# NATURAL RESISTANCE TO AND HOST-DIRECTED PREVENTION OF TUBERCULOSIS

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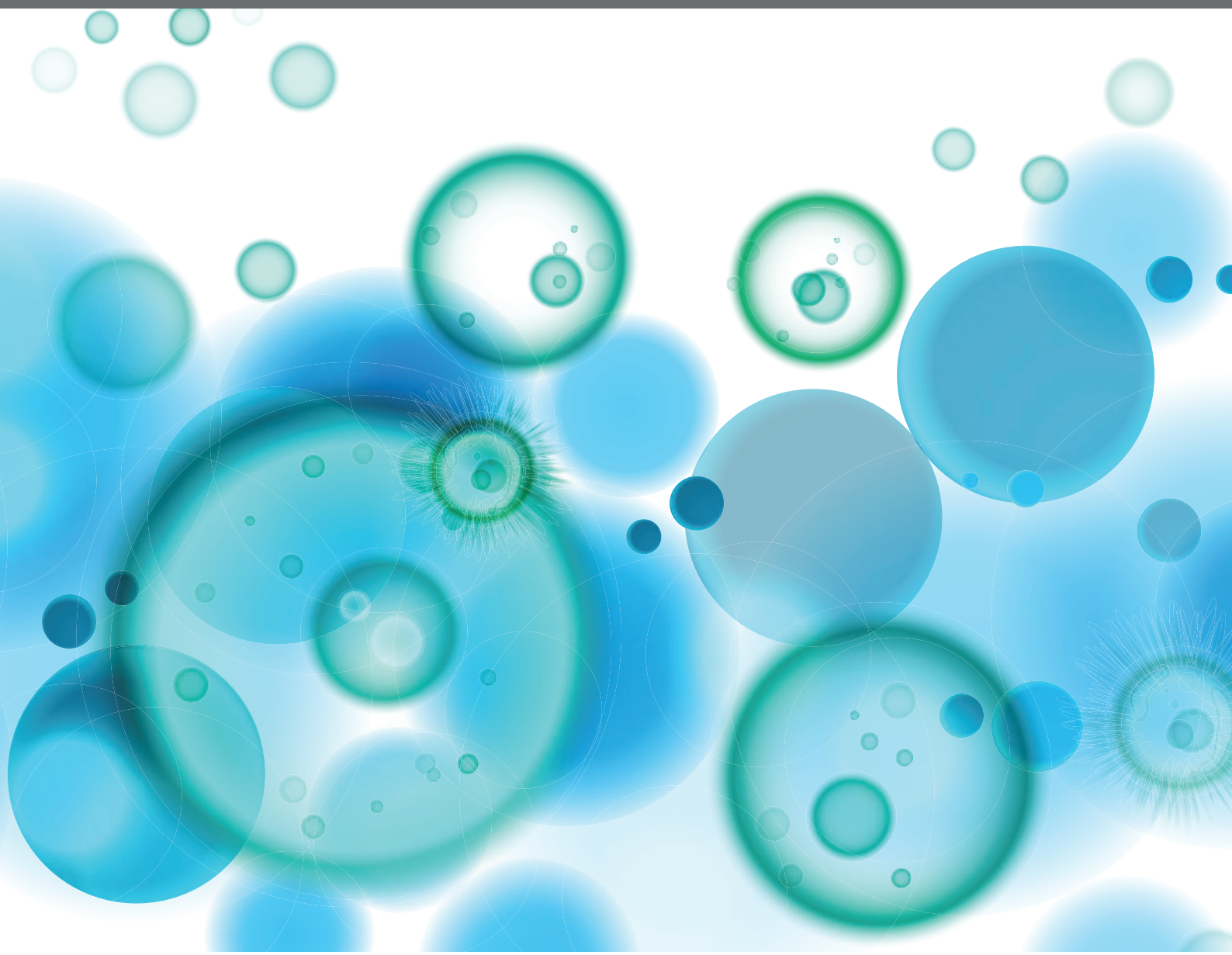

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# NATURAL RESISTANCE TO AND HOST-DIRECTED PREVENTION OF TUBERCULOSIS

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Tuberculosis remains an important bacterial disease responsible for more than one million deaths per year. The risk of overt disease is highest in the first year post infection, nevertheless, asymptomatic chronic infection (referred to as Latent Tuberculosis Infection, LTBI) may also be established. LTBI cannot be ascertained directly, it can only be inferred from a skin or blood test of immune sensitization. Nevertheless, it is often stated that one third of the world's population has LTBI. The central tenet of Tuberculosis control has therefore been antibiotic treatment of overt disease and the selective less intensive antibiotic treatment of patients considered at risk of progression of LTBI.

Much Tuberculosis research has been directed towards elucidation of the mechanisms of host susceptibility to disease. The best-characterized immune risk factor for Tuberculosis is HIV-1 co-infection. Others include anti-TNF therapies, Diabetes Mellitus, other forms of immunosuppression, and cigarette smoking. However in most clinical cases of Tuberculosis, no underlying immunological defect can be identified.

Since the general assumption is that most people infected with Tuberculosis never develop disease, this suggests that most people who are exposed and infected with Mycobacterium tuberculosis harbor immunity to Tuberculosis. This encourages the hypothesis that vaccination should be possible and indeed Bacille Calmette Guérin (BCG) vaccination confers protection against disseminated disease in children. However, BCG vaccination is not associated with reduced pulmonary disease in adults, which is a significant limitation. Furthermore it has been recognized that increased resistance to Tuberculosis occurs in specific populations. These include (i) heavily exposed persons in whom tests of immune sensitization nevertheless remain persistently negative; (ii) children aged between 5 years and puberty, and (iii) persons with documented persistent positive tests of sensitization who nevertheless never manifest disease.

As progress towards the elimination of Tuberculosis is insufficient under current antibiotic-based strategies, the idea to enhance immune resistance either via improved vaccination or enhanced natural immunity is important. Recent research interest has therefore increased attention on the analysis of resistance in humans. The current BCG vaccine is conventionally thought to prevent progression of established infection. However, vaccination strategies now also envisage the prevention of infection and relapse. There has been a rapid growth of interest in adjunctive host-directed immune interventions which aim to either enhance protective immunity or to regulate pathological tissue-damaging immunity. However, the idea of host-directed prevention is less widely discussed.

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MINI REVIEW
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# New Players in Immunity to Tuberculosis: The Host Microbiome, Lung Epithelium, and Innate Immune Cells

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Tuberculosis (TB) is a highly contagious infection and devastating chronic disease, causing 10.4 million new infections and 1.8 million deaths every year globally. Efforts to control and eradicate TB are hampered by the rapid emergence of drug resistance and limited efficacy of the only available vaccine, BCG. Immunological events in the airways and lungs are of major importance in determining whether exposure to *Mycobacterium tuberculosis* (*Mtb*) results in successful infection or protective immunity. Several studies have demonstrated that the host microbiota is in constant contact with the immune system, and thus continually directs the nature of immune responses occurring during new infections. However, little is known about its role in the eventual outcome of the mycobacterial infection. In this review, we highlight the changes in microbial composition in the respiratory tract and gut that have been linked to the alteration of immune responses, and to the risk, prevention, and treatment of TB. In addition, we summarize our current understanding of alveolar epithelial cells and the innate immune system, and their interaction with *Mtb* during early infection. Extensive studies are warranted to fully understand the all-inclusive role of the lung microbiota, its interaction with epithelium and innate immune responses and resulting adaptive immune responses, and in the pathogenesis and/or protection from *Mtb* infection. Novel interventions aimed at influencing the microbiota, the alveolar immune system and innate immunity will shape future strategies of prevention and treatment for TB.

**Keywords: tuberculosis, microbiota/microbiome, innate lymphoid cells, alveolar immune system, mucosa-associated invariant T**

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## INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is responsible for over one billion deaths in the last 200 years, more than any other single pathogen. Despite the increased global attention, the expansion of therapeutic drug regimens, and the widespread use of existing vaccine, ~1.8 million people still die every year as a result of this devastating disease (1). Furthermore, increasing outbreaks of drug-resistant TB and TB/HIV co-infection pose a significant threat to treating and preventing further transmission (2). An epidemiological model has estimated that without adequate treatment and prophylactic measures, TB will infect ~225 million and kill 79 million people between the years 1998 and 2030 (3).

Available anti-TB drugs have a profound effect on drug-susceptible TB with >90% cure rates. But resistance to almost all of the available drugs is rapidly emerging in the form of multi-, extremely- and

totally drug-resistant TB (MDR, XDR, and TDR-TB), and the development of new anti-TB drugs severely lags behind (4). The current vaccine, bacille Calmette–Guérin (BCG), has been available for ~70 years, but it is not very effective and provides only partial and inconsistent protection (0–70%) (5). Furthermore, the incidence of concurrent infection with different strains of *Mtb* and exogenous re-infection following *Mtb* drug-treatment suggest that adaptive immunity to *Mtb* is not solely protective. Extensive clinical and animal studies have examined an essential role of adaptive immunity in controlling mycobacterial growth or replication. In addition, in the last 20 years, a concerted worldwide effort has been prompted to develop a new preventive and/or therapeutic TB vaccine. Unfortunately, none of them showed sufficient efficacy through clinical trials. Clearly, something is missing. Development of a new effective vaccine against TB remains challenging due to a poor understanding of immune-correlates of protection and disease pathogenesis (6).

