected mice. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the establishment of type 1 immune response in the 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

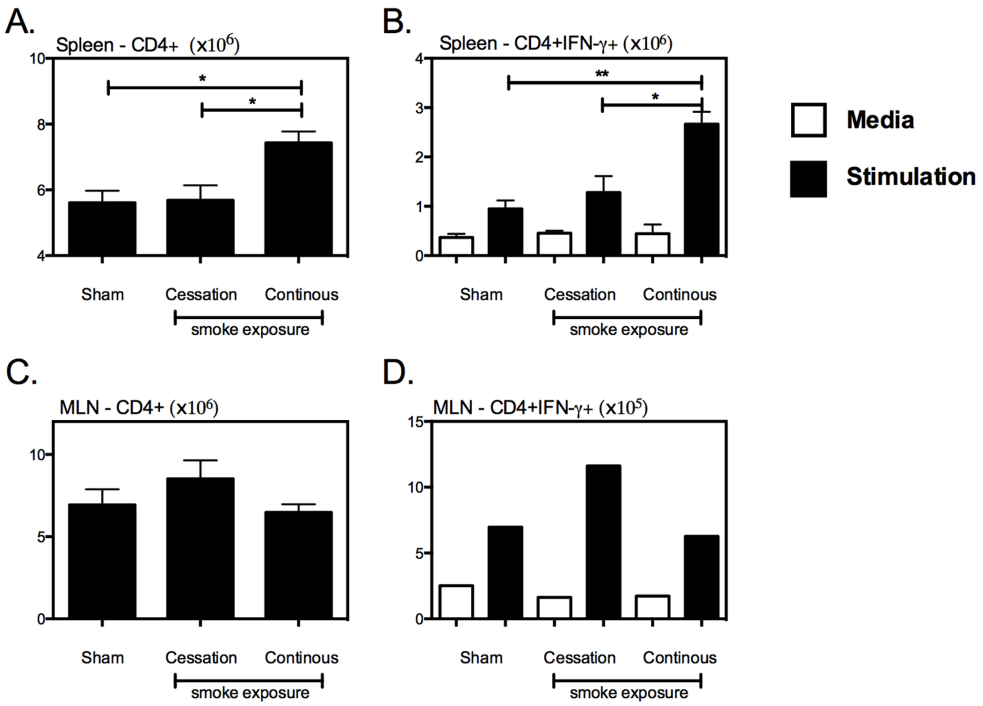

Figure 4. Continuous cigarette smoke exposure does not impair the generation of type 1 immunity in the in the spleen or MLN. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the establishment of type 1 immune response in the spleen 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

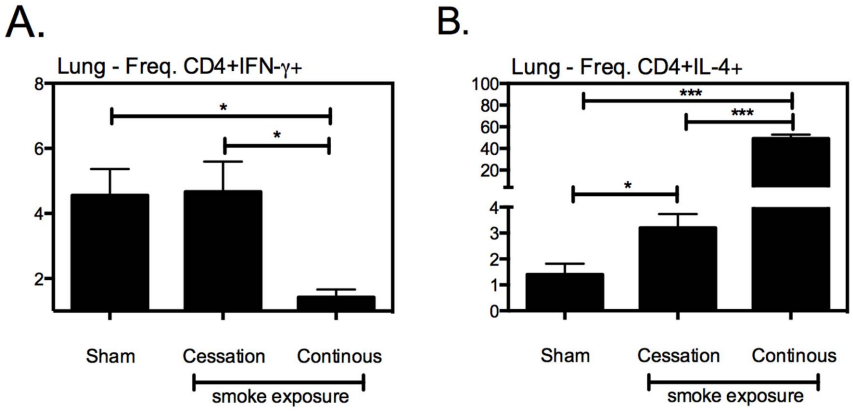

Figure 5. Continuous cigarette smoke exposure alters the balance of Th1 and Th2 CD4 T cells in the lung. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the frequency of CD4+IFN-γ+ (A) and CD4+IL-4+ (B) T cells in the airway lumen 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.
doi:10.1371/journal.pone.0059185.g005

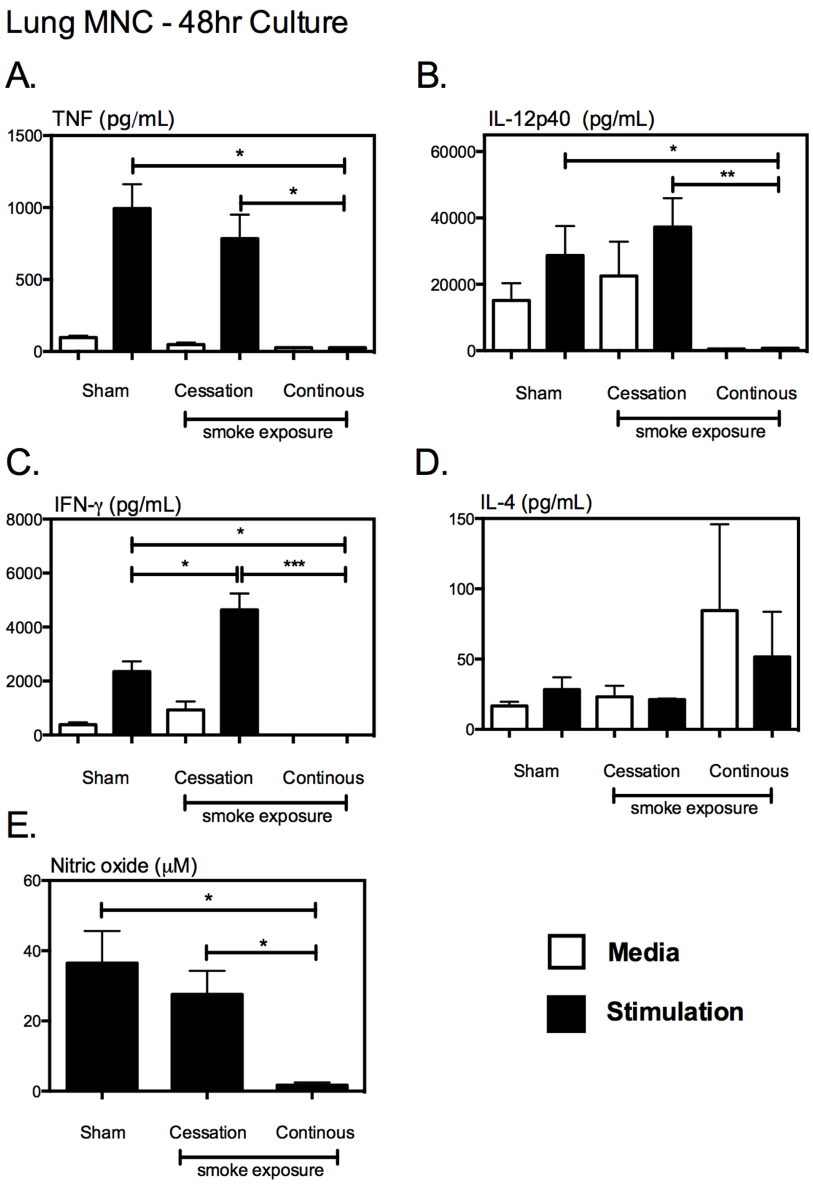

Figure 6. Continuous, but not discontinuous smoke exposure, impairs the production of type 1 cytokines while enhancing the production of IL-4, and reducing the production of bactericidal nitric oxide by lung MNCs following mycobacterial infection. Following the exposure-challenge model described in Figure 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.
doi:10.1371/journal.pone.0059185.g006

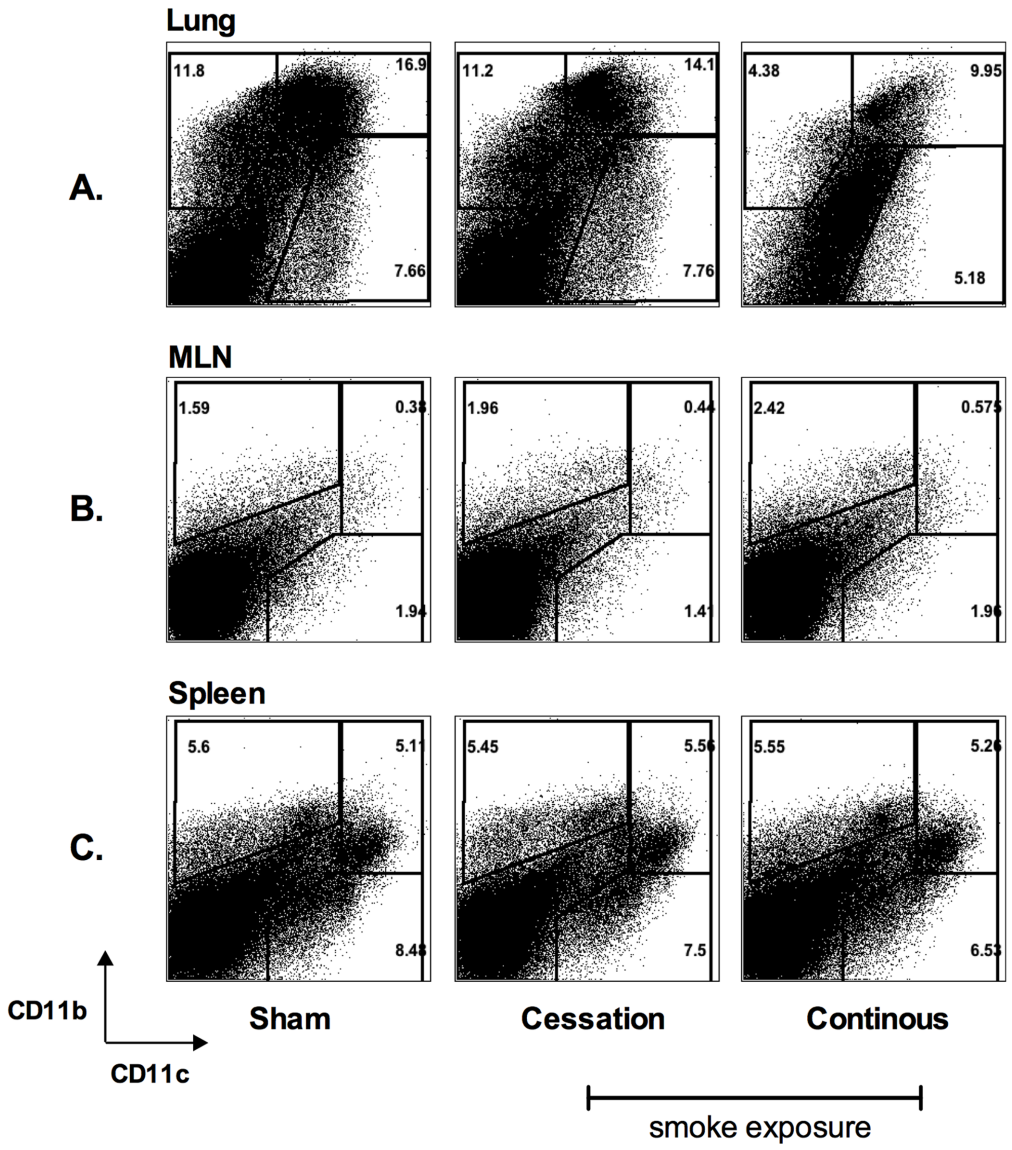

Figure 7. Continuous cigarette smoke exposure alters the surface marker expression of lung, but not spleen or MLN APC populations following mycobacterial infection. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the expression of 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

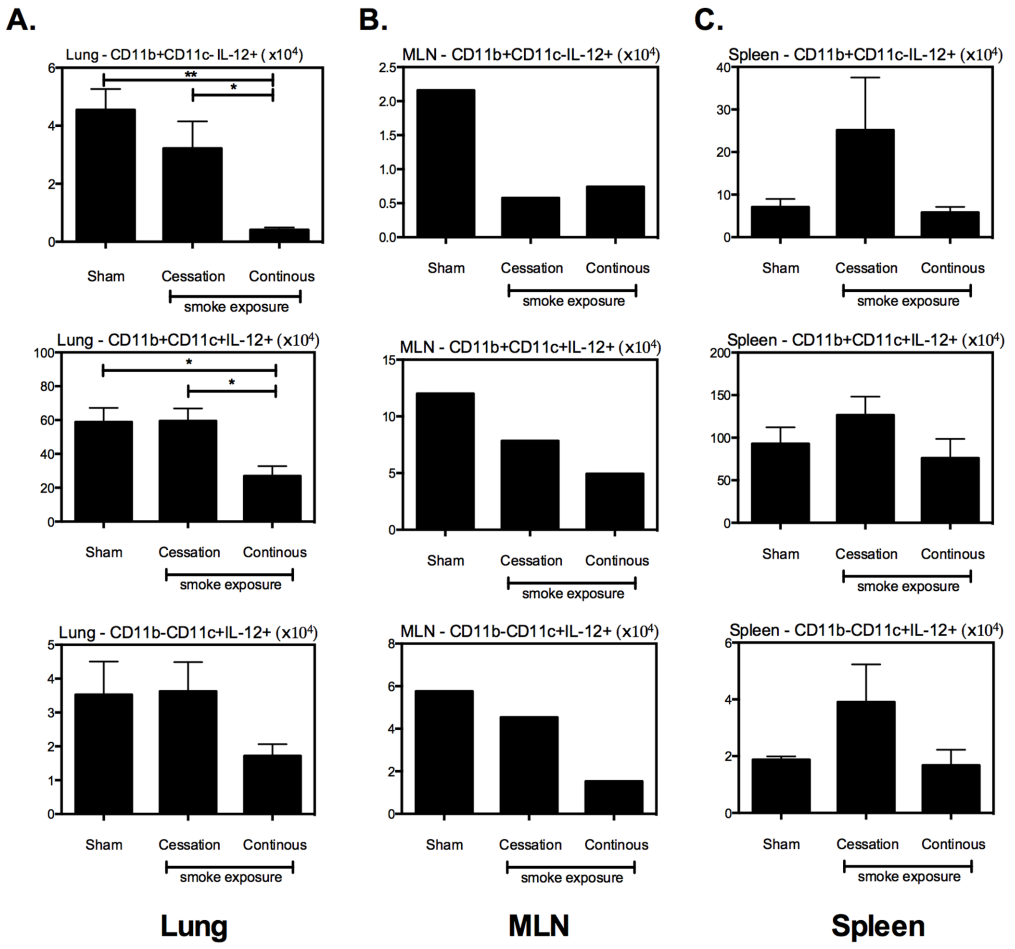

Figure 8. Continuous cigarette smoke exposure suppresses the ability of local, but not systemic, APCs to produce Th1 polarizing cytokines following mycobacterial infection. Following the exposure-challenge model described in Figure 2A, we evaluated the impact of cigarette smoke exposure on the total 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