Consequently, novel therapeutic strategies, which could control ongoing infection and associated pathogenesis, reduce or prevent recurrence, and effectively deal with increasing drug resistance, are needed to control the global epidemic of TB. A positive aspect to this is that 90% of immunocompetent individuals exposed to *Mtb* do not develop active disease, clearly suggesting a critical role of host immunity to prevent and/or clear the early infection. Most research targeting host immunity has so far focused on generating and maintaining antigen-specific adaptive immune responses against *Mtb* as an effective way to prevent and/or treat *Mtb* infections. Despite significant effort and resources, they have not been very successful yet. There is a serious need to more comprehensively understand the network of immunological mechanisms underlying protection and/or clearance of TB infection, which allow a precise balance between host protective responses and immune pathogenesis. This extensive undertaking must take into account relatively newly identified but dominant players in host immunity: the microbiome/microbiota, the epithelium and the innate immune cells, in addition to adaptive immunity (**Figure 1**). In this review, we provide a brief overview of our current understanding of the gut–lung microbiota, the airway

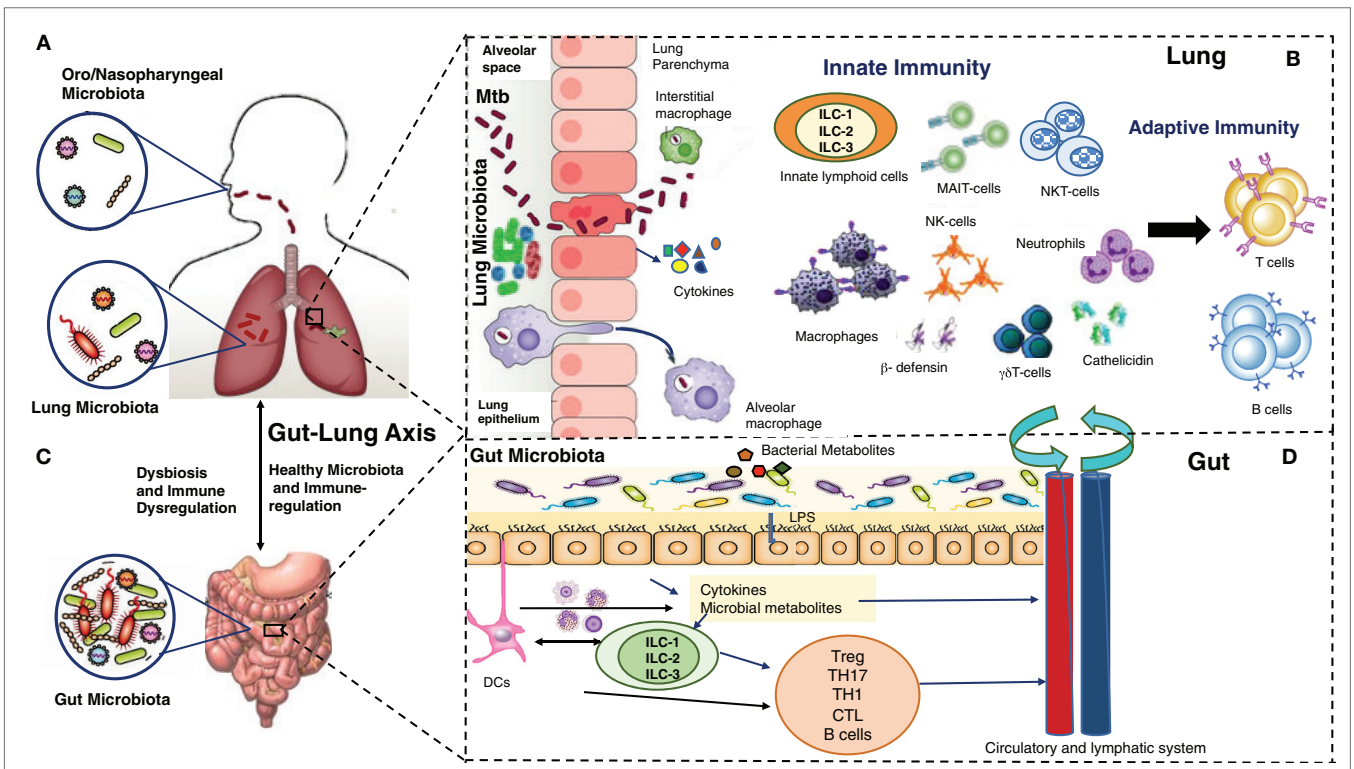


**FIGURE 1** | Multiple players in *Mycobacterium tuberculosis* (*Mtb*) infection and immunity: lung microbiota, lung epithelium, gut–lung axis, gut microbiota, innate and adaptive lymphocytes. **(A)** The upper (oro/nasopharyngeal) and lower respiratory (lung) microbiota. **(B)** Alveolar epithelial cells secrete cytokines, opsonins, and antimicrobial peptides upon mycobacterial infection. Alveolar macrophages/interstitial macrophages constitute the first line of immune defense and also the first port of entry during mycobacterial infection, but their interaction with lung microbiota is not yet known. Innate lymphocytes, such as MAIT, NK, NKT, γδ T cells, and innate lymphoid cells (ILCs) become activated, and their coordination leads to subsequent expansion/modulation of adaptive T and B cells. Dendritic cells transport *Mtb* antigens to draining lymph nodes to promote *Mtb*-specific immunity. **(C)**. In a healthy state, the gut microbiota regulates lung immunity and influences the lung microbiota. Dysbiosis caused by anti-TB therapy in the gut can lead to dysregulation of immune responses in the lung. **(D)**. The intestinal microbes and their metabolites regulate ILCs directly, or through cytokines produced by gut epithelium or DCs. ILCs and DCs in turn regulate adaptive T and B cells in the gut which migrate systemically and to lungs. Also, a combination of signals from microbes leads to migration of DCs to the draining lymph nodes, where DCs promote activation of various T cell subsets and B cells. During mycobacterial infection, cells activated in gut-associated lymphoid tissue (GALT) and mesenteric lymph nodes migrate to the lungs where they promote protective immunity and influence the lung microbiota. Bacterial metabolites are also directly transported to the lungs to influence the lung immunity against mycobacterial infection. The exact pathways of interactions between the components of **(A–D)** are still being explored.

epithelium, innate immune cells, and their collective interaction with *Mtb*.

## MICROBIOTA AND TB

The microbiota have emerged as a new biomarker of human health as it plays a vital role in maintaining normal health, developing and educating the immune system, and providing protection against pathogens. There is an elegant mutualistic interaction between the human host and microbiota. Recent studies have revealed that changes in the microbiota of healthy individuals parallel various pathological conditions (7–12). How to apply this knowledge to improve human health is at the very least "convoluted." Still, it is an important beginning that needs a dedicated effort to succeed.

Early studies of human microbiota fundamentally focused on the roles of intestinal commensals and their metabolites in regulating various inflammatory and metabolic disorders. It is now becoming apparent that the immunological function of gut microbiota extends far beyond the local environment of the GI tract, immune homeostasis, and immune defense against enteric bacterial and viral infections (13–15). Evidence is mounting in support of a dominant and decisive role of gut as well as lung microbiota in shaping and modulating immune responses in the prevention, pathogenesis, and treatment of respiratory diseases (16–21). Alteration in gut microbiota, resulting in immunological dysregulation, is associated with the development of chronic respiratory diseases, such as allergy, asthma, COPD, and cystic fibrosis (21–28).

The role of microbiota during mycobacterial infection remains largely unexplored. To date, only a few studies have focused on studying the changes in the gut and/or lung microbiota during *Mtb* infection and the risk of progressive TB. The cross-talk between the lung and gut microbiome, as well as innate and adaptive immune cells that may link these two mucosal sites, appear to be important in the prevention, pathogenesis, and treatment of TB.

There are several studies that point toward a prominent role of gut microbiota in stimulating and fostering the development and maintenance of immune responses during *Mtb* infection.

*Helicobacter pylori* are commensal bacteria inhabiting the stomach of ~50% of the world's population. Usually *H. pylori* are harmless gut habitants, but in ~10% of the people harboring them, they can lead to gastritis, peptic ulcer, and gastric cancer. The acidic gastric environment has long been considered to be a sterile environment, but now it is well recognized that stomach also has a distinct microbial community, albeit with much lower bacterial density than intestine and colon. Interestingly, *H. pylori* are uniquely adapted to colonize in the human stomach, by generating ammonia and $HCO_3^-$, which can neutralize the gastric acid. The infection with *H. pylori* results in the induction of inflammatory responses, which are unable to clear the infection but which drive the chronic gastric immunopathology. Long-term colonization and neutralization of gastric acid by *H. pylori* may also contribute to the alteration in the microbiota leading to dysbiosis (29). Perry et al. have reported that *H. pylori* seropositive individuals with latent TB had high TB antigen-specific Th1 responses and IFN-γ production and were less likely to develop active TB disease, compared to *H. pylori* seronegative individuals (30). Furthermore, it has been shown in a mouse model that alteration of gut microbiota in early life dominated by the bacterium *Helicobacter hepaticus* intensely influences the magnitude and protective efficacy of immune responses to subunit vaccine Ad85A (31). In addition, stool microbiota rich in *Bifidobacterium* spp. was associated with increased PPD-specific T cell responses after BCG vaccination in infants. This study suggested a role of the neonatal gut microbiome in modulating vaccine-induced immunity and the effectiveness of the BCG vaccination right after birth (32).

Antibiotic-induced alteration in gut bacterial composition before and after *Mtb* challenge has been shown to promote higher susceptibility to *Mtb* infection and dissemination of mycobacteria in liver and spleen (33). Disruption of gut microbiota has also been demonstrated to modulate adaptive immune responses to TB, with increased numbers of regulatory T cells and reduced frequency of IFN-γ and TNF-α-secreting $CD4^+$ T cells upon *Mtb* challenge. Interestingly, fecal transplantation was shown to reconstitute the gut microbiota, restore anti-TB immunity, and prevent dissemination of TB to other organs (33). Recently, a meta-analysis of published studies has shown that narrow-spectrum first line anti-TB drugs have dramatic effects on microbiome diversity and immunity, which persists even after the completion of TB drug therapy. Further, the persistent dysbiosis that accompanies curative anti-TB treatment could contribute to post-treatment susceptibility to reinfection, not only mycobacterial but also with other diseases linked with altered immune responses (34, 35).

The studies described above clearly establish the role of gut microbiota on the quality of TB immunity in the lungs. Although previously unrecognized and unappreciated, the respiratory tract also harbors a rich microbiota, albeit at smaller levels than gut. Interestingly, there is a continuum of bidirectional cross-talk between gut and lungs through the so-called gut–lung axis, mediated by passage of bacteria, bacterial products, and inflammatory mediators through blood and lymphatics and directly through aspiration. The lung microbiota is more dynamic and transient than in the gut as the lung is a low bacterial burden organ and is continually influenced by microbial immigration and elimination (36–38). In the upper respiratory tract (URT), nasal and oral cavities contain distinct microbiota: the nasal cavity is enriched with *Streptococcus*, *Acinetobacter*, *Lactococcus*, *Staphylococcus*, and *Corynebacterium* whereas *Prevotella*, *Streptococcus*, *Fusobacterium*, *Neisseria*, *Leptotrichia*, and *Veillonella* dominate in the oral cavity (39). Furthermore, the microbiota in the URT is constantly exposed to airborne and ingested diet-associated microbes.

The limitation in obtaining lower respiratory tract (LRT) samples remains a major impediment in studying the composition of the lung microbiome in health and disease. Previously, the LRT or lungs were considered a sterile environment. But a number of investigations have conclusively demonstrated that a healthy LRT also has abundant microbiota similar to the predominant phyla detected in the healthy intestine: *Firmicutes, Bacteroidetes,* and *Proteobacteria*. However, the LRT has 100- to 10,000-fold fewer bacteria than the URT (40).

The role of the lung microbiota is beginning to be appreciated, although its contribution to pulmonary diseases still remains unclear. The lung microbiota in healthy humans more closely resembles that of the oropharynx than of the nasopharynx (41). Emerging evidence suggests that microbiota residing in lungs are crucial to immune fitness, and provide essential signals for the development and appropriate function of the immune system and resistance to inflammatory and infectious disease. Despite many advances, our understanding of the changes in the composition of the microbial communities in the lungs in the context of TB is only starting to emerge.

The data collected so far suggest the lung microbiome changes in disease pathogenesis, treatment failure and recurrent TB infection, however, several contradictory findings have been reported in the characterization of the microbial diversity associated with TB disease.

Cui et al. (42) reported that bacterial diversity was significantly higher among sputum isolates of TB patients than of healthy controls. In addition, healthy participants demonstrated a strong clustering pattern (235/614 total genera) while pulmonary TB patients had a more scattered pattern (564/614 total genera). Furthermore, many foreign bacteria, such as *Stenotrophomonas, Cupriavidus, Pseudomonas, Thermus, Sphingomonas, Methylobacterium, Diaphorobacter, Comamonas, Mobilicoccus,* and so on, were unique to, and widely distributed among, the pulmonary TB patients (42). By contrast, Cheung et al. (43) reported that there was no difference in microbial diversity among TB patients and healthy controls and no direct correlation between microbial diversity and TB disease. This study, however, had a small sample size and comparisons were made between sputums from TB patients and respiratory secretions from healthy controls (44). In a more recent study, Krishna et al. (45) reported that *Firmicutes* and *Actinobacteria* dominate the sputum of TB patients, while *Bacteroides* and Proteobacteria were significantly higher in sputum samples of healthy controls. Similar to Cui et al., they reported the presence of opportunistic bacteria in sputums of TB patients prior to anti-TB therapy (45). In another study of a relatively large cohort (total 95) of new TB patients, recurrent TB and treatment failure TB patients, sputum analysis suggested that the presence of foreign bacteria and changes in lung microbiome are not only associated with the onset of disease but also with the recurrence and failure of anti-TB therapy (44).

In addition to microbiota, microbial metabolic activity and their products may also influence the outcome of TB infection. Increased production of short-chain fatty acids such as butyric and propionic acids by anaerobic *Prevotella* in the LRT of HIV-infected individuals are positively correlated with increased incidence of active TB (46). Butyrate inhibits mycobacterial antigen-specific IL-17 and IFN-γ responses and causes an increase in *Mtb* antigen-specific FOXP3$^+$ regulatory T cells in the lungs, suggesting an active role of microbial metabolites in immunity to *Mtb* (47, 48).

Recent studies of the human microbiome have mainly been focused on the role of bacteria (bacteriome) and their components. But new evidence suggests that non-bacterial microbiota residing in gut and lungs, fungi (mycobiome) and viruses (virome), could be critical in modulating immune responses, and disease and treatment outcomes (49–51). Yet these relationships remain largely unexplored. In TB patients, two genera of fungus, *Candida* and *Aspergillus*, were found in abundance in both sputum and oropharyngeal samples (52).

## THE LUNG EPITHELIUM

The respiratory tract epithelium serves as the first protective barrier in defense against respiratory/mucosal pathogens. Non-hematopoietic airway epithelial cells (AECs) are now emerging to play a critical, active role in interacting with the microbiota, initiating and expanding local innate immune responses and subsequent adaptive immunity, thereby preventing pathogens from invading lung parenchyma and remodeling tissue after a pathogenic or inflammatory damage (53–56). AECs express a number of pattern-recognition receptors (PRRs), which bind to pathogen-associated molecular patterns of various pathogens. Upon sensing pathogens, AECs secrete antimicrobial effector molecules, peptides, enzymes, reactive nitrogen and oxygen species and a range of cytokines, chemokines, and growth factors, which help in the recruitment and communication with immune cells and contribute to the initiation of innate immune responses critical for early control of an infection (57–61). The current knowledge of the contribution of AECs in the induction of innate immune responses and their possible role in pathogenesis or protection in context of mycobacterial infection is described in the following section.

Airway epithelial cells are the very first host cells encountering *Mtb* bacilli after aerosol inhalation and play the most prominent role in the binding, recognition, and internalization of mycobacteria followed by initiation of an immune response (62). They express a variety of PRRs, such as TLRs, RIG-1-like receptors, NOD-like receptors, and C-type lectins, as well as surfactant proteins that bind to the components of the mycobacterial cell wall (63–66). Epithelial recognition of *Mtb* activates several signaling pathways and induces production of cytokines (TNF-α, IFN-γ, GM-CSF, IL-6, IL-10, etc.) and chemokines (IL-8, IP-10, IL-27, MCP-1, MIG) (67–71). Early secretion of these soluble immune mediators allows communication between these immune AECs and other immune cells to subsequently initiate recruitment and activation of monocytes, phagocytes, lymphocytes, and polymorphonuclear leukocytes to the lungs (72). Recently, a study using human primary bronchial epithelial cells *in vitro* revealed that epithelium was inert to direct *Mtb* infection but was a potent responder to cytokines (IL-1β and type I interferons) released by infected macrophages, allowing an efficient cross-talk (73). Stimulation of AECs by BCG also leads to early activation of neutrophils that positively affect the protective efficacy against pulmonary TB through the induction of Th1 and Th17 cells (74). Interestingly, AECs express MHC I molecules and can directly present intracellular antigens to resident CD8$^+$ T cells. It has been shown that *Mtb* are localized in the late endosomal vacuole of lung epithelial cells, and their antigens are efficiently presented to CD8$^+$ T cells to stimulate IFN-γ production (75). Thus, AECs may play a critical role in initiating protective adaptive immunity to mycobacterial infections.

In response to mycobacterial infections, AECs were shown to produce antimicrobial peptides and nitric oxide (NO) in several *in vitro* and *in vivo* studies. AECs secrete antimicrobial peptides cathelicidin (LL-37), β-defensin-2, and hepcidin that have been shown to play a critical role in innate immunity against mycobacteria (76–78). Human alveolar epithelial cell line A549 has also been shown to produce LL-37, hepcidin, and NO upon stimulation with BCG. Human epithelial cells have also been shown to produce β-defensin-2 upon exposure to BCG, which enhances host defense to control *Mtb* infection (79–81). Airway epithelium, therefore, plays a non-redundant role in initiating and shaping the innate immune response at the very first site of exposure and influences the outcome of *Mtb* infection (73, 82). In addition, through TGF-β production, alveolar epithelial cells also play an important immunoregulatory role in maintaining epithelial integrity and preventing immune-mediated destruction by limiting inflammation (83). Recent studies have also revealed a comprehensive interaction of airway epithelium with lung microbiota. How these interactions are affected during *Mtb* infection, and also whether or how they play a significant role in active TB, latency and reactivation from latency, needs to be investigated.

## INNATE IMMUNITY

The early innate immune interactions between mycobacteria and the host are crucial and predictive of the eventual outcome of infection as well as maintenance of long-term memory responses. And yet they are poorly understood due to a historic emphasis on adaptive immunity as a major player in TB immunity. It is now being recognized that early immune events after exposure to *Mtb* are not "silent" in humans but are rather robust, and characterized by inflammatory processes and thoracic lymph node involvement, regardless of infection trajectory. Notably, these initial events are successful in restraining the infection to a large extent since most infections do not progress to active TB disease.

Several case contact studies have confirmed that exposure to *Mtb* does not always lead to TB infection. In high TB epidemic areas, half of the exposed people never get infected with *Mtb* and remain negative to the tuberculin skin test (TST) and IFN-γ release test, while half become TST positive with the absence of Th1-type adaptive immunity against *Mtb* antigens. In these settings, it is highly likely that *Mtb* was inhaled, contained and cleared before the development of adaptive immunity. In this regard, quantitative assessment of innate immune responses in whole-cell *Mtb* stimulation assays revealed an unexpected cytokine signature: TST-negative individuals demonstrated lower TNF-α induction in response to LPS stimulation compared to TST-positive people. These results clearly demonstrated that measurement of a single parameter such as TNF-α is not sufficient and there is a need for deeper understanding of the roles played by various innate immune functionalities (84–87). Activation of TLR2 on human and mouse macrophages by microbial lipoproteins has been shown to kill intracellular *Mtb*, providing direct evidence of innate immune-mediated clearance of *Mtb* (88). These studies suggested that innate immune responses are associated with the early clearance of *Mtb* before the onset of adaptive immunity. Furthermore, it is conceivable that examining gut–lung microbiota and early immune events in the subjects with resistance to and/or self-clearing infection with *Mtb* would provide essential information regarding protective immunity. Growing evidence suggests that the innate immune system can also produce pathogen-specific responses and mount resistance to secondary infections through "innate immune memory" or "trained immunity" (89–91). Successful treatment for mycobacterial infection requires complete clearance of mycobacteria, resolution of infection-induced inflammation and repair/remodeling of lung epithelium. Multiple studies have examined the role of various innate immune cells recruited to lungs following *Mtb* exposure/infection, e.g., neutrophils, NK cells, NKT cells, and γδ T cells (92–97). However, the role of mucosal (lung)-resident innate lymphocytes in *Mtb* pathogenesis and clearance remains to be established. We will briefly discuss the possible role of some of these potential new players in protective immunity against TB in the following section.

Neutrophils are among the first immune cells that migrate to the infection site during *Mtb* infection and play a crucial role in the development of innate and acute inflammatory responses (98). During *Mtb* infection, neutrophils produce and secrete antimicrobial enzymes (α-defensins, matrix metalloproteases, lactoferin, and lipocalin) to restrict the growth of mycobacteria within macrophages, and promote apoptosis of infected macrophages, thereby limiting *Mtb* survival within the host. Upon stimulation with *Mtb*, they also secrete chemokines (IP-10, MCP-1, MIP-1α/β) and pro-inflammatory cytokines (IFN-γ and TNF-α) to recruit and activate other immune cells (99, 100). However, these effector molecules also mediate lung tissue damage and a sustained, hyper-activated inflammatory response. Neutrophils are the second most abundant cells, after lymphocytes, found in bronchoalveolar lavage (BAL) and sputum samples of active pulmonary TB patients (101, 102). Furthermore, neutrophils have been reported to highly express programmed death ligand-1 (PDL-1) and type I IFN-inducible genes in the blood of active TB patients (103, 104). It is still controversial whether the increased expression of PDL-1 on neutrophils is associated with suppression of protective immunity or with the resolution of inflammation.

NK cells are prominent cellular components of innate immunity that play a central role in clearing the intracellular pathogens. NK cells mediate their function through cellular cytotoxicity and production of a range of cytokines (105, 106). In acute mycobacterial infection, NK cells have been shown to possess increased cytotoxic activity, IFN-γ and TNF-α production, and upregulation of activation marker NKG2D/NKp46 (107–109). They have been also shown to lyse infected monocytes, alveolar macrophages, and *Mtb*-expanded T regulatory cells, induce γδ T cell proliferation, and promote IFN-γ production from $CD8^+$ T cells (110, 111). It has been shown that depletion of NK cells in mice at the time of BCG vaccination enhances the expansion of T regulatory cells and impedes the vaccine-induced protective immunity against challenge with Mtb H37Rv (112). In another study, vaccination of mice with BCG was shown to expand memory-like NK cells in an antigen-dependent manner,

which was suggested to provide protection against subsequent *Mtb* infection (113). Expansion of IL-21-dependent memory-like NK cells was also seen in people with latent TB (114). By contrast, in patients with active TB, NK cells have been reported with reduced cytotoxicity, depressed IFN-γ production, and lowered expression of NKp30- and NKp46-activating receptors (115, 116). In orchestrate acute inflammation highlighted the importance of NK cells during mycobacterial infection, especially in TB-HIV co-infected patients (93). A full understanding of the role of NK cells in antimycobacterial immunity may open new possibilities for the development of immunotherapeutic strategies against TB.