5.1 Contribution to the field:

Throughout my Ph.D, I have made significant contributions to understanding the mechanisms maintaining the balance between immunity and immunopathology in the context of pulmonary mycobacterial infection. Specifically, I have furthered the notion that the mycobacterial granuloma may not be only to the host's benefit, but also contribute to the persistence of the invading mycobacteria. In **Chapter 2**, we documented the critical role of IL-10 in functionally altering the immune cells within the granuloma structure, noting that both infected macrophages as well as infiltrating T cells were significantly altered. Moreover, we demonstrated that while APCs isolated from within the granuloma were defective in the production of bactericidal products such as nitric oxide, APCs isolated from outside the granuloma within the lung remained competent. Furthermore, we were able to demonstrate that the removal of IL-10 through the use of IL-10KO mice resulted in enhanced IFN-γ production and better bacterial control. Moreover, in **Chapter 3**, we were able to demonstrate that the integrity of mycobacterial granuloma is dependent on TNF. It was determined that TNF regulates the integrity of the granuloma by limiting T cell activation, and in doing so maintains the critical balance between bacterial control and immunopathology. Within this study, it was demonstrated that TNF was essential to the production of nitric oxide, demonstrating that in addition to its established bactericidal function, nitric oxide is also critically required to regulate T cell activation. We further established that this was achieved via nitric oxide mediated

induction of Tim-3 expression on the surface of T cells, limiting their activation and preventing lethal pathology.

At the other end of the spectrum, in **Chapter 4**, we were able to demonstrate that cigarette smoke exposure enhances the susceptibility of mice to mycobacterial infections by limiting T cell and APC recruitment to lungs and limiting granuloma formation. Under the environmental exposure to cigarette smoke, it was found that the lack of immune recruitment was indeed responsible for the impaired granuloma integrity. This was confirmed by the observation that with cigarette smoke cessation, immune cells begin to infiltrate the lung, granuloma structures begin to form, and bacterial control is established. We further demonstrated that limited T cell recruitment to the lung following cigarette smoke exposure altered the bactericidal capacity of infected APCs within the lung, thus preventing bacterial control. This attenuation of T cell infiltration was thought to be attributed to the reduced production of RANTES, a key chemokine required for T cell recruitment to the lungs following *M.tb* infection, but was significantly blunted in cigarette smoke exposed mice. Therefore this study confirms the role of the granuloma in achieving bacterial control following mycobacterial infection. In summary, my Ph.D work has emphasized the dichotomy of the mycobacterial granuloma, where the very structure required for controlling mycobacterial dissemination may actually be a key means by which mycobacteria can evade immune mediated clearance.

5.2 The controversial role of the granuloma in TB control

Tuberculosis has been a plague on human society for thousands of years. The ability of *Mycobacterium tuberculosis* to parasitize its host is largely due to its ability to

manipulate and evade the host immune response. To date, we have only a rudimentary understanding of how *M.tb* is capable of effectively manipulating the host immune response in order to ensure survival within the host for decades, if not a lifetime. Moreover, very little is known as to why *M.tb* is rarely cleared, despite the robust immune response induced by the host upon infection.

One of the most enigmatic responses seen following a pulmonary mycobacterial infection is the formation of the granuloma, a theme that forms the common thread throughout my Ph.D work. While undoubtedly a key hallmark of pulmonary tuberculosis, the role of mycobacterial granuloma has recently been called into question. The formation of granuloma is a dynamic process that begins shortly after infection and continuously evolves over time. Temporally, the granuloma can be divided into three distinct phases: (1) the 'innate granuloma', a loose aggregate composed primarily of recruited macrophages and neutrophils; (2) the 'adaptive immune granuloma' formed following the emergence of antigen specific T cells; and (3) the 'chronic granuloma', resulting from distinct morphological changes in granuloma structure (for details see intro section). In each of the three studies described above, we have observed the three distinct states of the pulmonary granuloma, and addressed critical attributes that contribute to adequate bacterial control and limited immunopathology.

Originally identified post mortem in individuals that had succumbed to tuberculosis disease, it was thought that the mycobacterial granuloma was a "last ditch" attempt by the host immune system to control or limit bacterial dissemination [127,236,237]. This notion implied that the immune response was incapable of adequately clearing the invading

mycobacteria, and was forced to wall the bacteria off into these granuloma aggregates. Indeed, early evidence suggested this was the function of the granuloma, as large amounts of live bacilli can be isolated from the granuloma, but not the lung parenchyma in postmortem histological studies [158,229]. While it does appear that the majority of bacteria is restricted to the granuloma, more sophisticated techniques were able to demonstrate live bacilli residing within the lung parenchyma, indicating that not all bacteria is restricted to those formations following mycobacterial infection [238]. Nevertheless, the majority of live mycobacteria is restricted to the granuloma, reinforcing the notation that granuloma represents the central control mechanism following pulmonary mycobacteria infection. However, it was not until recently, that this function was called into question and the notion that the granuloma may actually play a role in the persistence of mycobacteria was suggested.

In our first study, we demonstrated that the microenvironment of the granuloma, contrary to the classical view of a Th1 dominated environment, actually housed a subset of uniquely activated or altered APCs and T cells. Within this environment, we observed decreased production of Th1 cytokines, specifically, TNF, IL-12 and IFN-γ while observing increased production of the immunosuppressive cytokine IL-10. The production of these suppressive cytokines was accompanied by a reduced capacity of APCs to uptake mycobacteria, and produce bactericidal nitric oxide. Moreover, the reduced capacity of granuloma-derived APCs to produce nitric oxide was associated with the reduced capacity of the host to eliminate mycobacteria. Indeed, if IL-10 is removed from the system, there is a restoration of Th1 immunity and improved bacterial control.

Previous studies have demonstrated a resident T regulatory population in human histological studies, and IL-10 production within the liver granulomas of mice infected intrahepatically [126,141,142]. It should be noted that while Th1 immunity within the granuloma is suppressed, it appears that the chemotactic response of the granuloma remains. Most notably, both MCP-1 and MIP1β were significantly elevated in the culture supernatants of granuloma cells compared to those isolated from the airway lumen [154]. It would appear that while the type 1 immunity within the lung is impaired, there is continuous requirement of macrophages to the granuloma environment. Indeed, previous studies have demonstrated that the mycobacterial granuloma is dynamic, not static immune compartment, with frequent movement of macrophages into and out of the granuloma structures [126,141,142].

While the findings of study one provide some explanation as to why mycobacterial species are capable of surviving long term within the host, there is little explanation as to why the host mounts or maintains a granulomatous response to begin with. Recently, much controversy surrounding the perceived role of the granuloma has been documented. As was described in the introductory section of this document, there is a growing notion within the field that the granuloma may indeed be a pathogen mediated or manipulated event, rather than the long perceived protective function that has dominated the field since the discovery of the granuloma. Our work has begun to explore some of the mechanisms responsible for maintaining this formation and the outcomes associated with granuloma disintegration.

Interestingly, as shown in study two, when mice are exposed to cigarette smoke

there is a marked defect in the recruitment of Th1 CD4+ T cells to the lung, and an almost complete reduction in the formation of granuloma structures that results in only a modest increase in bacterial loads. In stark contrast, when a critical immune regulatory cytokine, TNF is removed, there is remarkable immune activation, however a complete loss of bacterial control. Moreover, there is a loss of the structural organization of the granuloma, and destruction of the lung that ultimately results in death. These contrasting situations demonstrate a paradox of the functional role of the granuloma, indicating that our preconceived notions about the role of the host immune response and the granuloma formation in the host protection against *M.tb* may be incorrect. Furthering this thought, it is remarkable to see that RAG KO mice, which completely lack functional T cell subsets, do not succumb to *M.tb* infection for several months, whereas TNF or IFN-γ KO mice do so within 6 weeks [199,239,240]. Moreover, when T cells were depleted from TNF KO mice, there is remarkable extension in survival in all treated mice. Therefore, it would appear that having no T cell response is better than an unregulated one. Moreover, when T cells were depleted there was a partial restoration in the granuloma structure that was not accompanied by a reduction in CFU. This further convolutes the system, and demonstrates that at least in some cases the host immune response is far deadlier than the infecting bacteria. While it appears that an organized granuloma is better for the host, the organization of the granuloma structure may actually be due to an immunosuppressive influence that the contained mycobacteria has on the microenvironment within the granuloma itself. This raises the question of whether the infecting mycobacteria influence the formation of the granuloma structure as a means of protecting itself from the host

immune response, or as a means of protecting the host from its own pathological immune response.

As has been recently documented, it appears that the infecting mycobacteria play a dominant role in the formation of the granuloma. As discussed above in the introduction section of this document, the lipid components of the mycobacterial cell wall are highly biologically active, and are responsible for manipulating many of the immune responses seen following mycobacterial infection. Given that *M.tb* is capable of selectively deploying these lipid components, it would appear that the immune status of the granuloma may be largely, if not completely controlled by the release of immune modulatory lipids by *M.tb*. Regardless of the reason, it would appear that the microenvironment within the granuloma is highly distinct from the surrounding tissues, and it is likely a direct result of modulation mediated by *M.tb* as a means to provide itself with a protective niche to survive for long periods within the host. Moreover, while the host immune response is essential to the long-term control of *M.tb*, there is growing evidence that the cytotoxic or tissue toxic effects of the host immune response are required for the eventual spread of *M.tb* for transmission to new hosts. The concept that either via host or bacterial factors the granuloma undergoes a suppressive transformation, strongly suggests that this microenvironment is capable of shielding the invading mycobacteria from host-mediated clearance.

Moreover, post-mortem pathological studies of human lung granulomas have demonstrated high levels of viable mycobacteria within these granuloma structures [156,157]. Detailed examination also reveals a number of healed granuloma lesions that

contain no mycobacteria [156,157]. These healed lesions are largely considered to be of two possible origins; first, they may represent granulomas in which the immune response was able to mediate effective clearance of the mycobacteria, or second they may represent a granuloma in which the viable mycobacterium has escaped in order invade other tissues [156,157]. In either case, it remains unclear whether the mycobacterium is mechanism of immune control or immune ignorance.

Further, despite the traditional protective view of granuloma, we and others [141,142,154,241] have shown an immune environment within granuloma that is more favorable to the persistence of *M.tb* than its elimination. While animal models do not accurately replicate structures of the human granuloma, several murine studies have provided invaluable insight into the role of granuloma in preventing *M.tb* dissemination control. Early studies demonstrating the role of critical cytokines such as TNF and IFN-γ perpetuated the notion that the granuloma was essential for bacterial containment as in the absence of either cytokine, mice display ill-formed granulomas and overwhelming bacterial dissemination [242,243,244,245,246]. Furthermore, disruption of CD4+ T cell functionality rapidly results in a loss of the granuloma structure, and extensive bacterial dissemination as seen in both man and mouse [126,186,247]. Interestingly, while essential to the control of *M.tb*, the role of the CD4+ T cells within the granuloma's structure is somewhat species-dependent. Specifically, in humans, T cells surround and wall-off infected macrophages, and do not infiltrate the granuloma, but rather form a defined lymphocytic cuff [157,182,229]. Conversely, murine CD4+ T cells associate directly with infected cells infiltrating throughout the granuloma forming lymphocytic

aggregates or pseudo-granulomas [76,157,229,230,245,248]. Nevertheless, while the loss of CD4+ T cell mediated immunity is detrimental to the host, it is impossible to separate the relative contribution of the two processes to the resulting impaired bacterial control: loss of Th1-mediated immunity and loss of granuloma structure [247,249].

Regardless of whether the granuloma functions to limit bacterial dissemination, much evidence suggests that *M.tb* is especially adept at altering the immune response within the granuloma, creating a uniquely suppressed environment largely through the induction of IL-10 [83,96,150,154,250,251,252,253]. Functionally, the infected macrophages within the granuloma are altered, showing a reduced capacity to produce bactericidal products such as nitric oxide, while showing enhanced IL-10 production [83,96,150,154,250,251,252,253]. Interestingly, while the macrophage populations of the granuloma have reduced bactericidal function, they continue to produce large amounts of chemokines, facilitating the continuous recruitment APC populations into the granuloma [154,241]. Recent studies utilizing *intravital* microscopy have revealed significant movement of inflammatory APCs both into and out of the granuloma [141]. It is this movement of infected APCs that has been speculated to facilitate the early dissemination of *M.tb*. Likewise, human granuloma contains a high frequency of foxp3+ T regulatory cells [254]. In addition, murine studies have confirmed that T cells residing within the granuloma display a highly altered, and functionally suppressed phenotype. Despite the central role of IL-10 in suppressing T cell and macrophage activation within the granuloma, IL-10 neutralization or infection of IL-10 KO mice results in only marginally reduced bacterial loads [252,253,255,256]. Given *M.tb's* long evolution with humans, it is

not surprising that *M.tb* targets multiple pathways to interrupt the host immune response. Moreover, while conventionally immune suppression would appear to benefit only the pathogen, the induction of IL-10 may actually be a host-mediated event required to limit unwanted immunopathology.