NKT cells are innate immune cells expressing both NK and T cell markers and possess effector as well as regulatory functions. NKT cells are classified as type 1 or invariant NKT (iNKT) with restricted TCRs and type II or heterogeneous NKT with less restricted TCRs (117–120). Growing evidence suggests that NKT cells mediate protection against *Mtb* in both humans and mouse models (121). In mice, administration of α-GalCer (a known iNKT agonist), both alone and in combination with anti-TB drugs, improved the outcome of *Mtb* infection (122). Incorporation of α-GalCer in BCG vaccine has been shown to enhance the induced immune responses (123). Also, patients with active TB were found to have dysfunctional NKT cells with increased expression of inhibitory molecule PD-1 (94). Recently, it has also been shown that NKT cells isolated from pleural fluid of TB patients produce IFN-γ, TNF-α, IL17, IL-2, and IL-21 upon *ex vivo* stimulation with antigen (124, 125). It has been suggested that NKT cells become activated during the early infection with pulmonary TB, and actively participate to resolve *Mtb* infection (126). Whether, and to what extent NKT cells are associated with early innate resistance to mycobacterial infection is not clear yet.

γδ T cells are a distinct subset of $CD3^+$ T cells, which carry a T cell receptor encoded by Vγ and Vδ gene segments. They recognize unprocessed, non-peptide phosphate antigens in a non-MHC restricted manner (127). γδ T cells represent an early defense against pulmonary TB and serve as a link between innate and adaptive immunity. During the initial phase of *Mtb* infection, γδ T cells are recruited in the lungs, which express IFN-γ and IL-17 along with cytotoxic effector function (128). Increased frequency of γδ T cells has been shown in lungs in patients with active TB (129). Studies in both mice and humans suggest that γδ T cells, expanded after BCG vaccination, are capable of restricting mycobacterial growth in a perforin- and granulysin-dependent manner (130). γδ T cells also elicit protective immune responses through their interaction with NKs, DCs, and $CD8^+$ T cells (131). Further studies are needed to define a precise role of γδ T cells in protective immunity to *Mtb* infection.

Mucosa-associated invariant T (MAIT) cells are prevalent in blood and mucosal sites in humans. They are unique innate cytotoxic T cells that emerge from the thymus as effectors, and thus act as immediate effectors in response to pathogens. MAIT cells have a limited T cell receptor repertoire, act in a non-classical MR1-restricted manner, get stimulated by vitamin B metabolites common in bacteria and yeast, and respond to host cells infected with bacterial pathogens with cytokine production and cytotoxicity, without prior priming and stimulation (132). MAIT cells have been shown to produce IFN-γ and TNF-α in response to *Mtb*-infected cells and also induce target cell lysis through secretion of cytotoxic granules (133). Mice lacking MR1 or MAIT cells were shown to have increased mycobacterial loads upon aerosol challenge with *M. bovis* (134). In patients with active TB, the frequency of MAIT cells was decreased in peripheral blood but increased in the lungs compared to healthy individuals, suggesting that MAIT cells migrate from the periphery to the mucosal site of infection to provide protection during infection (135). Also, MAIT cells from the peripheral blood of active TB patients exhibited impaired cytokine (IFN-γ, TNF-α, IL-17) and cytotoxic response (granulysin and granzyme B) upon stimulation with *Mtb* antigens. Furthermore, MAIT cells from peripheral blood of active TB patients had elevated expression of programmed death-1 (PD-1) molecules and blockade of PD-1 resulted in enhancement in antigen-stimulated IFN-γ production (136). Early clearance of mycobacteria after exposure has been associated with MAIT memory cells that have previously encountered non-tuberculous environmental mycobacteria (137). Therefore, MAIT cells appear to be an important component of innate immunity against TB, which need further exploration.

In recent years, innate lymphoid cells (ILCs) have emerged as a new family of innate counterparts of T helper lymphocytes. ILCs are derived from an Id2-dependent lymphoid cell progenitor cell population abundant at mucosal surfaces, and play a significant role as a first line of defense against pathogens as well as in immune homeostasis. ILCs rapidly respond to microbial and cytokine signals and are potent innate cellular sources of multiple pro-inflammatory and immunoregulatory cytokines (138). ILCs have also been shown to play a critical role in modulating adaptive immunity toward tolerance and/or protective immunity (139). There is considerable phenotypic and functional heterogeneity in the mature ILC family, and broadly three groups of ILCs (ILC1, ILC2, and ILC3) have been defined based on shared expression of surface markers, transcription factors, and effector cytokines (140). ILCs orchestrate acute inflammation to promote immunity to infection as well as promote the resolution of infection-mediated inflammation and damage of tissues in lungs and intestine (141). They have also been shown to promote the barrier function of lung epithelium and lung tissue homeostasis in multiple chronic infectious and inflammatory diseases of the respiratory tract. Lung tissue destruction and/or remodeling is a key process in the development of TB disease. However, the role of ILCs in pathogenesis and/or clearance of mycobacterial infection remains largely unknown.

A significant reduction in all ILC populations was reported among 44 subjects diagnosed with active drug-susceptible and drug-resistant TB infections, in comparison to healthy controls ($p < 0.0001$) (142). Treatment of drug-susceptible TB was reported to restore the levels of ILC1 and ILC3 but not ILC2. Furthermore, ILC populations isolated from lungs of TB-infected individuals expressed high levels of activation markers CD69, CD25, and CCR6 compared to NK and T cells (142). This study suggests that modulation of the ILC population during *Mtb* infection might possibly have a significant pathogenic and/or protective role in TB disease.

Innate lymphoid cells also interact with the microbiota and the mucosal epithelium in a major way that induces tolerance or active adaptive immunity, and thus could shape the success or failure of a pathogen such as *Mtb* in establishing an active long-term infection. However, our understanding of the details underlying the pivotal role of microbiota–epithelium–ILCs–T cell interactions during *Mtb* infection still remains scarce. Such knowledge would be critical in understanding protective immunity against *Mtb* and developing effective and selective host-directed therapeutics.

## CONCLUSION AND FUTURE DIRECTIONS

Our understanding of microbiota and their widespread role in infectious, inflammatory, metabolic diseases, and homeostasis has expanded in recent years, but numerous challenges and unanswered questions remain. Detailed analyses of the role of microbiota in active TB disease, latency, reactivation from latency and clearance with or without antibiotic treatment remains to be thoroughly investigated. In addition, instead of examining microbiota in TB disease on its own, a more in-depth understanding of their interactions with airway epithelium and the innate and adaptive immune systems will be required. Such studies will help translate these intricate interactions to support the development of new therapeutic interventions that target the dynamic association among these processes.

## AUTHOR CONTRIBUTIONS

NG, BA, and RK contributed equally to the writing of this review article. RK and BA are co-corresponding authors.

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# A Serum Circulating miRNA Signature for Short-Term Risk of Progression to Active Tuberculosis Among Household Contacts

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Biomarkers that predict who among recently *Mycobacterium tuberculosis* (MTB)-exposed individuals will progress to active tuberculosis are urgently needed. Intracellular microRNAs (miRNAs) regulate the host response to MTB and circulating miRNAs (c-miRNAs) have been developed as biomarkers for other diseases. We performed machine-learning analysis of c-miRNA measurements in the serum of adult household contacts (HHCs) of TB index cases from South Africa and Uganda and developed a c-miRNA-based signature of risk for progression to active TB. This c-miRNA-based signature significantly discriminated HHCs within 6 months of progression to active disease from HHCs that remained healthy in an independent test set [ROC area under the ROC curve (AUC) 0.74, progressors < 6 Mo to active TB and ROC AUC 0.66, up to 24 Mo to active TB], and complements the predictions of a previous cellular mRNA-based signature of TB risk.

**Keywords: tuberculosis, microRNA, household contact, biomarker, correlate of risk, machine learning**

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## INTRODUCTION

Almost one-fourth of the global population carries a latent *Mycobacterium tuberculosis* (MTB) infection (1) and is at risk of progressing to active tuberculosis. Known risk factors for progression, such as co-infection with HIV and potentially age of first exposure (2) can only explain a fraction of active disease, thus novel diagnostic and prognostic tests are needed to identify those most likely to progress (3). Accurate identification of individuals likely at high risk of active TB would facilitate prophylactic treatment strategies, potentially curing the TB infection before it progresses to its highly infectious symptomatic stage. As a first step toward this objective, we recently described a blood RNA-based correlate of risk (RNA-CoR) for progression to active TB based on splice-junction abundance from

16 interferon-response genes (4). This RNA-CoR was discovered in a South African cohort of MTB latently infected adolescents and validated using samples from South African and Gambian cohorts of household contacts (HHCs) of MTB index cases. While the results for the RNA-CoR are promising, the sensitivity and specificity of the signature were limited and there is a need to determine whether performance can be augmented using alternative approaches. The predictive power of the RNA-CoR is improved for patients close to progression to active TB. This is consistent with detection of subclinical incipient TB prior to the onset of disease symptoms. Other effective biomarkers could reflect underlying long-term risk factors that predispose individuals to develop active, rather than latent, TB after an exposure event. Exploring alternatives to whole-blood mRNA expression measurements may facilitate the discovery of these factors.

MicroRNAs (miRNAs) are small, non-coding RNAs that, as part of enzymatic protein complexes, execute post-transcriptional regulation of gene expression (5). Recent studies have demonstrated important roles for specific miRNAs during MTB infection (6). Although the established functions of miRNAs are intracellular, numerous studies have detected highly stable extracellular circulating miRNAs (c-miRNAs) in blood (7). These c-miRNAs have been explored as biomarkers for infectious diseases, including TB (8).

In this study, we evaluate c-miRNAs as candidate biomarkers for risk of TB disease progression in HHCs. These analyses make use of serum samples collected from prospective HHC cohort studies carried out in South Africa and Uganda as part of the Bill and Melinda Gates Foundation-funded Grand Challenges 6-74 program (GC6-74).

# MATERIALS AND METHODS

## Study Recruitment and Sampling

Within GC6-74, 1,197 HIV-negative South African HHCs of 209 index cases were enrolled between February 27, 2006 and December 14, 2010, and 499 HIV-negative Ugandan HHCs of 181 index cases were enrolled between June 1, 2006 and June 8, 2010. HHCs from Uganda were offered INH preventative treatment. For all sites, adult participants, or legal guardians of participants aged 10–17 years old, provided written or thumb-printed informed consent to participate after careful explanation of study aims and any potential risks. All sites adhered to the Declaration of Helsinki and Good Clinical Practice guidelines in treating study participants. Ethical approvals were obtained from the relevant institutional review boards, for the South African study site, the Stellenbosch University Institutional Review Board (N05/11/187), and for the Ugandan study site, the Uganda National Council for Science and Technology (MV 715), and University Hospitals Case Medical Centre (12-95-08).

Serum samples were collected from HHCs at enrollment (within 2 months of exposure) and at 6 and 18 months after enrollment if participants remained disease free. TB progressors were defined as individuals who developed intrathoracic TB within the study period based on one of the following two criteria: (1) positive TB sputum culture coupled with at least one of the following: positive chest X-ray, positive acid-fast bacilli (AFB) sputum smear, a second positive TB sputum culture from an independent sample or clinical symptoms consistent with active TB; or (2) positive AFB sputum smear coupled with a positive chest X-ray or a second positive AFB sputum smear from an independent sample. Co-incident TB cases, defined as HHC who developed TB within 3 months of exposure, were excluded from all further analyses. At study end, controls were selected from the individuals who had remained free of active TB for the 2-year study period and matched to cases by study site, sex, age (four age groups: <18, 18–25, 25–36, >36), and year of enrollment (three enrollment groups: 2006–2007, 2008, 2009–2010). Two to three matched controls were included for each progressor. Case–control assignment was performed prior to quantification of c-miRNA levels to ensure a blind case–control design. Prior to analysis, South African samples were split into discovery and validation sets; all Ugandan samples were apportioned to the validation set.

## Serum c-miRNA Profiling and Selection

Quantification of serum c-miRNA levels was performed by Exiqon Inc. (Vedbaek, Denmark) using qRT-PCR with locked-nucleic acid primers as previously described (9). Briefly, total RNA was extracted from serum using the miRCURY™ RNA isolation kit—biofluids (Exiqon, Inc., Vedbaek, Denmark) as follows. Serum/plasma was thawed on ice and centrifuged at 3,000 × *g* for 5 min in a 4°C microcentrifuge. An aliquot of 200 µL of serum/plasma per sample was transferred to a new microcentrifuge tube and 60 µL of Lysis solution BF containing 1 µg carrier-RNA per 60 µL Lysis Solution BF and RNA spike-in template mixture was added to the sample. The tube was vortexed and incubated for 3 min at room temperature, followed by addition of 20 µL Protein Precipitation solution BF. The tube was vortexed, incubated for 1 min at room temperature and centrifuged at 11,000 × *g* for 3 min. The clear supernatant was transferred to a new collection tube, and 270 µL isopropanol was added. The solutions were vortexed and transferred to a binding column. The column was incubated for 2 min at room temperature, and emptied using a vacuum-manifold. 100 µL wash solution 1 BF was added to the columns. The liquid was removed using a vacuum-manifold, and 700 µL wash solution 2 BF was added. The liquid was removed using a vacuum-manifold. 250 µL wash solution was added and the column was spun at 11.000 × g to dry the columns entirely. The dry columns were transferred to a new collection tube and 50 µL RNase free $H_2O$ was added directly on the membrane of the spin column. The column was incubated for 1 min at room temperature prior to centrifugation at 11,000 × *g*. The RNA was stored in a −80°C freezer.

2 µL RNA was reverse transcribed in 10 µL reactions using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation, and cDNA synthesis kit (Exiqon, Inc., Vedbaek, Denmark). cDNA was diluted 50× and assayed in 10 µL PCR reactions according to the protocol for miRCURY LNA™ Universal RT microRNA PCR; each microRNA was assayed once by qPCR on the microRNA Ready-to-Use PCR, Pick-n-Mix using ExiLENT SYBR® Green master mix. Negative controls excluding template from the reverse transcription reaction was performed

and profiled like the samples. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analyzed using the Roche LC software, both for determination of Cq (by the second derivative method) and for melting curve analysis. Two technical replicates were performed for each sample, and mean $C_t$ values for each c-miRNA in each sample, along with experimental metadata are provided in Table S1 in Supplementary Material.

An initial panel of 608 c-miRNAs were considered for analysis, based on miRNA primers suggested by Exiqon, Inc. including c-miRNAs previously suggested as potential biomarkers (Table S2 in Supplementary Material). This panel was down-selected to 164 c-miRNA (Table S2 in Supplementary Material) based on detectable expression in >80% of samples and association with progression in a subset of 40 discovery set samples. The technical replicability of each of the 164 initial candidate miRNAs was then assessed by rerunning the PCR quantification of the candidate miRNA, resulting in two technical replicates for each sample. The quality of the replicates was assessed by measuring the Pearson correlation of individual miRNAs between technical replicates. We observed a strong, non-linear relationship between miRNA expression (as measured by $C_t$) and technical replicability. In particular, a sharp decline in replicability was observed for miRNAs with mean $C_t$ values greater than 32, indicative of low levels of c-miRNA (Figure S4 in Supplementary Material). A final panel of 47 candidate miRNAs was thus selected, comprised of miRNAs expressed at reliably detectable levels ($C_t < 32$) in serum. PCR quantification of these 47 miRNAs was then run on all samples, including the pilot study samples.

## Normalization of PCR c-miRNA Data

As the abundance of c-miRNAs in serum is relatively low and varies across conditions, there is currently no universally accepted set of reference "housekeeping" c-miRNAs or universally accepted approach for standardizing c-miRNA profiles in order to maximize comparability across samples. To address this issue, we explicitly evaluated multiple normalization approaches within the suite of machine-learning approaches employed to generate predictive signatures. If a particular normalization strategy was strongly superior or inferior than others, this difference would be evident as increased or decreased predictive accuracy when assessed during cross-validation of the discovery set. The normalization strategies that we investigated were variants of two classes. In the first class, subsets of potential reference c-miRNAs were selected by ranking the final panel of 47 c-miRNAs by the magnitude of Spearman rank correlation between the c-miRNA and the overall sample mean of the $C_t$s of all 47 miRNAs. The assumption behind this approach is that any universal difference in c-miRNA abundance between samples would be due to technical reasons (like smaller or less concentrated plasma aliquot) as opposed to biological reasons. The c-miRNAs with the top 1, 3, 5, 10, 20 rank correlations to the overall sample mean would be selected as reference c-miRNAs and then averaged within each sample to generate per-sample normalization constants. Alternatively, for the second class of approaches, the per-sample normalization constants were generated by taking the mean, median, or 25% trimmed-mean computed from all 47 assayed c-miRNAs. The $C_t$s for a given sample were then normalized by subtracting the value of the normalization constant from the $C_t$ of each c-miRNA. This gave a total of eight normalized datasets: trimmed-mean, trimmed-median, 1-ref, 3-ref, 5-ref, 10-ref, 20-ref, or 47-ref (i.e., mean) normalized.

## c-miRNA Signature Development

The predictive potential of candidate c-miRNA signatures of risk was estimated by leave-one-donor-out-cross-validation (LOOCV) of the discovery set measurements of the 47 c-miRNAs. To ensure unbiased cross-validation, all samples relating to one donor were held out, the machine-learning algorithm was fit to the remaining data, and the resulting fit used to make blind predictions on the held-out samples. This step was done for each donor, and repeated for every combination of machine-learning algorithm and normalization approach. Using the R package caret (10), a variety of machine-learning algorithms were assessed (**Figure 1**).

Five machine-learning algorithms were used to train predictive models on the miRNA datasets, with models trained using the R caret (10) package as an interface: Random Forest [R randomForest package (11)]; Support Vector Machine using RBF kernel [R kernlab (12) package]; Neural Networks [R nnet (13) package]; Elastic-net Logistic Regression [R glmnet (14) package]; and Linear Discriminant Analysis (13). Initial performance was assessed using LOOCV during training. During LOOCV, all samples relating to a single donor were held out and predicted on together, i.e., samples taken at differing timepoints from a single donor. In the discovery analysis, the optimal model was selected by examining LOOCV predictive performance considering only the sample most proximal to TB diagnosis.

The R pROC (15) package was used to calculate ROC curves by applying a set of thresholds to numeric predictions from predictive models to predict the progressor or control status of the samples, and then calculating the sensitivity and specificity of the predictor at each threshold. ROC curves were plotted using the R ggplot2 (16) package. Accompanying positive and negative predictive values were calculated using the model prediction threshold that maximized the sum of sensitivity and specificity.

Prediction performance, as measured by ROC statistics, was assessed using the sample for each participant that was most proximal to TB diagnosis. The combination of algorithm and normalization that maximized the area under the ROC curve (AUC) was selected to construct the final signature and was then used to make blind predictions on the validation set. *p*-Values associated with each signature were calculated using a one-tailed Mann–Whitney *U*-test comparing signature scores for cases compared with controls and were adjusted for multiple testing using the Benjamini–Hochberg algorithm. Bootstrapping was used to estimate 95% confidence intervals (CIs) of the AUC.

## Prediction Performance of Combined RNA + c-miRNA Signature

To determine whether combining the c-miRNA signature with the existing RNA-based risk signature (RNA-CoR) led to significant improvement in prediction accuracy, a