Recent studies suggest that the granuloma may be dispensable for preventing bacterial dissemination and may be central to *M.tb's* persistence. Moreover, in the absence of intracellular adhesion molecule-1 (ICAM-1), there is also a failure of granuloma formation, and despite this defect, mice are protected for the first 90 days post-infection, with no increase in bacterial growth compared to wild type mice within this time frame [148]. Similarly, zebrafish models have shown that in the absence of early granuloma formation, there is no defect in the ability of the host to limit bacterial replication and dissemination, and that the granuloma may actually facilitate early dissemination [138,257]. Furthermore, in the absence of IL-27 in mice, there is a substantial defect in the ability of the host to form granuloma in response to *M.tb* infection, and yet infected mice exhibit markedly enhanced bacterial control when compared to their wild type counterparts [147]. Indeed, recent studies indicate that granuloma does not always function to limit bacterial dissemination. For example, CCR2 deficient mice form exaggerated granuloma structures when infected with *M.tb* and paradoxically have a decreased capacity to control bacterial growth [149]. While the specifics as to why the granuloma remains immunologically active remains unknown, one may speculate that this is the means by which *M.tb* recruits new targets in which to infect and propagate itself.

5.3 The essential need for balance between immunity and immunopathology

Classically, TNF is known for its role in the initiation of the inflammatory response acting as an alarm cytokine to alert surrounding cells to infection. Indeed, during an *M.tb* infection, TNF is critical to the initiation of the immune response, where TNF KO mice display delayed cellular influx and adaptive immune activation and impaired bacterial control. Furthermore, recent literature has identified TNF as a biphasic molecule, linked to both the initiation of inflammation, and later regulation of active immune response [74,200,243,258,259,260]. Previously, we have identified that in a murine mycobacterial model TNF is essential to limiting pathogenic type 1 immunity, where in the absence of TNF mice develop exaggerated T cell responses resulting in lethal lung pathology [146,201,261]. However despite this, little is known about how TNF regulates the development of pathogenic T cell responses.

Recently, the use of certain chemotherapeutics for the treatment of inflammatory disease such as rheumatoid arthritis, specifically targeting TNF, has been linked to a rapid progression to active disease in previously latently infected humans [201]. Moreover, TNF deficiency is uniformly fatal in mice infected with mycobacteria (virulent or avirulent), with 100% mortality achieved within 6 weeks of infection [146]. Indeed, TNF plays an indispensible role in preventing the development of active disease both in humans and mice.

Using TNF KO mice, we were able to determine that TNF plays an essential role in both controlling mycobacterial growth, as well as limiting the development of lethal immune mediated pathology. Our study demonstrated that the mechanism by which TNF

limits the development of pathogenic T cells was indirectly through its contribution to the production of nitric oxide, and the immune regulatory attributes of this potent radical. Notably, we were able to demonstrate that the TNF-Nitric oxide pathway was responsible for the induction of Tim-3 and SOCS-3 on exposed T cells. It was the induction of Tim-3 that resulted in the regulation of pathogenically activated T cells, dampening their IFN-γ production capacity, reducing tissue damage, and promoting survival of the host. Interestingly, in the absence of TNF there is profound loss in the structure and organization of the pulmonary granuloma, which can be restored by reconstitution of TNF, and blockaded by the inhibition of nitric oxide or by Tim-3.

In study one, we demonstrated that nitric oxide production by infected APC populations is impaired within the granuloma, calling into question its role in maintaining the granuloma integrity. In light of our study on the role of TNF, it can be speculated that the activated APCs within the granuloma may have produced high levels of nitric oxide, which may have contributed to the suppressive nature of the granuloma. This would result in the dampening of the Th1 response within the granuloma that we observed, and in turn suppress any further nitric oxide production. Given our finding that in the absence of nitric oxide the integrity of the granuloma is lost, and T cell activation is significantly enhanced, it would appear that nitric oxide plays a central role in the regulation of immunity and immunopathology. Indeed, in our third study, we provided significant evidence that nitric oxide plays a key role in communicating the activation status of an infected APC to the recruited T cell. Our study demonstrates that nitric oxide is central to the activation status of recruited Th1 cells through the induction of SOCS-3, a key

suppressor of cytokine signaling. Moreover, recently, Carow *et al* demonstrated the essential role of SOCS-3 in CD4+ T cells following *M.tb* infection. In line with our current study, they have demonstrated that a deficiency in SOCS-3 expression in T cells results in a loss of granuloma integrity, bacterial control, and is unilaterally fatal by 6 weeks [262]. Moreover, in the absence of SOCS-3, mice demonstrate enhanced IFN-γ responses but highly impaired bacterial control, furthering the notion that the integrity of granuloma can only be maintained by a carefully balanced immune response [262]. These results indicate that a degree of immune suppression or immune regulation is required by the host in order to maintain granuloma structure and protective efficacy.

Central to TNF's role during an *M.tb* infection is its synergy with IFN-γ to induce the production of nitric oxide; a critical bactericidal molecule produced by infected macrophages to kill internalized *M.tb.* The production of nitric oxide is a highly regulated process involving the coordinated release of multiple cytokines. Undeniably, the arrival CD4+ T cells to lung of an *M.tb* infected host and the subsequent production of IFN-γ is fundamental to the production nitric oxide, however, IFN-γ cannot up-regulate the inducible nitric oxide synthase (iNOS) alone [245,259,263]. Therefore, despite high levels of IFN-γ production, in the absence of TNF, nitric oxide production is extremely attenuated and bacterial control is significantly impaired.

While previous studies have demonstrated that macrophage-produced nitric oxide, a critical bactericidal agent in mycobacterial immunity, can dampen T cell activation, little is known about the underlying mechanism by which nitric oxide mediates its suppressive effects. Undeniably at high concentrations, nitric oxide can cause cell damage

and induce apoptosis, however, this is not the case at the concentrations seen physiologically. Furthermore, nitric oxide has been shown to selectively suppress Th1, but not Th2 immune responses, indicating that the function of nitric oxide is not simply to kill T cells through toxicity, but to rather selectively target them for removal. Moreover, the production of nitric oxide does not induce T cell suppression unless the producing macrophage is in direct contact with the T cell, indicating a contact dependent mechanism. Together, these observations indicate that the function of nitric oxide in the regulation Th1 immunity is more complex than previously considered, and likely involves the up-regulation of suppressive molecules on both the T cell and interacting APC.

While the majority of infected individuals control *M.tb*, sterile clearance is rarely achieved, and a highly delicate balance between maintaining immunity and preventing pathology must be maintained. Previously, it has been demonstrated that mice lacking T cell regulatory molecule programmed death receptor -1 (PD-1) rapidly succumb to an *M.tb* infection due to extensive immunopathology, rather than bacterial dissemination. Similarly, T cell Ig and mucin domain protein-3 (Tim-3) is up-regulated on Th1 CD4+ T cells and is responsible for coordinating their contraction following bacterial clearance/control, however little is known about its regulatory role during an *M.tb* infection. Specifically, despite its established role in regulating Th1 immunity, little is known about how Tim-3 is regulated, and what signals the up-regulation of Tim-3 during the contraction stage of an immune response.

In summary, we demonstrate that the extreme susceptibility of TNFKO mice to *M.tb* is due to the development of pathogenic T cells, rather than bacterial dissemination

as TNF is essential to the production of nitric oxide; critical to both bacterial control and T cell regulation. Furthermore, we demonstrate a novel mechanism whereby infected macrophages signal the recruited T cell to 'stand down' through the production of nitric oxide, an effect mediated by the selective up-regulation of Tim-3, and production of SOCS-3 within responding T cells. Overall, our study highlights the importance of balance between bacterial control and immunopathology, demonstrating the detrimental outcome that occurs when both are lost.

5.4 Cigarette smoke and TB immunity: a failure to mobilize effectors cells to the lung

While epidemiological data has long shown a link between cigarette smoke and tuberculosis risk, to date little experimental data has demonstrated how cigarette smoke alters the host immune response resulting in such impairment. In our study, we attempt to unravel the alterations in the host immune response altered by cigarette smoke exposure. Our data has demonstrated two key impairments in mycobacterial immunity. Firstly, the recruitment of Th1 CD4+ T cells into the lung, and secondly the formation of the mycobacterial granuloma. Interestingly, despite the significant attenuation of both processes, the bacterial loads within the cigarette smoke exposed lungs were only modestly increased. This in and of itself calls into question the requirement of both Th1 CD4+ T cells and granuloma formation. Indeed, to date no experimental evidence exists that can conclusively demonstrate that the granuloma is essential to mycobacterial control. Moreover, in light of our studies and others, there may be justification to question whether the granuloma may actually promote mycobacterial resistance.

It is interesting to note that despite the known inflammatory properties of cigarette smoke exposure, it would appear in at least the context of type 1 immunity, that cigarette smoke may actually attenuate lung inflammation rather than promote it. This may be due to the inflammatory nature of cigarette smoke, as it has been documented to induce a type 2 immune response. Given the opposing nature of type 1 and type 2 immunity, it may be that exposure to cigarette smoke following mycobacterial infection that mutually attenuate each other. Indeed, we have seen a response that is neither type 1 or type 2 dominated within the lungs of mycobacterial-infected mice exposed to cigarette smoke, although both responses were present.

In this study we also noted a remarkable reduction in the formation of granulomas within the lungs of mice exposure to cigarette smoke which coincides with the reduced recruitment or retention of CD4+ T cells within the lung. It is very interesting that despite an almost complete lack of type 1 immunity in the lung of cigarette smoke exposed mice, there was only a modest increase in the bacterial burden. Notably it appears that the defect lies in the recruitment or retention of T cells in the lung rather than their priming in the secondary lymphoid tissues. This was noted in particular by the normal activation profiles within the mediastinal lymph nodes and the spleens of cigarette exposed mice, and the contrasting attenuated T cell profiles in the local environment of the lung interstitium and airway lumen. These findings call into question the role of type 1 T cells in protective immunity to *M.tb*. Indeed, we see that at least in early studies of RAGKO mice, T cells are somewhat dispensable to host protection [264].

Unexpectedly, our study revealed that continuous cigarette smoke severely attenuates lung inflammation resulting in less lung pathology. This observation is particularly dangerous, as epidemiological data suggests 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 onset of disease [226]. Given that the majority of TB symptoms are due to the inflammatory responses generated by the host, cigarette smoke mediated inflammatory suppression may allow for the infected host to remain asymptomatic despite active bacterial growth. The notion that cigarette smoke may mask TB symptoms, allowing a critical bacterial threshold to be reached long before diagnosis may explain why lethal disease rapidly ensues, and the likelihood of mortality is high in TB infected smokers. Together TB and cigarette smoke exposure has the capacity to be particularly deadly; not only impairing bacterial control, but by simultaneously preventing the development of classical clinical symptoms.

While cigarette smoke exposure profoundly impacts local immunity within the lung, our study reveals cigarette smoke exposure does not significantly alter the generation of CD4+IFN-γ+ T cells in the peripheral lymphoid organs of mycobacterial infected mice. We demonstrate that continued cigarette smoke exposure alters the local lung immune environment attenuating the release of critical anti-mycobacterial cytokines, IL-12, TNF, IFN-γ, and the T cell chemokine RANTES. While a loss of any one of these cytokines will severely compromise mycobacterial control, the phenotype seen following cigarette smoke exposure is unique to any one deficiency. To draw a comparison, in the absence of IL-12, mycobacterial infected mice fail to mount Th1 responses, control

bacterial growth, and localize T cells to lung, an observation shared with cigarette smoke exposure [145]. 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 [102]. Despite their similarities, it should be noted that the development of lung pathology significantly differs between deficiencies, with IL-12 and RANTES deficient mice displaying less lung pathology, while TNF and IFN-γ deficient mice displaying exaggerated lung pathology [199,239]. The difference lies in the recruitment of T cell immunity to lung, where IL-12 and RANTES deficient mice recruit far fewer active T cells, TNF and IFN-γ deficient mice recruit far more cells in an attempt to compensate for the impaired ability of the infected APCs to produce nitric oxide. It is interesting that cigarette smoke targets multiple anti-mycobacterial immune pathways, reducing an exposed 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 were 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 cigarette smoke attenuate the establishment of Th1 immunity, it augmented Th2 immunity further impairing the host's ability to control mycobacteria. The unique influence of cigarette exposure on the development of mycobacterial immunity cannot be directly attributed to its effect on a single component of mycobacteria, but rather its broader impact on the innate, and ensuing adaptive components of the immune system.

Given the overwhelming epidemiological and experimental data demonstrating the associated effect of cigarette smoke exposure on TB acquisition, disease, and mortality; global TB control strategies must incorporate additional measures to combat the growing problem of cigarette smoke in regions endemic to TB. Furthermore, in light of our experimental data, it should be stressed that the best way to avoid and combat active TB is to stop smoking. These recommendations are underscored by the observation that continued cigarette smoke exposure not only suppresses host immunity, it also hinders antibiotic therapy, delaying disease treatment [265]. Our findings strongly indicate that smoking cessation should be considered and reinforced as part of the first line treatment protocols for both active and latent TB.

Conclusions:

While bacterial control is essential to host survival, this control comes at a cost, one that must be carefully weighted to ensure the best possible outcome for the infected host. This raises the notion that while it is commonly believed that latency is a failure of the host to eliminate the pathogen, this may not be the case. It remains plausible that the host may be capable of eliminating the pathogen, however the price for this clearance may be too great, leading the host to compromise and permit the establishment of latency. We must revisit how we think about chronic *M.tb* infections, and the strategies we employ to treat them.