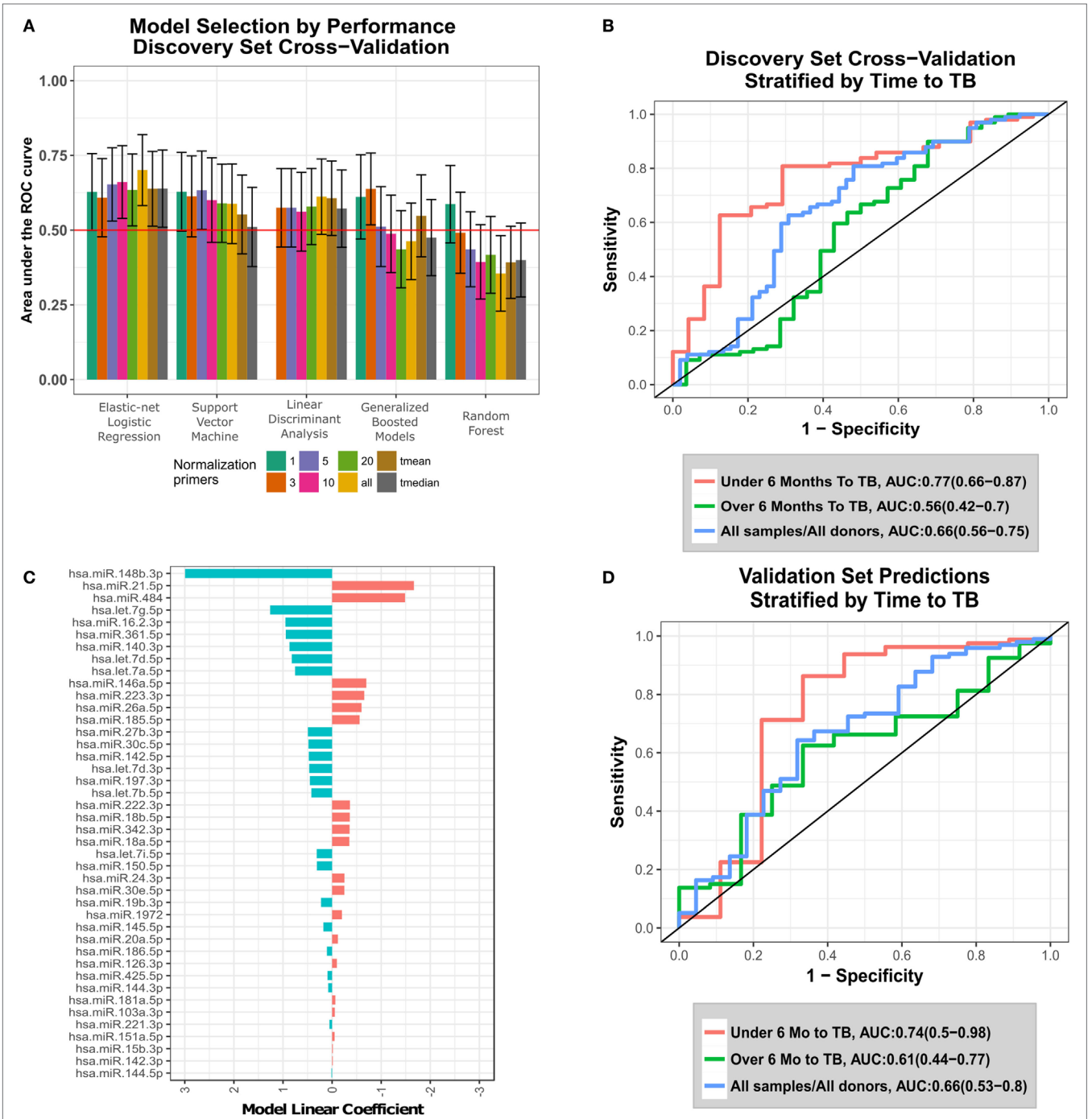


**FIGURE 1** | Development and validation of the circulating microRNA (c-miRNA) TB risk signature. **(A)** ROC area under the ROC curves (AUCs) from discovery set leave-one-donor-out-cross-validation (LOOCV) for five different machine-learning algorithms applied to data generated using eight different normalization approaches. Error bars represent the 95% confidence intervals. Normalization primers indicate the numbers of reference primers used to normalize the data ("all" = all 47 primers, and "*t*mean" and "*t*median" = 25% trimmed-mean or median of all primer expression, respectively). Horizontal red line indicates non-discrimination (AUC = 0.5). The machine-learning algorithms employed are indicated on the *x*-axis. **(B)** LOOCV ROC curves for the optimal algorithm (elastic-net logistic regression-all), stratified by the time between collection of the sample and TB diagnosis (time to TB). **(C)** Values of fitted linear coefficients for each c-miRNA in the final logistic regression signature. Red and blue indicate c-miRNAs detected at higher and lower levels in the serum of progressors compared with controls, respectively. **(D)** Validation set blind prediction ROC curves for the optimal TB risk signature with progressors stratified by time to TB as in **(B)**.

$\chi^2$ test was performed comparing two logistic regression models: (1) *Progression = f(RNA-CoR + c-miRNA)* and (2) *Progression = f(RNA-CoR)*. This approach takes into account the nested nature of these models. The significance of the improvement in the combined models' AUC was also evaluated using the highly conservative (17, 18) DeLong (19) test, which assumes

the independence of the models. These analyses were performed using samples for which both RNA-CoR scores (4) and c-miRNA signatures scores were available (34 progressor samples, 79 control samples) from both the training and test sets. To conservatively estimate c-miRNA signature performance, c-miRNA scores from the cross-validation analysis were used for training set samples and from the blind prediction analysis for the test set samples. Spearman correlations between normalized RNA-CoR PCR data (4) and normalized c-miRNA data were also calculated using matching samples.

# RESULTS

## Establishment of Study Cohorts

43 and 11 HHCs from the South African and Ugandan cohorts, respectively, progressed to active TB ("progressors") and were matched to HHCs that had remained healthy ("controls") during the 2-year study period (summarized in Table S3 in Supplementary Material). Tuberculin skin test (TST) measurements at enrollment found 91% of participants to have TST indurations ≥10 mm and 75% ≥15 mm, suggesting that the vast majority of HHCs have a latent TB infection. TST induration size did not differ significantly between progressors and controls (*U*-test $p = 0.78$), indicating that the TST is an ineffective predictor of TB risk in these cohorts. This ineffective prediction is unlikely to be related to false positives caused by BCG vaccination or TST cross reactivity with non-tuberculous mycobacteria (20) and the large TST indurations are more likely to reflect latent *M. tuberculosis* infection. Compared with our previous study of progression in South African adolescents with latent TB where 0.7% of individuals progressed to active TB over the course of 2 years (4), 3.6% of South African HHCs progressed to active TB.

A panel of 47 high expression, technically replicable c-miRNAs were selected from 608 candidate miRNAs. These 47 c-miRNAs were then analyzed in parallel on the discovery (151 samples) and validation (120 samples) sets.

## Generation and Validation of the c-miRNA Signature of TB Risk

To identify an optimal c-miRNA signature of risk for TB among HHCs, we evaluated five different machine-learning algorithms using eight different normalization strategies (see Materials and Methods, **Figure 1A**; Table S4 in Supplementary Material). The top algorithm was elastic-net logistic regression normalized by the average of all 47 c-miRNAs, which achieved a cross-validation AUC of 0.7 (95% CI: 0.58–0.82, FDR-adjusted $p = 0.04$, negative predictive value = 81%, positive predictive value = 59%) (**Figure 1A**). **Figure 1B** shows ROC curves for LOOCV results stratified by the time between sample collection and TB diagnosis ["Time To TB", as in Ref. (4)]. Predictions for samples within 6 months of progression (AUC: 0.77, CI: 0.66–0.87, NPV = 92%, PPV = 47%) were superior compared with those at all times to progression (AUC: 0.66, CI: 0.56–0.75, NPV = 76%, PPV = 59%). Significant predictions were also observed when considering baseline samples only (AUC: 0.63, CI: 0.5–0.77, Figure S1 in Supplementary Material). The optimal final signature selected was trained on the entire discovery set (**Figure 1C**; Table S5 in Supplementary Material). Blind prediction of TB progression by the signature when applied to the validation set was successful (ROC AUC = 0.66, CI: 0.53−0.8, NPV = 90%, PPV = 30%) when applied to all samples; **Figure 1D**. Stronger performance was observed on samples under 6 months to TB (ROC AUC = 0.74, CI: 0.5−0.98, NPV = 96%, PPV = 35%), consistent with the discovery set. While the signature was not significantly predictive on the baseline validation samples, i.e., samples taken close to study enrollment (AUC: 0.55, CI: 0.32–0.77, NPV = 83%, PPV = 37%), Figure S1 in Supplementary Material, very strong significant predictive performance was seen on baseline validation set samples within 6 months of TB progression (AUC: 0.95, CI: 0.88–1, NPV = 100%, PPV = 50%), Figure S1 in Supplementary Material. These results demonstrate that a c-miRNA derived signature significantly predicts TB risk for HHCs within 6 months of progression.

## Drivers of the c-miRNA Signature of TB Risk

Having validated the c-miRNA signature of TB risk, we performed a retrospective analysis to determine which c-miRNAs were the drivers of prediction accuracy. By sequentially removing c-miRNAs with the smallest model weight, retraining on the discovery set, and predicting on the validation set, we were able to identify the most parsimonious predictive signature (Figure S2 in Supplementary Material, Table S6 in Supplementary Material). Although prediction performance fluctuated stochastically with an overall decline as the signature was reduced, a three-c-miRNA signature predicted comparably to the full signature (AUC: 0.67, CI: 0.55–0.80, NPV = 78%, PPV = 64%), indicating potential for model reduction. **Figure 2A** shows the combined discovery and validation set expression of the three c-miRNAs. Thus, it appears signature predictions are dominated by the contribution of the three most important miRNAs.

## The c-miRNA Signature of TB Risk Complements the RNA-CoR Predictions

The c-miRNA signature of TB risk includes c-miRNAs up- and down-regulated in TB progression, in contrast with the transcriptomic RNA-CoR (4) which was composed of genes upregulated during progression. These distinct kinetics suggest that the c-miRNA and RNA-CoR signatures may contain independent information for predicting TB among HHCs. The South African samples used to validate the RNA-CoR form part of this study cohort, facilitating a direct comparison of the c-miRNA signature with the published qRT-PCR RNA-CoR measurements. A linear combination of the c-miRNA, including all 47 miRNAs, and RNA-CoR signatures shows a modest increase in predictive power, from an AUC of 0.77 (CI: 0.68–0.87, NPV = 88%, PPV = 48%) using RNA-CoR alone to 0.78 (CI: 0.69–0.88, NPV = 87%, PPV = 52%) for the combined signature (**Figure 2B**), and we observed wide overlap of the 95% CI between the RNA-CoR alone and the RNA-CoR + c-miRNA model. Although the AUCs of the RNA-CoR + c-miRNA did not significantly improve on the RNA-CoR when compared using the conservative DeLong