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APPENDIX 1

Understanding delayed T cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis

Christopher R. Shaler, Carly Horvath, Rocky Lai and Zhou Xing

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Understanding Delayed T-Cell Priming, Lung Recruitment, and Airway Luminal T-Cell Responses in Host Defense against Pulmonary Tuberculosis

Christopher R. Shaler, Carly Horvath, Rocky Lai, and Zhou Xing

McMaster Immunology Research Centre; Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada L8S4K1

Correspondence should be addressed to Zhou Xing, xingz@mcmaster.ca

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Mycobacterium tuberculosis (*M.tb*), the causative bacterium of pulmonary tuberculosis (TB), is a serious global health concern. Central to *M.tb* effective immune avoidance is its ability to modulate the early innate inflammatory response and prevent the establishment of adaptive T-cell immunity for nearly three weeks. When compared with other intracellular bacterial lung pathogens, such as *Legionella pneumophila*, or even closely related mycobacterial species such as *M. smegmatis*, this delay is astonishing. Customarily, the alveolar macrophage (AM) acts as a sentinel, detecting and alerting surrounding cells to the presence of an invader. However, in the case of *M.tb,* this may be impaired, thus delaying the recruitment of antigen-presenting cells (APCs) to the lung. Upon uptake by APC populations, *M.tb* is able to subvert and delay the processing of antigen, MHC class II loading, and the priming of effector T cell populations. This delay ultimately results in the deferred recruitment of effector T cells to not only the lung interstitium but also the airway lumen. Therefore, it is of upmost importance to dissect the mechanisms that contribute to the delayed onset of immune responses following *M.tb* infection. Such knowledge will help design the most effective vaccination strategies against pulmonary TB.

1. Introduction: Current Challenges and Global Impact

Despite extensive vaccine coverage in endemic areas, pulmonary tuberculosis (TB) remains one of the top three infectious causes of death worldwide [1]. With an alarming 9 million new cases annually, it is estimated that one third of the world's population is latently infected with *Mycobacterium tuberculosis* (*M.tb*), the causative bacterium of TB [1]. Following primary infection, greater than 90% of infected individuals enter into an asymptomatic latency period, showing no clinical sign of disease (Figure 1). The ability of the host to control *M.tb* is accomplished through bacterial restriction and segregation, rather than clearance. *M.tb*, a facultative intracellular pathogen, is spread person-to-person through infected aerosols generated by coughing or sneezing. Once deposited in the airways, *M.tb* primarily infects the alveolar macrophage, the resident macrophage of the airway lumen [2, 3]. *M.tb* has a transmission rate of 30% or less but the relative susceptibility of an exposed individual to infection is determined by a number of factors including the living conditions, contact time with infected individuals, and immune status [4–7]. It is estimated that under the correct conditions a single bacillus could establish a successful infection [2]. The fact that *M.tb* is spread via aerosols, and can be infectious in low numbers makes it a major health concern in regions such as those commonly seen in developing world with high population densities, poor living conditions, and immune compromising diseases.

While many infections remain asymptomatic, the sheer number of infected individuals makes TB the number one bacterial killer worldwide, responsible for nearly 2 million deaths annually [1]. The majority of TB-related deaths are seen in the developing world where infected individuals

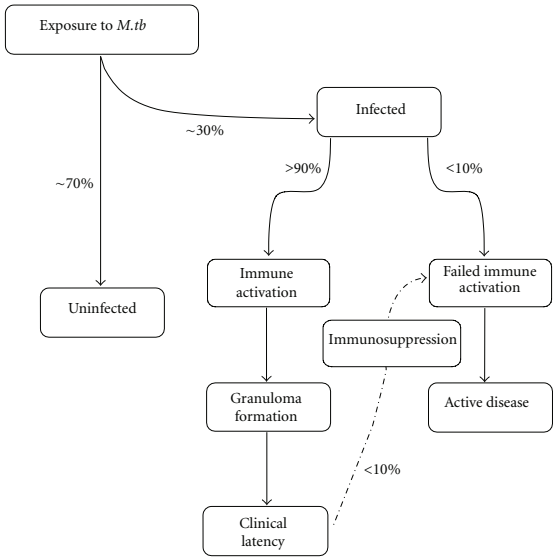

Figure 1: Flow chart of TB disease progression and major events leading to protection. Major steps are outlined for the progression of and infected or uninfected hosts from the point of exposure to development of active disease or clinical latency (protection). The relative percentage of individuals to progress between steps is shown beside the appropriate progression line.

cannot afford the lengthy antibiotic regime required to treat *M.tb* [1]. Compounding the problem, the only licensed TB vaccine, Bacillus Calmette-Guerin (BCG), has shown highly variable efficacy (0–80%) [8]. Even with the highest efficacy, BCG is only effective in limiting severe disseminated forms of TB in children, not preventing lung disease or providing sterile immunity [8]. Moreover, the usefulness of BCG is further limited as protective immunity typically wanes by adolescence [9] and cannot be boosted by repeated BCG vaccination [10]. Further compounding the problem, many of the regions with the highest incidence of TB also coincide with those endemic to HIV-AIDS [11]. The ability of HIV-AIDS to dramatically suppress cellular immunity has made coinfections with *M.tb* particularly deadly. Of the estimated 2 million deaths by TB per year, approximately 400,000, nearly one quarter, are of HIV-TB-coinfected individuals, highlighting the significance of this deadly coinfection [11].

As one of the most persistent global health concerns, the success of *M.tb* as a human pathogen can be attributed to its ability to parasitize the host-pathogen microenvironment. Studies of ancient DNA and skeletal remaining have traced the coevolution of *M.tb* and prehuman lineages for nearly 3 million years [12–16]. As such, *M.tb* has evolved multiple mechanisms to evade, elude, and subvert the host immune system. For instance, compared to many other respiratory pathogens, *M.tb* infection retards adaptive T-cell activation by eliciting much delayed T-cell priming and lung recruitment [17, 18]. Temporally, *M.tb* targets both early immune initiation as well as chronic bacterial control preventing the host from ever achieving sterile immunity. While much research has been done to understand the various ways *M.tb* suppresses established immunity, little progress has been made in understanding the mechanisms underlying delayed early adaptive immune activation.

To this end, it has been proposed the impairment in adaptive immune activation may be attributed to a combination of underlying defects in the immune initiation cascade. Specifically, the limited availability of antigen due to the slow replication rate of *M.tb* is thought to account for a weak early inflammatory response, delaying the recruitment of innate immune cells into the lung. The impaired entry of immune cells into the lung coupled with active immune suppression driven by *M.tb* are the major mechanisms thought to delay the migration of antigen-presenting cells (APC) to the lymph node. Puzzling and poorly understood, the contributions of both host and pathogen to the relative delay in T-cell activation still remain largely unresolved.

2. Initiation of Innate Immunity in the Lung Following *M.tb* Infection

2.1. Infection. *M.tb* is spread through aerosols generated by an infected individual [19]. Coughing or sneezing is the primary method of transmission, and persons with active disease are highly contagious [11]. Infected aerosols are

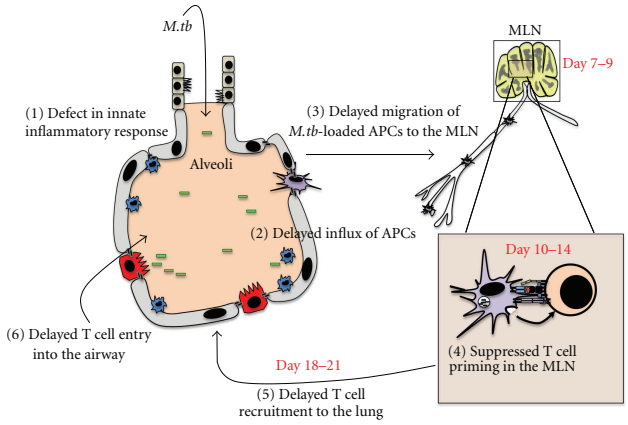

Figure 2: Illustration of the speculated major immunologic setbacks seen in the early course of pulmonary *M.tb* infection. The major defects are numbered in the diagram according to the sequence of events. APC: antigen-presenting cells; MLN: mediastinal lymph nodes.

taken into the lung and deposited in the alveolar space, where *M.tb* is actively taken up by the resident alveolar macrophage (AM) via phagocytosis [20]. Once engulfed by the macrophage, *M.tb* becomes highly resistant to clearance. This is achieved by evading immune detection and elimination through a variety of immune evasion strategies, including blocking phagolysosome fusion and detoxifying oxygen and nitrogen radicals [21]. Although the primary cell type to be infected is the AM, *M.tb* can also actively infect and replicate within recruited neutrophils [22], dendritic cells [23], and alveolar type II epithelial cells [24].

2.2. Innate Recognition. Upon entering the airway lumen, *M.tb* is thought to "silently" enter the resident AM. Infection of the AM occurs through receptor-mediated phagocytosis. Utilizing the complement receptors (CR3 and CR4), the mannose binding receptor, surfactant molecules, and DC-SIGN, *M.tb* rapidly facilitates its uptake by the AM [25–27]. Upon entry, recognition of *M.tb* is mediated through the engagement of pattern recognition receptors (PRRs). While the toll-like receptors (TLRs), specifically TLR-2, 4, and 9, have long been recognized as the primary PRRs required for the detection of *M.tb* [28, 29], recently a member of the NOD family of receptors, NOD2, has been shown to play a critical role in the intracellular recognition and activation by the *M.tb*-infected macrophage [30]. Typically, the AM acts as a sentinel, detecting and alerting surrounding cells to the presence of an invader. However, in the case of *M.tb*, this function is thought to be impaired. In particular, *M.tb* has been shown to uniquely engage the mannose receptor (MR) of responding macrophages. A major cell wall component of *M.tb,* lipoarabinomannan (LAM), is alternatively capped with mannose, which signals through the MR, inducing an anti-inflammatory program; impairing the secretion of both proinflammatory cytokines (TNF-α and IL-1β) and chemokines (MCP-1 & IP-10) [27, 31], thereby deferring the recruitment of innate immune cells to the lung. Interestingly, *M.tb* appears to simultaneously induce both pro- and anti-inflammatory effects as it has recently been shown that *M.tb* interacts with the airway epithelium to induce the production of MMP-9, a mechanism to attract macrophages to the site of infection facilitating its own propagation [32].

2.3. Recruitment of Antigen Presenting Cells (APCs) to the Lung. The entry of responding immune cells into the lung involves cell migration across the vascular endothelium and the airway epithelium that separates the lung interstitium and airway luminal space. Although not much has been studied in the context of TB, it is understood that crossing the endothelium requires appropriate activation, involving tight junction modification and the expression of addressin molecules on the luminal surface. These processes are significantly inducible by inflammatory cytokines such as TNF-α and IL-1β [33–36]. Preliminary findings from our group suggest that there is a minimum recruitment of innate immune cells to both the lung interstitium and airway lumen for first 5 days postmycobacterial infection, which is associated with a lack of both TNF and IL-1β induction in the lung during this time period [unpublished data]. It is thus our belief that the absence of an early innate inflammatory response in the lung represents an important mechanism delaying the upregulation of chemotactic and adhesion signals and the subsequent recruitment of innate immune cells to the lung, which in turn delays T-cell priming in the draining lymphoid tissues (Figure 2).

2.4. Mycobacterial Antigen Acquisition. Under noninflammatory conditions, immune surveillance of the airway lumen is passive and mediated primarily by a limited number of

intraepithelial dendritic cells (DCs) [37–39]. However, upon the initiation of an inflammatory response, there is a rapid recruitment of DCs to the various lung compartments [37]. The precise timing of these inflammatory events is not fully understood in the context of *M.tb* infection. However, as discussed above, it is believed that the major influx of APCs into the lung is delayed for the first 5–7 days following infection [40] and the trafficking of immune cells from the vasculature to the airway lumen is a two-step process. The majority of DCs first exit the vasculature into the interstitium and then migrate through the interstitial matrix and reach the alveolar epithelium [34, 41]. Rather than fully entering the airway lumen, the majority of recruited DCs interdigitate into the epithelial wall, extending their dendrites into the luminal space where they acquire antigen or microbial organisms while maintaining access to the collecting lymphatics located in the interstitium [37, 42].

While TNF has many functions, it is considered central to the appropriate control of an *M.tb* infection. During the initial stages, TNF acts primarily as an alarm cytokine alerting surrounding cells to the presence of an infection. It is believed that AM-derived TNF commences the recruitment of innate immune cells by activating the type II alveolar epithelial cells (AEII). This initiates the production of chemokines such as MCP-1, upregulates critical adhesion molecules, and reduces tight junction adhesion [43–45]. Following *M.tb* infection, it is unclear how the vascular endothelium becomes activated. However, given their geographic location and based on the knowledge from other models, it is plausible that the AEII relay the inflammatory signal from the airway to the interstitium, thereby activating the endothelium. AEII are central to the initial recruitment of APCs to the lung, functioning as the "gatekeepers" of the airway lumen controlling the production of chemokines such as MCP-1 and regulating the expression of addressin molecules [38, 46]. The early mobilization of APCs to the lung is critical to the timely control of an *M.tb* infection as it has been shown that in the absence of MCP-1's receptor, CCR2, APC recruitment to the lung is significantly delayed, impairing the downstream adaptive immune activation and bacterial control [47]. Upon entering the lung, it is currently unknown which subsets of DCs are primarily responsible for acquiring and transporting antigen or mycobacteria to the draining lymph node. Further complicating our understanding, *M.tb* actively infects macrophage, DC, and neutrophil populations, all of which have been shown to be capable of transporting antigen to the MLN [22, 48–50].