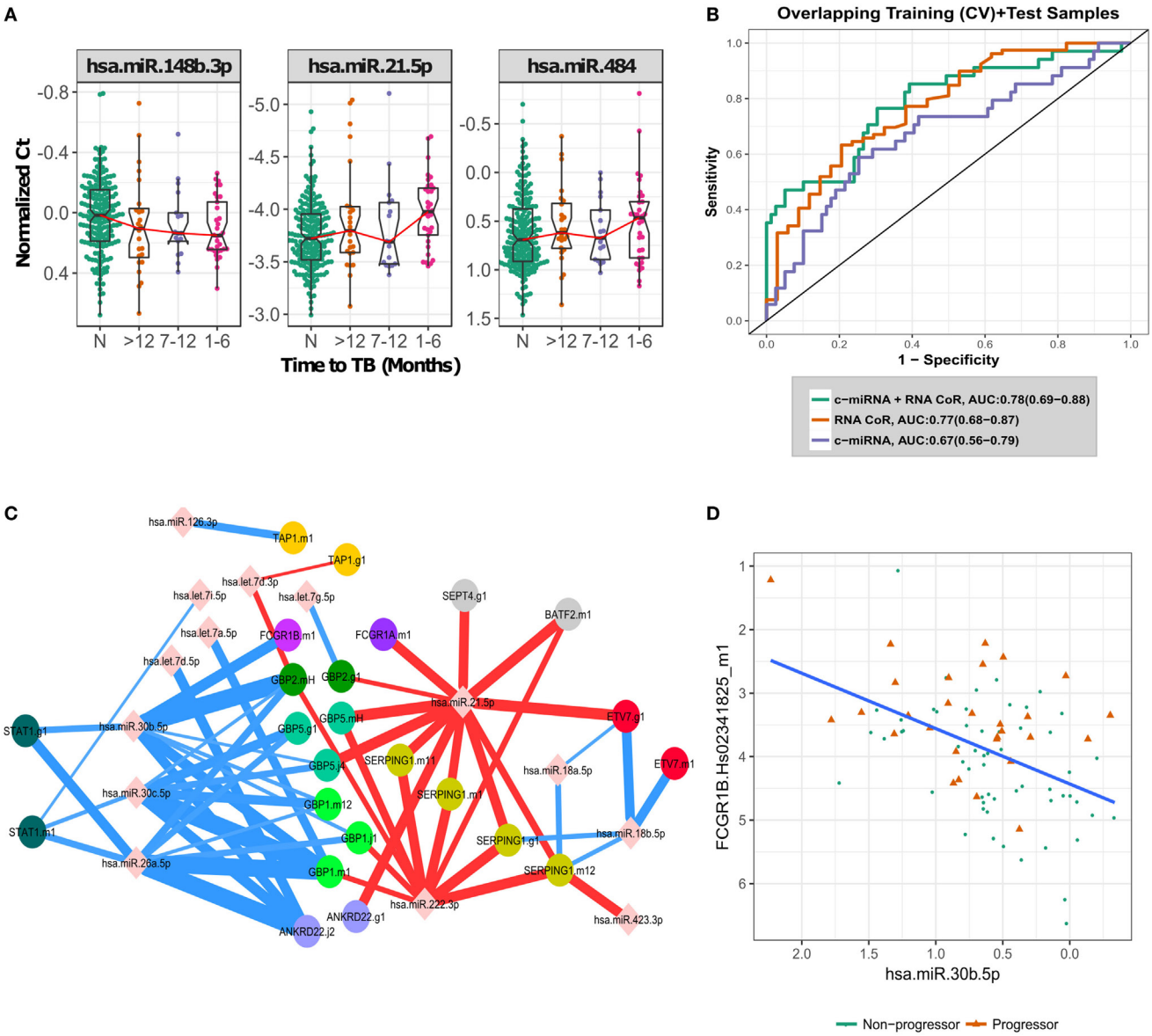


**FIGURE 2** | Biological signal underlying the circulating microRNA (c-miRNA) signature. **(A)** Normalized PCR $C_t$ values for the three most important c-miRNAs in the signature, with progressors stratified by time to active TB disease. "N" indicates non-progressor control samples. **(B)** ROC curves illustrating RNA-based correlate of risk (RNA-CoR) prediction score, c-miRNA (leave-one-donor-out-cross-validation + blind prediction scores for the 47 miRNA model) and combined score performance at classifying the shared set of discovery and validation samples. **(C)** Correlation network of c-miRNA and RNA-CoR gene PCR primers. c-miRNA—gene correlations calculated using Spearman's rho with FDR < 0.05 are indicated by edges connecting miRNAs to genes. Edge thickness is proportional to significance of the correlation. Positive correlations are indicated in red, with negative correlations in blue. **(D)** Correlation between FCGR1B and miRNA hsa-miR-30b-5p, linear best fit line shown in blue.

test ($p = 0.43$), significant ($p = 0.03$) improvement in predictive performance was observed when the linear combination of RNA-CoR + c-miRNA was compared with RNA-CoR alone using the $\chi^2$ test, which takes into account the nested nature of the models. Notably, predictions were strongly improved in the high-specificity region of the ROC curve, at a specificity of 90%, where RNA-CoR shows a sensitivity of 41%, which improves to a sensitivity of 50% when the c-miRNA scores are added.

To further explore the relationship between the c-miRNA and cellular RNA expression changes, we performed a correlation analysis between the constituents of the two signatures. **Figure 2C** shows a network of significant (FDR < 0.05) correlations between the components of the c-miRNA and RNA-CoR signatures (Table S7 in Supplementary Material). Both positive and negative correlations between c-miRNAs and the interferon-response genes in the RNA-CoR were observed in a manner consistent with previous functional studies of the implicated RNAs (21–25) (**Figure 2D**). These results demonstrate that elements of the c-miRNA signature are correlated with the previously identified RNA-CoR, and that the c-miRNA signature may provide information complementary to the RNA-CoR.

## DISCUSSION

Several previous studies have identified c-miRNAs that are differentially expressed in active TB disease (8), but to our knowledge, this is the first to have prospectively validated a c-miRNA-based signature of risk of TB in an independent cohort. The c-miRNAs comprising the signature are abundant in blood and have established roles in inflammatory and infectious conditions (21, 23–25). This signature is highly predictive of HHCs likely to progress within 6 months of testing, including tests performed close to exposure, although predictive power is diminished for more distal samples. This increase in signal close to diagnosis suggests that the c-miRNA signature is likely to be detecting an immune response to subclinical or incipient TB, prior to the development of symptomatic active disease. We observed that most progressors developed TB within 6 months of exposure (Figure S3 in Supplementary Material), suggesting that the temporal resolution of this test may be sufficient for practical application. As our analysis was limited to previously characterized c-miRNAs, we could not have identified potentially important uncharacterized c-miRNAs. Future improvements in sequencing approaches have potential to identify additional c-miRNAs that may be important in the context of TB progression.

The RNA-CoR signature has been shown to have over double the positive predictive value of an interferon-gamma release assay alone and meets the Stop TB Partnership's performance criteria for a prognostic TB test (26). Combined with the RNA-CoR, the c-miRNA signature displays only a slight improvement in AUC vs the RNA-CoR alone. However, the predictive performance shows a strong improvement in sensitivity at high specificities, suggesting that combination of the RNA-CoR and c-miRNA signature would act as an improved "rule-in" test to identify HHCs at higher risk and likely to benefit from INH prophylaxis.

Correlating the components of the c-miRNA signature with components of the RNA-CoR signature suggest how the interferon response to TB disease may be regulated by c-miRNAs. miR-21, which is induced by mycobacteria (21), and is a marker of immune cell activation (24), was positively correlated with genes in the RNA-CoR. In contrast, miR-26a, which has been shown to suppress macrophage responsiveness to IFN-γ (23), and miR-30b, which has been shown to suppress pro-inflammatory cytokine secretion and Fc-receptor expression (25), were both negatively correlated with RNA-CoR genes, including *FCGR1B* (**Figure 2D**).

Recently, blood transcriptional signatures have been developed capable of evaluating TB risk (4) and effective response to TB treatment outcome (27), although the sensitivity and specificity of the risk signature is limited. Investigating alternative platforms to whole-blood transcription holds out the possibility of augmenting the performance of this initial work. The c-miRNA signature developed here demonstrates the potential of serum c-miRNAs to predict TB risk, despite being limited by a preselected pool of candidate miRNAs, and the difficulty of accurately quantifying low-abundance miRNAs in serum. In the future, the development of accurate, sensitive, and unbiased sequencing approaches for c-miRNAs would hold much promise for further improving prediction of TB risk.

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## ETHICS STATEMENT

This household contact study included participants from two African sites: South Africa and Uganda, under the Bill and Melinda Gates Grand Challenges 6-74 (GC6-74) program. All clinical sites adhered to the Declaration of Helsinki and Good Clinical Practice guidelines, and ethical approvals were obtained from institutional review boards at both sites. Ethics review

committee names and protocol numbers for each are listed in Materials and Methods.

## AUTHOR CONTRIBUTIONS

FD, ET, and DZ carried out the computational analyses and drafted the manuscript. WB, SK, HD, TO, TS, and GW conceived the experimental and study design. KD, SS, HK, BT, JW, DD, DT, GT, DZ, and GW designed, oversaw and performed subject recruitment, biological sample collection and experimental procedures. All authors contributed to writing and revising the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00661/full#supplementary-material.

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# Differential Effect of Viable Versus Necrotic Neutrophils on *Mycobacterium tuberculosis* Growth and Cytokine Induction in Whole Blood

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Neutrophils exert both positive and negative influences on the host response to tuberculosis, but the mechanisms by which these differential effects are mediated are unknown. We studied the impact of live and dead neutrophils on the control of *Mycobacterium tuberculosis* using a whole blood bioluminescence-based assay, and assayed supernatant cytokine concentrations using Luminex™ technology and ELISA. CD15+ granulocyte depletion from blood prior to infection with *M. tuberculosis*-lux impaired control of mycobacteria by 96 h, with a greater effect than depletion of CD4+, CD8+, or CD14+ cells ($p < 0.001$). Augmentation of blood with viable granulocytes significantly improved control of mycobacteria by 96 h ($p = 0.001$), but augmentation with necrotic granulocytes had the opposite effect ($p = 0.01$). Both augmentations decreased supernatant concentrations of tumor necrosis factor and interleukin (IL)-12 p40/p70, but necrotic granulocyte augmentation also increased concentrations of IL-10, G-CSF, GM-CSF, and CCL2. Necrotic neutrophil augmentation reduced phagocytosis of FITC-labeled *M. bovis* BCG by all phagocytes, whereas viable neutrophil augmentation specifically reduced early uptake by CD14+ cells. The immunosuppressive effect of dead neutrophils required necrotic debris rather than supernatant. We conclude that viable neutrophils enhance control of *M. tuberculosis* in blood, but necrotic neutrophils have the opposite effect—the latter associated with induction of IL-10, growth factors, and chemoattractants. Our findings suggest a mechanism by which necrotic neutrophils may exert detrimental effects on the host response in active tuberculosis.

**Keywords: neutrophil, mycobacteria, tuberculosis, necrosis, viability**

## INTRODUCTION

Neutrophils interact with *Mycobacterium tuberculosis* (1, 2) and are important in the host immune response to this pathogen (3). However, their influence on the outcome of infection remains controversial: while they may have a protective role in early infection (3), evidence exists for a detrimental effect in established tuberculosis disease (4, 5).

Literature indicates that neutrophils have a profound impact on the development of innate and acquired immune responses (6, 7), but this has not been significantly studied in tuberculosis. *In vivo* data have revealed a lower percentage of interferon (IFN)-γ expressing CD4+ T cells in bronchoalveolar lavage samples from cavities, which are rich in neutrophils (8), but a causative relationship has not been established.