3. Initiation of Adaptive T-Cell Activation in the Mediastinal Lymph Node (MLN)

While much controversy surrounds the generation of adaptive immune responses following *M.tb*, it is now widely accepted that the earliest antigen-specific T-cells are not observed for at least 10 days postinfection, appearing first in the MLNs [51] (Figure 2). This delay is highly significant when compared to lung infection by other intracellular pathogens such as influenza or *Legionella pneumophila,* where adaptive responses are seen as early as 2-3 days in the MLN [52, 53] and in the lung 6-7 days postinfection [18, 54, 55]. This may suggest that such delay is due to insufficient bacteria or bacterial products in the lymph node required for T-cell priming [40]. Much controversy surrounds the precise arrival of *M.tb* to the MLN as some groups have identified *M.tb* in the MLN as early as 4 hrs postinfection, while others cannot detect *M.tb* for 7–9 days postinfection [40, 56]. Nevertheless, it is now well established that viable *M.tb* resides within the MLN several days prior to the emergence of the effector T-cells [23, 40]. We also detected viable mycobacteria in the MLN within a few days post-infection [unpublished data]. As *M.tb* is slowgrowing, it is possible that the antigenic threshold may not be reached in the MLN until days after mycobacterial arrival. Alternatively, the delayed T-cell priming could be due to insufficient APC activation and/or active suppression by *M.tb* of T-cell priming. Indeed, considered central to this delay, *M.tb* directly infects DCs, impairing both their capacity to migrate to the lymph node and activate naïve T-cells [23, 57].

3.1. Migration of DCs to the MLN. The ability of antigen-loaded DCs to home to the MLN is largely due to the upregulated expression of chemokine receptor CCR7. CCR7 expression allows activated DCs to rapidly migrate towards the CCL19/21 chemokine gradients generated by the MLN [39]. Khader et al. [58] have demonstrated the dependence of IL-12p40 for the expression of CCR7 by *M.tb*-infected DCs. To this end, impairing the production of IL-12p40 was found to delay the migration of infected DCs to the MLN, thus deferring T-cell priming by several days [58]. Furthermore, it has also been demonstrated that *M.tb* directly induces a splice variant of the IL-12 receptor, significantly enhancing the responsiveness of infected DCs to IL-12p40, augmenting the migratory capacity of these populations [59]. It is proposed that *M.tb* may utilize the induction of this high efficacy receptor to facilitate its dissemination away from the lung in the manner similar to a Trojan horse [59]. These data suggest that that the delay in T-cell priming is a result of impaired DC functionality in the lymph node rather than the impaired migration of DCs into this compartment [60, 61].

3.2. Migrating APC Subsets. While the intraepithelial DC may be the primary APC sampling mycobacteria or mycobacterial antigen in the airway lumen, it has been proposed that the AM may egress from the airway lumen into the interstitium, accessing the collecting lymphatics and transporting *M.tb* to the MLN [49]. Within the lung, it is difficult to appropriately classify the APC populations based on a single cell surface marker. To this end, the expression of conventional markers such as CD11c must be used in conjunction with other makers such as MHC class II expression to denote activated DC populations [39, 62]. In recent years, there has been a movement to classify the migratory potential of specific DC cell populations in the lung based on cell surface marker expression. While far from resolved, two distinct DC populations have been identified based on their potential to migrate to the MLN. The expression of $CD103^{+}$ ($CD11c^{hi}$

CD11b$^-$ MHC-II^{hi} CD103$^+$) has recently been shown to be important in transporting apoptotic bodies and mediating Ag cross-presentation to CD8$^+$ T-cells during many viral infections [63]. The expression of CD11b$^+$(CD11c^{hi} CD11b^{hi} MHC-II^{hi} CD103$^-$) has been shown to be key to the delivery of the majority of viable mycobacteria to the MLN [23]. From our preliminary studies, we have seen a surge in both DC subsets in the MLN following pulmonary mycobacterial infection [unpublished data]. As described above, complement represents one of the major mechanisms responsible for the uptake of mycobacteria by recruited APC populations. As such, it can be considered that the expression of both CD11b and CD11c, components of complement receptors 3 and 4, respectively, may allow for more efficient uptake of mycobacteria by these APC subsets. An enhanced capacity to uptake mycobacteria may provide a plausible, yet unconfirmed, explanation for why CD11c+ CD11b+ DCs represent the dominant APC population during *M.tb* infection. The relevance of these DC populations with regard to the efficiency of antigen presentation and subsequent T-cell priming is still currently unknown in the context of *M.tb* infection. Furthermore, it remains to be understood whether some of the T-cell-priming APCs in the MLN are actually AMs, the AMs that have differentiated into DCs, or *M.tb*-loaded neutrophils.

3.3. Passive Transport of M.tb to the MLN. In addition to the active transport of *M.tb*/*M.tb* antigen to the MLN by migratory DC or AM populations, it has been suggested that the passive transport of antigen could be accomplished via the lymphatic drainage of the lung. Whether the *M.tb* organism actively utilizes this system to mediate its "cell-free" dissemination from the lung to the MLN is unknown. It remains plausible that discrepancy in the time of bacterial arrival to the MLN and the time of T-cell priming could be attributed to cell-free transport of *M.tb*. Regardless of how *M.tb* arrives in the lymph node, the appropriate activation of naïve T-cells depends on the interaction between the antigen-loaded DCs and their cognate naïve T-cell. Critical to this interaction is the expression of sufficient levels of costimulatory molecules, a high density of MHC loaded with the cognate antigen, and the production of polarizing cytokines. The inflammatory microenvironment during the acquisition of antigen plays an integral role in the maturation of DC populations and subsequent T-cell priming.

3.4. Mechanisms of Delayed T-Cell Priming. It has long been recognized that *M.tb* utilizes the induction of IL-10 as a means to suppress effector cell function. Specifically, it has been demonstrated that infected macrophage and DC populations can produce high levels of IL-10 in response to live, but not heat-killed, *M.tb* [64, 65]. It has been demonstrated that upon infection, *M.tb* employs multiple secreted virulence factors to subvert host recognition, many of which actively impair antigen processing and loading, and the surface expression of MHC class II [66–68]. Most notably, the 19 kDa protein secreted by *M.tb* has been shown to inhibit the activation of several genes involved in antigen presentation, including the downregulation of MHC class II, HLA-DM, and CIITA [66, 67, 69]. In addition to impairing antigen processing, *M.tb*'s major cell wall component, cord factor (trehalose 6,6′-dimycolate) has been shown to significantly impair the upregulation and appropriate expression of costimulatory molecules such as CD28 [70]. Together, these impairments are thought to alter or delay T-cell priming [71]. Furthermore, the expression of high levels of IL-10 results in the preferential induction of an early T regulatory cell population that serves to delay the initiation of protective type 1 immune responses [72].

While virulence has long been considered an underlying mechanism for the relative delay in T-cell priming, the evidence from others and us suggests that this delay is independent of the relative virulence of the mycobacterium itself, as delayed T-cell priming has also been observed following infection with attenuated strains of *M.tb* or BCG [40, 73]. Rather, it is possible that delayed T-cell priming is due to factors that are inherent to slow-growing mycobacterial species. Many species of pathogenic and nonpathogenic mycobacteria exist in nature. It has been observed that "pathogenic" *mycobacterial* spp., such as *M.tb*, BCG, and *M. avium,* replicate slowly, lead to delayed immune activation, and are capable of persistent disease. On the other hand, "nonpathogenic" *mycobacterial* spp., such as *M. smegmatis* or *M. fortuitum,* replicate quickly, evoke a fast T-cell response, and are rapidly cleared [74, 75]. Slow-growing *mycobacteria* such as *M.tb* have developed many strategies to remain immunologically inert, fundamental to which are unique modifications to its cell wall [12, 76]. Compared with nonpathogenic mycobacteria, the capping of lipoarabinomannan (LAM), a key cell wall component, is unique. Following further examination, it was revealed that the cell wall of all pathogenic, slow-growing mycobacteria contained mannose capped LAM (manLAM). Further, it was shown that all nonpathogenic, fast-growing mycobacterial cell walls contained uncapped or phosphor-*myo*-inositol-(PI-) capped LAM [75, 77–81]. The mannose capping of LAM has been shown to facilitate many immunological events, including phagocytosis by the AM [20], impaired cytokine and chemokine production [27], delayed phagolysosome fusion [82], and suppressed DC activation [83]. It is now commonly accepted that manLAM is highly immunosuppressive, while uncapped or PI-capped LAM is strongly immunogenic [75, 80, 84]. While it represents an interesting postulate, the role that immunesuppressive manLAM plays in delayed T-cell priming following *M.tb* infection remains unknown.

The ability of *M.tb* to survive in the cell relies heavily on its unique ability to subvert the innate and adaptive immune systems. Its unique cell wall structure composed of lipids and glycoproteins mediates its survival in the phagosome, primarily through arresting fusion with the lysosome. One of the major components of the cell wall is mannose-capped LAM which is thought to be critical to arresting phagolysosome fusion [85, 86]. The ability of LAM to arrest phagosome fusion relies on its ability to prevent the phosphorylation of phosphatidylinositol 3-phosphate (PI3P), a required step in the conversion of an early phagosome to

a late phagosome [85]. The ability of LAM to prevent the phosphorylation of PI3P is mainly attributed to its ability to prevent the cellular influx of Ca^{2+}, a required step in the activation of phosphatidylinositol kinase (PI3K) [85]. In addition to LAM, the activation of PI3P is further prevented by SapM, a secreted PI3P-phosphatase, further ensuring that the phagosome is arrested at the early stage [85]. In addition to preventing phagosome maturation, *M.tb* encodes a number of proteins directed at survival in an activated phagolysosome. The ability to combat reactive nitrogen intermediates and reactive oxygen species is critical to *M.tb*'s survival following the induction of adaptive immunity and correlates with strain virulence [86, 87]. *M.tb* encodes two superoxide dismutases, sodA and sodC, which catalyse the conversion of superoxide anions to H_2O_2, and a catalase-peroxidase katG to combat the increased levels of H_2O_2 [87]. Furthermore, *M.tb* encodes a combined NADH-dependent peroxidase and peroxynitritesreductases which is composed of four protein components; an alkyhydroperoxidereductase, a thioredoxin-related oxidoreductase, a dihydrolipoamideacyltransferase, and a lipoamide dehydrogenase [87]. These four components function to detoxify both RNI and ROS and protect *M.tb* from the harsh environment of an activated phagolysosome, limiting the availability of *M.tb* antigen [87]. The ability of *M.tb* to survive within the APC, coupled with its slow replication rate, functions to limit the amount of antigen available to prime required T-cell responses. To support this notion, we have noted that a 10-fold increase in the infectious dose of mycobacteria could enhance the arrival of bacteria to the MLN and accelerate T-cell priming [unpublished data], indicating that the antigen load in the lung may be responsible for the delayed T-cell priming seen in the MLN.

Alterations to the inflammatory microenvironment can significantly impair the ability of responding DCs to appropriately initiate adaptive immune responses that are central to the control of *M.tb* infection. The ability of *M.tb* to live intracellularly shields it from the host's humoral response. Thus, controlling bacterial dissemination and curtailing its replication is largely the responsibility of activated T-cells subsets. Owing to the intracellular and intraphagosomal nature of *M.tb*, antigen loading is primarily through the MHC class II pathway. As such, the dominant T-cell subset induced is that of a $CD4^+$ IFN-γ^+. Additionally, mycobacterial antigen is loaded on the MHC class I pathway by either cross-presentation mediated by the uptake of apoptotic bodies or phagosomal escape, allowing for the priming of antigen-specific CD8 T-cell [88–91]. Studies using MHC class II and class I deficient mice have demonstrated the relative contribution of CD4 and CD8 T-cells to *M.tb* immunity. While a deficiency in MHC class I has a limited impairment on bacterial control, deficiency in MHC class II results in extensive impairment, signifying the relatively greater importance of CD4 T-cells [90]. Given the central role of IFN-γ in macrophage activation and nitric oxide production, a greater impairment is seen in iNOS-deficient mice then in either MHC class I or II deficient mice, thus indicating that IFN-γ from both type I CD4 and CD8 T-cells plays a critical role in protection [90]. While a type 1 immune response eventually ensues, the delayed T-cell priming by early immune evasion strategies employed by *M.tb* provides a critical window for *M.tb* to grow completely unchecked in the lung.

4. Effector T-Cell Recruitment to the Lungs

As expected, delayed T-cell priming in the lymph nodes of *M.tb*-infected animals results in delayed arrival of effector T-cells at the lung, the primary site of infection (Figure 2). This permits *M.tb* to increase logarithmically within the lungs of the host for approximately 20 days, thus establishing a robust "foothold" prior to the arrival and abundant presence of antigen-specific T-cells at the site of infection [92–94]. The mass arrival of T-cells to the lungs occurring between 18 and 20 days postinfection is associated with the ultimate plateau of bacterial growth [40, 92, 94, 95].

4.1. Recruitment of T-Cells to the Different Lung Compartments. The lung can be divided into two main compartments: the interstitial tissue existing between the alveoli, and the mucosal surface of the lung known as the airway lumen (Figure 2). While the timing of T-cell priming in the MLN ultimately determines the kinetics of effector T-cell migration to the infected lung, there is growing evidence to suggest that the coordinated upregulation of several molecules is essential to the homing of T-cells to the lung. Specifically, the most recent focus has been on the kinetics of chemokine production as well as the coordinated upregulation of specific adhesion molecules. Both the expression of integrins on T-cells and their respective addressin molecules on the vascular endothelium and alveolar epithelium are essential to appropriate recruitment of effector T-cells to lung. The differential upregulation of these molecules dictates whether a T-cell traffics to the lung interstitium or airway lumen and can dramatically effect bacterial control. While it is believed that T-cell trafficking to the airway lumen is a required process for the control of *M.tb* [96], little work has been done to understand the differential inflammatory signals required to recruit T-cells into the airway lumen. Based on this, most studies have focused on the molecules required for recruiting T-cells into the lung as a whole rather than the differential lung compartments.

4.2. Critical Chemotactic Molecules. It has been demonstrated that CCL5 (RANTES) dramatically increases between day 10 and day 20 post-*M.tb* infection [97]. However, the specific role of this chemokine in T-cell homing to the lungs has only recently been elucidated. Through the use of CCL5 knockout (KO) mice, Vesosky et al. [98] have shown that CCL5 is critically required for the early recruitment of CCR5-expressing CD4 T-cells to the lung in *M.tb*-infected mice. The delay in effector T-cell recruitment in CCL5 KO mice caused a significant reduction in IFN-γ production and impaired granuloma formation, resulting in significantly higher bacterial burden within the lungs of these animals when compared to wild type controls [98].

Best known for their critical role in DC homing to the MLN, CCL19 (MIP-3β), and CCL21 have been recently shown to be essential in the trafficking of IFN-γ^+ T-cells from the MLN to the lungs of *M.tb*, infected mice. In the study conducted by Khader [99], CCL19 was shown to increase in the lungs of infected mice between 15 and 18 days, correlating with the arrival of effector T-cells and the initiation of granuloma formation. Mice deficient in CCL19 and 21 showed significantly impaired $CD4^+$ IFN-γ^+ T-cell kinetics to the lungs prior to day 30 post-infection [99]. The blunted T-cell recruitment in the lungs of CCL19/CCL21-deficient mice resulted in delayed IFN-γ and iNOS production, macrophage activation, and bacterial control [99]. This leads to severely impaired granuloma formation and increased bacterial loads for at least 80 days postinfection, indicating the critical role of timely T-cell trafficking to the lung [99].

4.3. Adhesion Molecules. In addition to chemokine expression within the lung, several studies have focused on identifying the required adhesion molecules and specific integrins which mediate the entry of effector T-cells into the lung. Vascular cell adhesion molecule 1 (VCAM-1) expression is upregulated in *M.tb* infected lungs by day 21 and is associated with the recruitment of the majority of IFN-γ-producing T-cells [100]. The preferential expression of $\alpha_4\beta_1$ or $\alpha_4\beta_7$ on activated CD4 T-cells makes VCAM-1 essential to efficient recruitment of T-cells into lung [100]. Furthermore, depletion of either α_4 or $\alpha_4\beta_7$ results in a significant decrease in the number of lymphocytes within the lung, the consequence of which manifests in granulocyte-dominated granulomas consisting of disorganized infiltrates and heightened necrosis [100]. Similar defects in granuloma formation were seen in the lungs of mice deficient CD11a/18, where a 3-4 fold reduction in the number of antigen-specific T-cells recruited resulted in increased neutrophilia, necrotic foci, and poorly formed granulomas [101]. It is therefore apparent that the timing of effector T-cell trafficking into the lungs following *M.tb* infection is critical to the establishment of granuloma formation as well as appropriate bacterial control.

5. Effector Functions of Recruited T-Cells in the Lung

Upon arrival in the lung, effector T-cells are thought to 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 cleared.

5.1. Macrophage Activation. The airway lumen is largely considered the primary site of infection. With effector T-cells being recruited to the airway lumen, T-cell-derived IFN-γ activates infected AMs to mediate enhanced phagosome lysosome maturation, upregulation of MHC class II loading, and the induction of highly toxic antimicrobial substances. 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 [2, 102–105]. Following IFN-γ mediated activation, the infected macrophage generates both reactive oxygen substances (ROSs) and reactive nitrogen intermediates (RNIs) [106, 107]. 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 antimycobacterial action is through the generation of RNI, specifically nitric oxide (NO) by the inducible nitric oxide system iNOS-NOS2 [106, 107]. Limited resistance to RNIs is a common feature of mycobacteria, with the most virulent strains such as *M.tb* and *M.bovis* being almost completely resistant [106, 107]. Although sterile clearance is never achieved, the activation of an infected macrophages is thought to be strongly bacteriostatic, facilitating the persistence of *M.tb* within the host [107]. The key role of IFN-γ in this process is without question and was conclusively shown with murine IFN-γ-deficiency models [108]. In the absence of IFN-γ, mice fail to upregulate NOS2 and are unable to control bacterial dissemination, succumbing to the infection within the first few weeks [108]. Despite the unprecedented role of IFN-γ, it cannot function alone. IL-12 was shown to be essential to the optimal induction of both NO and TNF [109]. Other studies have demonstrated an essential role for TNF and it is now believed that IL-12, IFN-γ, and TNF must be present for optimal NO production [103, 105].

5.2. Granuloma Formation. The induction of what is termed the "immune" granuloma is the hallmark of immune mediated control and is thought to represent the primary mechanism of long-term protection. The formation of the "immune" granuloma is a very operose process, which follows the arrival of the adaptive immune cells to the lung, an event not normally seen until 2-3 weeks postinfection as aforementioned [60]. The "immune" granuloma induces a number of defined histopathological changes to the innate granuloma structure. The innate granuloma is further fortified by the arrival of effector T-cells, and the ability of infected macrophages to kill internalized bacteria is enhanced by the release of IFN-γ [99]. The addition of effector T-cells to the granuloma produces what is termed the lymphocytic cuff, where entering lymphocytes surround the infected macrophage populations forming a barrier, adjoining with the focal infection [2, 89, 104, 110–112]. The formation of the lymphocytic cuff and the ensuing production of inflammatory mediators results in defined structural changes to the partitioned macrophage populations. Two major morphological changes occur in the infected macrophage populations, within the type I granuloma: first, the induction of an epithelial-like appearance, producing epithelioid macrophages, and second, the fusion of macrophage populations to form multinucleated giant cell populations [2, 89, 104, 110–112]. The arrival of effector

T-cells and the formation of an "immune" granuloma correlates with the cessation of bacterial growth and a plateau is reached [2, 89, 104, 110–112]. Type 1 cytokines including IL-12, IFNγ, and TNFα required for macrophage activation are also essential to granuloma formation [113] or the maintenance of granuloma [114].

5.3. Granuloma as a Symbiotic Microenvironment for Mycobacterial Persistence. Recently, many groups have begun to compartmentalize the granuloma away from the lung parenchyma and airway lumen. Mounting evidence from us and others suggests that the granuloma represents an immunologically suppressed or dampened zone that the mycobacterium prefers to dwell in [115–117]. The immunological suppression of the granuloma was determined to be due to high levels of IL-10, which functioned to suppress both APC and T-cell populations within the granuloma [116]. Granuloma-derived IL-10 suppresses many effector functions of the resident APC populations. Within the granuloma, APCs show an impaired ability to phagocytose mycobacteria, drive T-cell priming, and produce nitric oxide [116]. Granuloma resident T-cell populations are also phenotypically distinct, having a regulatory-like function and producing high levels of IL-10 and low levels of IFN-γ [116]. Removing IL-10 from the model was shown to reverse the suppression on the APC and T-cell populations in the granuloma and overall better bacterial control was achieved [116]. Thus, the renewed knowledge of mycobacterial granuloma biology helps us further understand why the host immune system can hardly eliminate infection and often the latency is the best outcome.

6. Role of Airway Luminal T-Cells (ALTs) in Anti-TB Immunity

Primary mycobacterial infection studies have demonstrated that the delayed effector response in the lung is a direct result of retarded T-cell priming in the MLN. As described above, the lung can be divided into two main compartments: the interstitial tissue residing in between the alveoli, and the mucosal surface of the lung known as the airway lumen. Although the current literature describes the kinetics of effector T-cell responses in the whole lung, the role of ALTs in the airway lumen have been largely neglected to date [96, 118]. Recently, our group and others have begun to characterize the T-cell kinetics in these two lung tissue compartments. Despite the earlier arrival of effector T-cells in the lung interstitium, following *M.tb* infection, it is the arrival of ALTs to the airway lumen that is associated with the plateau of bacteria replication [119], (Figure 2). Inspired by the plethora of TB vaccine studies understanding the role of T-cells in the differential lung compartments, this is the first study to characterize the T-cell responses in both the lung interstitium and airway lumen following a primary challenge.

6.1. Mucosal versus Parenteral Vaccination Against M.tb . Over the past decade, the development of novel TB vaccine candidates has produced a wealth of knowledge on the ways in which TB vaccination can be improved. Arguably the most significant finding has been the repeated observation that vaccinating mucosally provides enhanced protection over parenteral immunization against pulmonary *M.tb* infection. In particular, work from our laboratory has demonstrated that a recombinant adenoviral vector expressing *M.tb* antigen 85a (AdAg85a) when delivered intranasally was seen to provide remarkably enhanced protection against virulent *M.tb* challenge compared to intramuscular administration [120]. Furthermore, intranasal vaccination was able to provide superior protection over the "Gold Standard" subcutaneous BCG immunization, with at least an additional log reduction in bacterial burden following *M.tb* infection [120]. However, the AdAg85a work does not stand alone, as several others have reported that mucosal immunization provides greater protection against *M.tb* infection compared to parenteral vaccination [121, 122].

6.2. Requirement of ALTs. Investigation into the mechanism by which this enhanced protection is achieved revealed that mucosal but not parental vaccination with AdAg85a resulted in the generation of a population of antigen-specific T-cells that reside within the airway lumen [123]. The failure of parenteral vaccination to generate a population of ALTs can be largely attributed to the lack of an inflammatory response generated in the airway [123]. This defect is particularly evident in experiments where the delivery of soluble mycobacterial antigens to the airway elicits a potent inflammatory response characterized by heightened levels of TNF, MIP-1α, MCP-1, and IP-10 [124, 125]. The production of these inflammatory mediators functions to draw in peripherally primed T-cells, enhancing protection to a level comparable to that of mucosally vaccinated animals [124, 125]. The neutralization of IP-10 or MIP-1α at the time of soluble protein delivery significantly impaired the recruitment of peripherally primed T-cells into the airway, impairing protection, thereby demonstrating the critical role of these chemokines in populating this compartment [124, 125]. Specifically, ALTs were found to be critical for protection as they are capable of responding quickly upon *M.tb* infection, eliminating the delay of effector responses [124, 125]. These findings indicate that both the timing and geographic localization of T-cell responses is critical to the efficiency of bacterial control. 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 insight into the mechanisms of delayed or impaired T-cell trafficking to the lung, and thus provide ways by which protection against *M.tb* can be enhanced.

7. Concluding Remarks

Understanding the mechanisms of delayed T-cell priming in the MLN and delayed effector T-cell recruitment to the lung interstitium and airway lumen following pulmonary *M.tb*

infection is critical for us to develop effective anti-TB vaccine and therapeutic strategies. The current vaccine initiatives are significantly hampered by our limited knowledge of what a protective immune correlate looks like as following infection *M.tb is rarely cleared*. Although much progress has been made in understanding the kinetics of T-cell priming following *M.tb*, the mechanisms underlying this delay are only beginning to be understood. Given the intimate relationship between the host and bacteria, it is of paramount importance for us to further dissect the differential contributions of both the host and pathogen to the relative delay in T-cell priming and recruitment, and their impact on bacterial control.

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APPENDIX 2

Within the enemy's camp: contribution of the granuloma to the dissemination, persistence and transmission of *Mycobacterium tuberculosis*

Christopher R. Shaler, Carly N. Horvath, Mangalakumari Jeyanathan, and Zhou Xing

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Within the Enemy's Camp: contribution of the granuloma to the dissemination, persistence and transmission of *Mycobacterium tuberculosis*

Christopher R. Shaler, Carly N. Horvath, Mangalakumari Jeyanathan and Zhou Xing*

McMaster Immunology Research Centre, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada

Edited by:
Stephen Wesley Chensue, University of Michigan, USA

Reviewed by:
Junji Yodoi, Kyoto University, Japan
Kottarappat Dileepan, The University of Kansas Medical Center, USA

****Correspondence:***
Zhou Xing, McMaster Immunology Research Centre, Department of Pathology and Molecular Medicine, McMaster University, MDCL-Room 4012, 1280 Main Street West, Hamilton, ON, Canada L8S 4K1.
e-mail: xingz@mcmaster.ca

Pulmonary tuberculosis, caused by *Mycobacterium tuberculosis (M.tb)* represents a leading global health concern, with 8.7 million newly emerging cases, and 1.4 million reported deaths annually. Despite an estimated one third of the world's population being infected, relatively few infected individuals ever develop active clinical disease. The ability of the host to remain latently infected while preventing disease is thought to be due to the generation of a robust type 1 immune response in the lung, capable of controlling, but not clearing, *M.tb*. A key feature of the type 1 immune response to *M.tb* is the formation of immune cellular aggregates termed granuloma. The granuloma structure has long been considered a hallmark of host's protective response toward *M.tb*. Historically, a correlative relationship between granuloma formation/maintenance and bacterial control has been seen in models where disrupted granuloma formation or structure was found to be fatal. Despite this established relationship much about the granuloma's role in *M.tb* immunity remains unknown. Recent publications suggest that the granuloma actually aids the persistence of *M.tb* and that the development of a necrotic granuloma is essential to person-to-person transmission. Our group and others have recently demonstrated that enclosed within the granuloma is a population of immunologically altered antigen-presenting cells and T lymphocyte populations. Of note, the ability of these populations to produce type 1 cytokines such as interferon-gamma, and bactericidal products including nitric oxide, are significantly reduced, while remaining competent to produce high levels immunosuppressive interleukin-10. These observations indicate that although the chronic granuloma represents a highly unique environment, it is more similar to that of a tumor than an active site of bacterial control. In this review we will explore what is known about this unique environment and its contribution to the persistence of *M.tb*.

Keywords: tuberculosis, immunopathology, immune regulation, granuloma, bacterial persistence, mycobacteria

INTRODUCTION

Pulmonary tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis (M.tb)* is the leading cause of death due to a bacterial pathogen and is responsible for 1.4 million deaths annually, latently infecting one third of the world's population (WHO, 2012). Despite the magnitude of individuals infected, the rate of mortality is relatively low with approximately 90% infected individuals controlling, but not clearing, *M.tb* (WHO, 2012). The ability of the host to "control" *M.tb* infection encompasses a number of immunological processes designed to restrain bacterial dissemination and persistence, and reduce person-to-person transmission. The classical hallmark of anti-TB host defense is the formation of type 1 immune granuloma in the lung. Historically, the granuloma has been perceived as essential to anti-TB host defense as the host is incapable of sterile clearance and thus is forced to segregate the infected cells as a means to preserve itself. However, experimentally little is known of the role of the granuloma in bacterial control.

THE HISTORICAL VIEW OF MYCOBACTERIAL GRANULOMA

First described in 1679, pathologists discovered unique structures in the lungs of TB patients (de le Boe, 1679; reviewed in Ramakrishnan, 2012). These structures were then termed tubercles and represent what we now know as granulomas. Commonly, it was observed that persons who had died of TB had a large number of these distinct pathological lesions, and the presence of tubercles became an associated hallmark of active TB disease. It was not until 1884 that tubercles were also characterized in individuals who had died from diseases other than TB. Upon post-mortem examination, a number of these individuals had lung lesions (granulomas) containing live TB bacilli, giving the first indication that TB latency may relate to the formation of granuloma (Dejerine, 1884). However, upon further microbiological examination, it was revealed that live bacilli persisted not only within the granuloma itself, but also in the surrounding lung tissue, albeit to a lesser degree (Wang, 1916; Opie and Aronson, 1927; Robertson, 1933; Feldman and Baggenstoss, 1938, 1939). It was around this time that the

protective view of the granuloma began to gain public acceptance and it was proposed that the recruitment of activated lymphocytes and the formation of a lymphocytic cuff served to wall-off infected macrophages as a means of limiting dissemination. However, the role of granuloma in TB has remained enigmatic largely because of the unavailability of reliable animal models and appropriate techniques to observe the dynamic process of granuloma evolution. Although different experimental models (mice, guinea pig, rabbit, cattle, and macaque) have been developed, only cattle and macaque monkeys form the type of granuloma that closely resembles those seen in humans (Capuano et al., 2003; Flynn et al., 2003; Tsai et al., 2006; Hunter et al., 2007; Via et al., 2008). Despite being extensively used as a model of TB, the murine granuloma lacks many of the unique characteristic features of the human granuloma including centralized necrosis, giant multinuclear cells, and a defined "lymphocytic cuff" (Rhoades et al., 1997). Contrasting the classical notion of its protective role, a number of recent studies have demonstrated unaltered bacterial control even in the absence of granuloma formation, strongly arguing against the granuloma being essential to bacterial restriction (Johnson et al., 1998; Scott and Flynn, 2002; Pearl et al., 2004). Moreover, it is now known that the *Mycobacterium* can significantly alter the immune environment of the granuloma as means to facilitate its persistence (Ly et al., 2007; Scott-Browne et al., 2007; Marino et al., 2010; Castano et al., 2011; O'leary et al., 2011). Regardless, the common perception remains that the granuloma serves to limit bacterial growth and prevent dissemination by segregating infected cells, and the role of the granuloma in *M.tb* infection remains an issue of continued debate. In this review we will challenge the traditional view of the function of granuloma, exploring what is known about its progression and maturation, and how this unique environment may in fact contribute to the persistence and transmission of *M.tb.*

FORMATION OF THE TYPE 1 IMMUNE GRANULOMA DURING *M.tb* INFECTION

The formation of granuloma is a dynamic process that begins shortly after infection and continuously evolves over time. Temporally, the granuloma can be divided into three distinct phases: (1) the "innate granuloma," a loose aggregate composed primarily of recruited macrophages and neutrophils; (2) the "immune granuloma" formed following the emergence of antigen-specific T cells; and (3) the "chronic granuloma," resulting from distinct morphological changes in granuloma structure (**Figure 1**).

FORMATION OF THE "INNATE GRANULOMA"

Shortly after aerosol exposure, *M.tb* infects the resident alveolar macrophage (AM) initiating the early inflammatory response. While amplifying the host immune response, the recruitment of innate immune cells inadvertently provides a large number of new targets for *M.tb* to infect and is thought to contribute to the early dissemination of *M.tb* (Doenhoff, 1997; Davis and Ramakrishnan, 2009). Augmenting this problem, the infected AM is unable to kill internalized mycobacteria due to impaired phagolysosome fusion, a process essential to the destruction of the phagocytosed bacteria (Welin et al., 2011). The efficiency by which a *Mycobacterium* species arrests phagolysosome fusion is directly attributable to its relative virulence, with highly virulent strains such as *M.tb* almost completely inhibiting fusion (Ferrer et al., 2010). Incapable of killing internalized *M.tb*, infected macrophages secrete an array of pro-inflammatory and chemoattractant cytokines including tumor necrosis factor (TNF), interleukin (IL)-6, and IL-8 which facilitate the recruitment of new macrophages and granulocytes to the site of infection and lead to the formation of the "innate granuloma" (Birkness et al., 2007). This initial recruitment is essential to establishing the macrophage-dominated center of the "immune" granuloma.

CONTRIBUTION OF *M.tb* TO THE FORMATION OF THE "INNATE GRANULOMA"

Historically the formation of granuloma has been considered to be a host-mediated event. Using an *M. marinum* model, real-time microscopic visualization has challenged this notion, revealing that virulent *Mycobacterium* drives the nascent formation of the early granuloma. A number of elegant studies conducted by Ramakrishnan's group have demonstrated the unique interplay between the *Mycobacterium* and the host immune system in the early stage of granuloma formation. To this end, the early release of 6 kDa early secretory antigenic target (ESAT-6) by *M. marinum* led to the activation of the epithelium, which facilitated the recruitment of macrophages to the site of infection through inducing the production of matrix metalloproteinase-9 (MMP-9) (Davis and Ramakrishnan, 2009; Volkman et al., 2010). In murine models, *M.tb* was also found to drive MMP-9 expression. Thus either broad MMP inhibition or MMP-9-specific depletion delayed granuloma formation, resulting in impaired macrophage recruitment to the site of infection and reduced granuloma size (Taylor et al., 2006). These findings indicate that *M.tb* may actually promote granuloma formation and utilizes the structure for its own benefit. Furthermore, it was demonstrated that virulent mycobacteria can utilize the innate granuloma as means of recruiting target cells allowing for the early dissemination of mycobacteria throughout the host (Davis and Ramakrishnan, 2009). This view is supported further by the observation that the early granuloma is not a static environment and there is a significant movement of antigen-presenting cell (APC) populations into and out of the early granuloma (Chiu et al., 2004; Egen et al., 2008, 2011; Schreiber et al., 2011).

FORMATION OF THE "IMMUNE GRANULOMA"

Following innate activation, dendritic cells are recruited to the lung and transport mycobacteria or mycobacterial antigens to the mediastinal lymph node (MLN). Within the MLN antigen-loaded APCs activate antigen-specific T cells. Due to the nature of *M.tb* infection, the majority of bacilli and antigen reside within an endosome, and are most efficiently loaded onto major histocompatibility complex (MHC) class II (Mogues et al., 2001; Anis et al., 2008; Yahagi et al., 2010). The loading of MHC class II facilitates the priming of Th1 interferon-gamma (IFN-γ)-secreting T cells, which rapidly home to the lung. While the dominant subset of T cells are $CD4^+$, cross-presentation also allows for the strong induction of $CD8^+$ T cells, collectively generating a type 1 polarized adaptive immune response (Winau et al., 2006). Although outside the scope of this review, it should be noted that there is a substantial lag period between *M.tb* infection and the initiation of antigen-specific T cell responses (reviewed in Shaler et al., 2012).

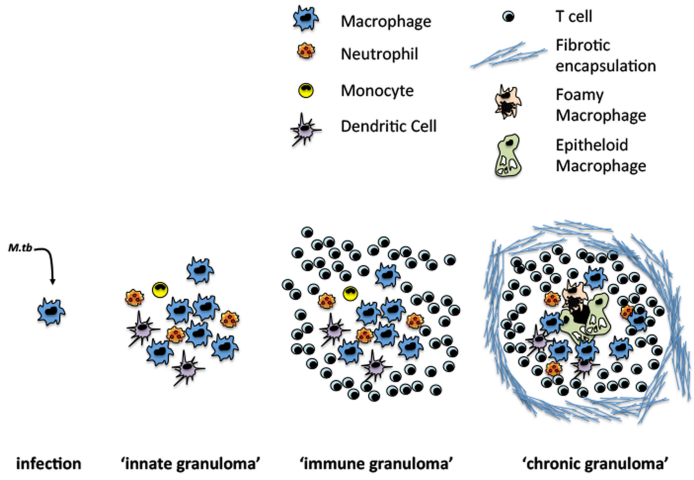

FIGURE 1 | Evolution of TB granuloma formation. Following infection sentinel macrophages cells facilitate *M.tb* uptake and the recruitment of other innate and adaptive immune cells.

The continuous production of chemokines by infected lung APCs efficiently recruits newly primed T cells into the lung. Once in the lung, recruited T cells surround and wall-off infected macrophages, activate them for enhanced bactericidal function, and physically limit their mobility to restrain bacterial dissemination. Indeed, the arrival of effector T cells and the establishment of the classical "immune granuloma" is associated with a plateau in bacterial growth (Mogues et al., 2001). While the prevailing immune response generated following *M.tb* infection is highly similar between the mouse and man, the structural formation of the "immune granuloma" differs significantly. In mice, many of the hallmark features of the human granuloma are missing, and thus the knowledge from murine granuloma research should be interpreted with caution. Despite these limitations, animal models have provided significant insight, and have been invaluable in delineating the stages of granuloma formation.

THE CONTROVERSIAL REQUIREMENT OF THE "IMMUNE GRANULOMA" IN LIMITING BACTERIAL DISSEMINATION

In spite of the traditional protective view of granuloma, it has recently been revealed that the immune environment within granuloma is more conducive to *M.tb* persistence than its elimination. Despite the fact that animal models do not accurately replicate human granuloma structures, several murine studies have provided invaluable insight into the role of granuloma in preventing *M.tb* dissemination control. Early studies demonstrating the role of critical cytokines such as TNF and IFN-γ perpetuated the notion that the granuloma was essential for bacterial segregation and limiting bacterial growth in the lung as the absence of either cytokine led to ill-formed granuloma and increased bacterial infection (Flynn et al., 1998; Kaneko et al., 1999; Algood et al., 2005; Beham et al., 2011; Gallegos et al., 2011). Moreover, any loss of $CD4^+$ T cell functionality results in a loss of the granuloma structure and extensive bacterial dissemination is seen in both man and mouse. While essential to the control of *M.tb*, the role of the $CD4^+$ T cell in the granuloma's structure is somewhat species-dependent. Specifically, in humans, T cells surround and wall-off infected macrophages, and do not infiltrate the granuloma, but rather form a defined lymphocytic cuff. Conversely, murine $CD4^+$ T cells associate directly with infected cells infiltrating throughout the granuloma forming lymphocytic aggregates or pseudo-granulomas (Mogues et al., 2001; Tsai et al., 2006; Hunter et al., 2007; Via et al., 2008; Gallegos et al., 2011; Geldmacher et al., 2012). Nevertheless, while the loss of CD4 T cell-mediated immunity is detrimental to the host it is impossible to separate the relative contribution of the two processes to the impaired bacterial control: loss of Th1-mediated immunity and loss of granuloma structure, these studies suggest a critical role for the granuloma in preventing bacterial dissemination (Saunders et al., 2002; Segovia-Juarez et al., 2004). The essential role of CD4 T cells to the control of mycobacterial growth has largely been attributed to their potent IFN-γ production and subsequent macrophage activation. While macrophage activation is essential to the control of *M.tb* in mouse and man, the role of nitric oxide has been an issue of some debate. Historically, studies have shown that human macrophages do not produce nitric oxide to the same degree *in vitro* as those isolated from mice (Aston et al., 1998). Recently, however, several groups have demonstrated that human macrophages from TB infected patients, as well as human macrophage cells lines are capable of inducing iNOS and producing nitric oxide in response

to *M.tb* antigen (Rich et al., 1997; Jagannath et al., 1998; Dlugovitzky et al., 2000). Indeed, it appears that while the timing of nitric oxide and its role in TB control may differ somewhat between mouse and man, nitric oxide remains important in both species.

Recent studies suggest that the granuloma may be dispensable for preventing bacterial dissemination and may actually contribute to *M.tb* persistence. Moreover, in the absence of intracellular adhesion molecule-1 (ICAM-1), there is also a failure of granuloma formation, and despite this defect, mice are protected for the first 90 days post-infection, with no increase in bacterial growth compared to wildtype mice within this time frame (Johnson et al., 1998). Similarly, zebrafish models have shown that in the absence of early granuloma formation, there is no defect in the ability of the host to limit bacterial replication and dissemination, and that the granuloma may actually facilitate early dissemination (Volkman et al., 2004, 2010). Furthermore, in the absence of IL-27 in mice, there is a substantial defect in the ability of the host to form granuloma in response to *M.tb* infection, and yet infected mice exhibit markedly enhanced bacterial control when compared to their wildtype counterparts (Pearl et al., 2004). Indeed, recent studies indicate that granuloma does not always function to limit bacterial dissemination. For example, C-C chemokine receptor type 2 (CCR2) deficient mice form exaggerated granuloma structures when infected with *M.tb* and paradoxically have a decreased capacity to control bacterial growth (Scott and Flynn, 2002).

While it remains debatable whether the granuloma is required for bacterial control, growing evidence supports the notion that the fate of *M.tb* within the granuloma is situation-dependent. For example, the initial inoculum size may influence the number of macrophages and granulocytes that are recruited to the site of infection. If a large number of cells are initially recruited, spatially it becomes difficult for effector T cells to interact with the infected cells residing at the core of the granuloma limiting their ability to activate these centralized macrophages to kill internalized *M.tb* (Segovia-Juarez et al., 2004). In comparison, a small initial inoculum size infects a small number of cells at the core of granuloma, which may increase the likelihood of interaction of infected cells with effector T cells (Segovia-Juarez et al., 2004). Based on this notion, it is tempting to speculate that a stronger initial innate immune response may perpetuate the infection and limit the host's ability to eliminate *M.tb*. Therefore, a small-size granuloma may favor host defense whereas a relatively large-size counterpart may favor the persistence of mycobacterial bacilli, regardless of the magnitude of T cells generated. The current limitation to diagnostic imaging makes studying the evolution of the granuloma in latently infected humans difficult and as a consequence, little is known about how its structure changes over the course of infection in otherwise healthy individuals.

THE "IMMUNE GRANULOMA": A NICHE FOR BACTERIAL PERSISTENCE

Regardless of whether the granuloma functions to limit bacterial dissemination, much evidence suggests that *M.tb* is especially adept at altering the immune response within the granuloma, creating a uniquely suppressed environment largely through the induction of IL-10 (de Waal Malefyt et al., 1993; Chiu et al., 2007, 2008; Higgins et al., 2009; Marino et al., 2010; Redford et al., 2010; O'leary et al., 2011; Shaler et al., 2011). Functionally, the infected macrophages within the granuloma are altered, showing a reduced capacity to produce bactericidal products such as nitric oxide, while showing enhanced IL-10 production (de Waal Malefyt et al., 1993; Chiu et al., 2007, 2008; Higgins et al., 2009; Marino et al., 2010; Redford et al., 2010; O'leary et al., 2011; Shaler et al., 2011). Interestingly, while the macrophage populations of the granuloma have reduced bactericidal function, they continue to produce large amounts of chemokines facilitating the continuous recruitment APC populations into the granuloma (Schreiber et al., 2010; Shaler et al., 2011). Recent studies utilizing *intravital* microscopy have revealed significant movement of inflammatory APCs both into and out of the granuloma (Schreiber et al., 2011). It is this movement of infected APCs that has been speculated to facilitate the early dissemination of *M.tb*. Likewise, human granuloma contains a high frequency of foxp3+ T regulatory cells (Rahman, 2009). In addition, murine studies have confirmed that T cells residing within the granuloma display a highly altered, and functionally suppressed phenotype. Despite the central role of IL-10 in suppressing T cell and macrophage activation within the granuloma, IL-10 neutralization or infection of IL-10 knockout (KO) mice results in only marginally reduced bacterial loads (de Waal Malefyt et al., 1993; Jacobs et al., 2002; Chiu et al., 2007; Higgins et al., 2009). Given *M.tb's* long evolution with humans, it is not surprising that *M.tb* targets multiple pathways to interrupt the host immune response. Moreover, while conventionally immune suppression would appear to benefit only the pathogen, the induction of IL-10 may actually be a host-mediated event required to limit unwanted immunopathology.

THE "CHRONIC GRANULOMA": A DYNAMIC INTERPLAY BETWEEN PERSISTING *M.tb* AND THE HOST IMMUNE RESPONSE

Following the establishment of the "immune granuloma," a period of immune quiescence is established. It is during this stage that chronic immune activation leads to significant alterations in the morphology and functionality of the granuloma, many of which are not typically seen in mice. Post-mortem studies of latently infected humans and non-human primates have revealed that the evolution of the granuloma structure is highly divergent, not only between individuals, but even within a single individual (Capuano et al., 2003; Flynn et al., 2003; Barry et al., 2009). Typically, within an infected individual, a spectrum of granuloma structures are seen. For instance, both the fully calcified lesions containing no bacilli and the fibrotically encapsulated necrotic granulomas containing large numbers of live bacteria can be seen in the lung of the same individual, indicating that the evolution of the granuloma is a highly dynamic process (Capuano et al., 2003; Flynn et al., 2003; Barry et al., 2009).

While the granuloma has long been believed to be a protective host's response, it is now acknowledged to result from a dynamic and continuous interplay between the host's immune response and persisting *M.tb*. The continuous "battle" between chronic immune activation and bacterial persistence causes infected macrophages to adopt an irregular epithelial-like or "epithelioid" appearance, and to fuse together forming multinucleated giant cells in the core of granuloma (Hunter et al., 2007; Hunter, 2011). Indeed, the virulent *Mycobacterium* has been shown to induce macrophage cell

death frequently throughout the course of infection, which several studies have shown to be a potential mechanism of gaining access to new host macrophages (Davis and Ramakrishnan, 2009). Newly recruited macrophages quickly phagocytose the dead bodies and become saturated with mycobacteria and lipids (Peyron et al., 2008). These lipid-rich macrophages that accumulate within the granuloma are known as foamy macrophages due to their distinct appearance, and are now recognized as a major contributing factor in the persistence of *M.tb* (Peyron et al., 2008). Recent studies have shown that foamy macrophages isolated from humans have lost key functions including their ability to phagocytose and to produce essential bactericidal agents such as nitric oxide (Peyron et al., 2008). Moreover, the polarization of macrophage populations within the granuloma are thought to shift from being a classically activated (M1) population toward that of an alternatively activated (M2), with reduced bactericidal capacity (Redente et al., 2010). Thus, such foamy macrophages have been proposed to function as the reservoirs of *M.tb* whereby the bacterium is able to successfully manipulate the infected macrophage into not only a safe haven but also a source of nutrients required for the synthesis of its cell wall and replication.An infected host typically houses a highly heterogeneous mixture of granuloma types ranging from large necrotic granulomas containing large amounts of bacilli, to completely calcified structures devoid of any detectible bacteria (Turner et al., 2003; Hunter et al., 2007; Hunter, 2011). In the later stages of granuloma evolution, fibrotic encapsulation can be seen in cases of both active and latent infection. Currently, it is unclear whether encapsulation functions to prevent bacterial escape, or to limit immune infiltration into the granuloma. Although complete mycobacterial clearance is rarely seen, latently infected humans display the evidence of healed granulomas, marked by central calcification in conjunction with fibrotic encapsulation containing no detectible bacilli (Opie, 1917). Despite the observed absence of bacteria within highly calcified granulomas, it is currently unclear whether this represents immune-mediated clearance, or simply a structural artifact left behind following *M.tb* escape.

Mycobacterium tuberculosis FACILITATES PERSON-TO-PERSON TRANSMISSION THROUGH ALTERATIONS TO THE GRANULOMA

Despite the attempts of the host to contain *M.tb* within the granuloma, as the infection progresses, the majority of individuals will develop granulomas with a necrotic focus formed due to the caesation of the macrophage infused center (Kim et al., 2010; **Figure 2**). This eventual necrosis of the granuloma is now accepted as a necessary event in facilitating the transmission of *M.tb* by disrupting the lung structure and allowing *M.tb* to gain access to the major airways. It should be noted that, in addition to person-to-person transmission, the active granuloma leaking bacteria into the airways may also allow for intrapulmonary dissemination (Cardona, 2009, 2010; Cardona and Ivanyi, 2011). It is therefore likely that the heterogeneity in granuloma structure seen in different lung regions of the same host represents different evolutionary timelines. Although much remains to be understood, it is clear that the evolution of granuloma is the result of the dynamic interplay between persisting mycobacteria and the host immune response, continuously evolving throughout the course of *M.tb* infection. Interestingly, *M.tb* may actually utilize the host immune response to facilitate the structural changes required to facilitate person-to-person transmission. In line with this, while essential to preventing bacterial dissemination, paradoxically IFN-γ-producing Th1 cells may also play an integrate role in facilitating bacterial transmission (Ehlers et al., 2001). The ability of *M.tb* to manipulate the host immune response as means to facilitate central granuloma necrosis and facilitate its transmission while deterring immune-mediated bacterial clearance is a remarkable but poorly understood feature of *M.tb*. It is important to note that the processes of bacterial dissemination within a host, and transmission between hosts may be independently regulated. For instance, clinically it has been observed that despite exaggerated bacterial burdens and extensive dissemination, HIV-AIDS individuals co-infected with *M.tb* transmit *M.tb* person-to-person far less efficiently (Doenhoff, 1997; Ledru et al., 1999; Corbett et al., 2003; Glynn et al., 2008). The inability of the HIV-infected host to spread *M.tb* has been attributed to a failure of *M.tb* to drive central granuloma necrosis and cavitation, and the transport of bacilli to the airway. These observations argue that *M.tb* utilizes the necrotic granuloma as a portal for person-to-person transmission.

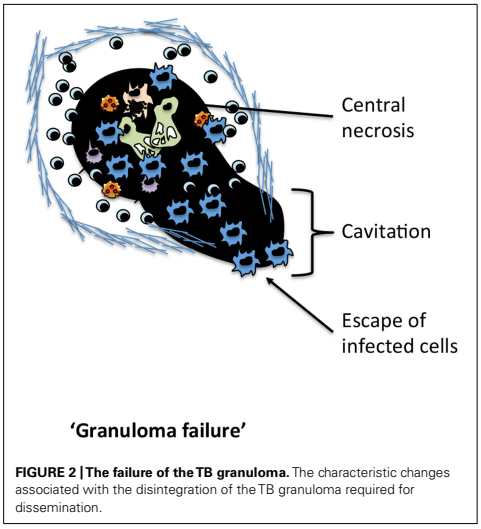

FIGURE 2 | The failure of the TB granuloma. The characteristic changes associated with the disintegration of the TB granuloma required for dissemination.

While it is well-known that changes to the granuloma structure are required for bacterial transmission, it is currently unknown whether the host or bacteria are responsible for these changes. Recently, studies have examined the granuloma at early and chronic stages of disease revealing a dramatic shift in the genes expressed by both *M.tb* and the host immune response. Notably, within the granuloma, the host immune response shifts from predominately pro-inflammatory during the early phases of infection, to immunosuppressive during the chronic stages (Karakousis et al., 2004; Ly et al., 2007; Mehra et al., 2010). Coincidentally, *M.tb* expresses a defined set of genes that function to facilitate immune

activation, while simultaneously expressing enzymes to combat immune-mediated clearance (Rohde et al., 2012). This is later followed by a shift in gene expression thought to facilitate immune senescence within the granuloma, allowing for *M.tb*'s persistence (Rohde et al., 2012). While traditionally *M.tb* is thought to lie dominant, it has recently been demonstrated that throughout the course infection *M.tb* will periodically "awaken," up-regulating a number genes and sample the immune environment (Karakousis et al., 2004). This sampling allows *M.tb* to identify the optimal conditions for facilitating person-to-person transmission. Moreover, during the caseous stage of granuloma formation there is a further shift in the genes expressed by *M.tb* with a significant up-regulation of genes associated with lipid metabolism (Kim et al., 2010). Notably, most mycobacterial species are rich in immunomodulatory lipids, which play a central role in immune evasion. Intriguingly, the distribution and release of certain lipids by *M.tb* varies significantly over the course of infection, providing a means by which *M.tb* directs the host immune response. To this end, *M.tb* can release toxic lipids and generate targeted tissue damage. The generation of central necrosis is essential to facilitating cavitation and promoting *M.tb* transmission. Trehalose 6,6′-dimycolate (cord factor) has potent cytotoxic effects and has been implicated in the generation of central necrosis of the granuloma and the transmission of *M.tb* (Hunter et al., 2006; Kim et al., 2010). Recently, it has been shown that neutrophils and AMs recognize mycobacterial cord factor through their surface c-type lectin receptor, mincle (Behler et al., 2012; Lee et al., 2012). The engagement of mincle leads to a pro-inflammatory cytokine pathway that aids in the early cellular recruitment and control of mycobacteria (Behler et al., 2012; Lee et al., 2012). Interestingly, however, the proportion of cord factor varies greatly throughout the course of infection with its synthesis heavily up-regulated by *M.tb* during the development of central necrosis and cavitation (Hunter et al., 2006; Kim et al., 2010). Indeed, studies have linked the amount of cord factor released by *M.tb* to the extent of necrosis and cavity formation (Hunter et al., 2006; Kim et al., 2010). Moreover, previous studies have documented mincle as a key receptor in the detection of necrosis and the development of an inflammatory response upon tissue damage (Yamasaki et al., 2008). Given that cord factor is known for its cytotoxic effects, one may speculated that engaging mincle may be central to the development of a pro-inflammatory response capable of aiding the formation of cavitation within the granuloma. Utilizing the host immune machinery *M.tb* facilitates the necessary structural changes to ensure its own transmission, which occurs at a time when the immune system is most vulnerable.

CONCLUDING REMARKS

While the true nature of the granuloma still remains to be defined, it is now clearly evident that the granuloma is not just a host-mediated entity of segregation and rather, it is a dynamic battlefield bearing the scars left both by the pathogen and the host immune response. While it may have been originally destined to restrain bacterial dissemination, *M.tb* efficiently hijacks the granuloma to provoke the generation of an immunologically sheltered niche to reside within and persist until the situation is favorable to bacterial transmission.

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