Neutrophils are also frequently demonstrated to associate with tuberculosis immunopathology in animals (9, 10) and this appears to be specifically associated with necrosis (11). We have also previously observed that neutrophil antimycobacterial activity is impaired during HIV-1 infection (12) and that this is associated with rapid neutrophil necrosis. Others have also noted a neutrophilic response with significant necrosis in the tuberculous lesions of HIV-1 co-infected persons, in association with impaired tumor necrosis factor (TNF) staining (13). Animal models link neutrophil necrosis with pathogen virulence and host susceptibility (14), or link overall necrosis of granulomas with bacillary burden (15), suggesting that this pattern of cell death may correlate with severity of disease. More recently, it has been demonstrated *via* immunohistochemistry that neutrophil markers in human tuberculous granulomas associate with areas of necrosis and with interleukin (IL)-10, while a pronounced neutrophilic necrosis response correlates with enhanced numbers of *M. tuberculosis* organisms (16).

We therefore investigated the influence of viable and necrotic neutrophils on the immune response to tuberculosis. We first examined the cytokine and chemokine profile of supernatants aspirated from whole blood which had been depleted of neutrophils or various other cell types before incubation with *M. tuberculosis*. We also examined the impact of augmenting blood with additional autologous neutrophils [as they are recruited in large numbers to sites of tuberculosis infection (1)] on both the control of infection and the cytokine response, using an *ex vivo* whole blood model (17, 18). We then investigated whether the effect of necrotic neutrophils contrasted with viable neutrophils in modulating the control of and response to infection. These experiments modeled a situation of neutrophilia with rapid cell death, as may be seen in necrotic granulomata, in tuberculosis-HIV co-infection or in severe tuberculosis disease.

# MATERIALS AND METHODS

## Patients and Recruitment

Participants for depletion experiments were recruited among people who had recently tested negative for HIV in Khayelitsha, South Africa, either at the Ubuntu Site B clinic or the Khayelitsha Youth Centre. Augmentation and phagocytosis experiments were performed using blood from healthy lab donors.

## Ethics

The studies were approved by the University of Cape Town Research Ethics Committee (HREC 545/2010) in South Africa and NHS Research Ethics Committee (REC 08-H0720-46) in the UK and performed in accordance with the Declaration of Helsinki. Participants provided written informed consent.

## Organisms and Labeling

The development of luminescent mycobacteria, including plasmid construction and electroporation, has been described previously (19). 1.5 ml vials of *M. tuberculosis*-lux stored at −80°C were thawed and added to 15 ml 7H9 (Becton Dickinson)/ADC (Becton Dickinson) growth medium containing 0.05% Tween 80 (Sigma) and 1 mcl/ml hygromycin B (Roche diagnostics). Organisms were cultured to mid-log phase (72 h) before use. Luminescence was measured as previously described (2). *M. bovis* BCG-lux organisms were processed similarly but cultured in 20 ml 7H9/ADC. Non-luminescent *M. bovis* BCG were cultured to mid-log phase in 7H9/ADC (monitored by optical density) and labeled with FITC when required for experiments, as previously described (2).

## Cell Depletion and Neutrophil Isolation

For comparative depletion experiments, heparinized blood was divided into aliquots and incubated with Miltenyi MicroBeads at 2–8°C. Volumes and incubation times were optimized for each antigen and achieved approximately 90% depletion of the target cell [anti-CD4: 100 mcl/ml, 30 min; anti-CD8: 50 mcl/ml, 15 min; anti-CD14: 100 mcl/ml, 30 min; anti-CD15: 50 mcl/ml, 15 min; Basic (unconjugated) MicroBeads used for controls: 50 mcl/ml, 15–30 min; see Figure S1 in Supplementary Material]. Blood was diluted 1:1 with RPMI-1640 and passed through Miltenyi Biotec LS columns supported in magnets (MidiMACS Separation Unit, Miltenyi Biotec); columns had been pre-"primed" with 3 ml MACS buffer [0.5% bovine serum albumin (Sigma) and 2 mM EDTA (Sigma)], all as previously described (2). Depleted blood was collected in Universal containers. Granulocyte isolation using discontinuous Percoll gradient was performed as previously described (2). Neutrophils were rendered necrotic (when required) *via* heat shock at 60°C for approximately 20 min, until all cells were trypan blue (Sigma) positive by microscopy, and then allowed to cool.

## Whole Blood Lux Assay

This was performed as previously described in detail (17, 18). Briefly, for comparative depletion experiments, approximately $5 \times 10^5$ cfu mid-log phase *M. tuberculosis*-lux in 100 mcl PBS was added to triplicate samples of 900 mcl specific cell-depleted or undepleted (Basic MicroBead-treated) blood, already diluted 1:1 with RPMI as described above. For augmentation experiments, the same inoculum was added to triplicate samples of 450 mcl venous blood pre-mixed 1:1 with either RPMI-1640, viable neutrophils in RPMI-1640 or necrotic neutrophils in RPMI-1640. For each donor, inocula were identical between different depletion/augmentation conditions. Samples were incubated at 37°C on a rocking platform for 96 h; mycobacterial luminescence was measured after removal of supernatants, cell lysis with 10 ml water, centrifugation at 2,000 × *g* for 10 min and resuspension in 1 ml PBS. Supernatants did not contain significant quantities of mycobacteria despite effective release from cells during lysis (Figure S2 in Supplementary Material). Uninfected samples served as controls for cytokine analysis. For comparison of the effects of necrotic neutrophil debris versus supernatants, samples were processed identically except that all Percoll-isolated

neutrophils were heat-shocked and the necrotic neutrophils were then centrifuged at 1,200 × *g* for 6 min. Supernatants were removed and the necrotic cell pellet resuspended; 450 mcl aliquots of reserved whole blood were then mixed 1:1 with necrotic neutrophils, necrotic neutrophil supernatant, or RPMI-1640 alone in duplicate (plus one uninfected sample per donor and condition). The infecting organism in these experiments was *M. bovis* BCG, added at the same multiplicity of infection.

## Phagocytosis Assay

Samples were prepared as for the lux assay, with the addition of viable, necrotic, or no autologous neutrophils to venous blood, in duplicate per experimental condition. Samples were infected with 100 mcl FITC-labeled BCG organisms diluted according to optical density to approximately $2 \times 10^6$ CFU/ml. For each donor, inocula were identical between different augmentation conditions. Samples were mixed and incubated at 37°C for 1 h.

Subsequently, two 200 mcl aliquots were taken from the samples, to which 10 mcl anti-CD14-PE (Becton Dickinson) and 1 mcl BD Horizon™ Fixable Viability Stain 450 (Becton Dickinson) were added. After 20 min, red blood cells were lysed *via* 15-min incubation with 2 ml 1× BD Pharm Lyse™ (Becton Dickinson). Samples were then centrifuged at 600 × *g* for 5 min, resuspended in 1 ml Stain Buffer (BD Pharmingen™) with 12.5 mcl filtered trypan blue (Sigma), centrifuged again, fixed in 500 mcl Cytofix (Becton Dickinson) for 15 min and finally resuspended in 1 ml Stain Buffer. Samples were processed on a BD Fortessa flow cytometer and data analyzed with FlowJo software (Treestar). Figure S3 in Supplementary Material details the gating strategy employed; results are presented as mean of duplicate results per donor/condition.

## TNF and IL-10 ELISA

These were performed on supernatants according to manufacturer's instructions (Peprotech, Rocky Hill, NJ, USA). Final dilutions were fivefold; results are presented as mean of duplicate samples per patient/condition. Undetectable readings were assigned an arbitrary value of 1 pg/ml (below the limit of detection of the assay).

## Quantiferon-TB Gold™ In-Tube IFN γ Release Assay

This was performed according to manufacturer's instructions.

## Luminex™

Supernatants from 96-h blood samples infected with *M. tuberculosis*-lux were pooled across triplicates per participant/condition, filtered twice through 0.22 μm filter plates (Merck Millipore, Billerica, MA, USA) with centrifugation at 3,000 × *g* and diluted appropriately according to preliminary results: final dilutions were either 4-fold or 10-fold depending on the experiment. The Invitrogen 30-plex kit (Invitrogen, Carlsbad, CA, USA) was used according to manufacturer's instructions, but we used a lower volume of beads: either 12.5 or 8.4 mcl. Plates were read on a calibrated Luminex 200™ reader and results calculated by the Luminex 200™ software.

## Statistics

Parametric data were analyzed using Student's *t*-tests when comparing two groups (paired when appropriate) or one-way ANOVA for comparing three or more groups with *post hoc* correction. Non-parametric data were analyzed using Mann–Whitney *U*-tests or Wilcoxon signed rank tests for two groups and Kruskal–Wallis or Friedman testing with *post hoc* Dunn's correction for three or more groups.

All statistical analyses were performed using SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism version 4.0 or later. All reported *p*-values are two-sided; $p < 0.05$ was inferred as significant.

## Principal Component Analysis (PCA)

Principal component analysis and associated three-dimensional plots were generated using Qlucore™ Omics Explorer v2.3 software. Multi-group comparison was used to identify Luminex™-measured cytokines or chemokines that were differentially abundant between the supernatants of three conditions (blood augmented with viable neutrophils, necrotic neutrophils, or medium only). A *p*-value of maximum 0.05 and *q*-value of maximum 0.1 were used as thresholds. Individual points in the plots represented one donor in one augmentation condition and their position in the plot was determined by the combined effects of all parameters measured for the sample that significantly contributed to the overall between-group difference. The distance between sample points represents Euclidean distance and was calculated using all parameters that significantly contribute to the overall between-group difference. Component vectors are displayed, along with a percentage figure signifying the proportion of the variability in the data that each component accounts for.

# RESULTS

## Depletion of Neutrophils From Whole Blood Impairs Mycobacterial Control More Than Depletion of Other Cell Types

Aliquots of blood from 20 healthy, HIV-uninfected persons (10 with positive results from Quantiferon-TB Gold In-Tube IFN-γ release assay) were depleted of CD4+, CD8+, CD14+, or CD15+ cells *via* Miltenyi MACS magnetic beads or were incubated with non-conjugated beads as controls. This blood was then infected with *M. tuberculosis*-lux and incubated in rocking tubes for 96 h, as per the previously described whole blood lux assay (17). The mean (SD) neutrophil count in mock-depleted blood [treated with Basic (unconjugated) Microbeads, diluted 1:1 with RPMI-1640] was $1.42 \pm 0.53 \times 10^9$/l and in CD15-depleted blood was $0.20 \pm 0.17 \times 10^9$/l. **Figure 1** details the impact of cell depletion on the 96-h luminescence of *M. tuberculosis*-lux. CD15+ cell (granulocyte) depletion had a greater effect than depletion of any other cell type on mycobacterial luminescence (a surrogate of mycobacterial metabolism and/or number) at 96 h (mean RLU per 100 mcl 88,686 ± 44,734 in mock-depleted blood versus 146,681 ± 72,663 in CD15-depleted blood, 102,262 ± 52,568 in CD4-depleted blood, 97075 ± 38993 in CD8-depleted blood and 111,338 ± 52,879 in CD14-depleted blood; $p < 0.001$ for

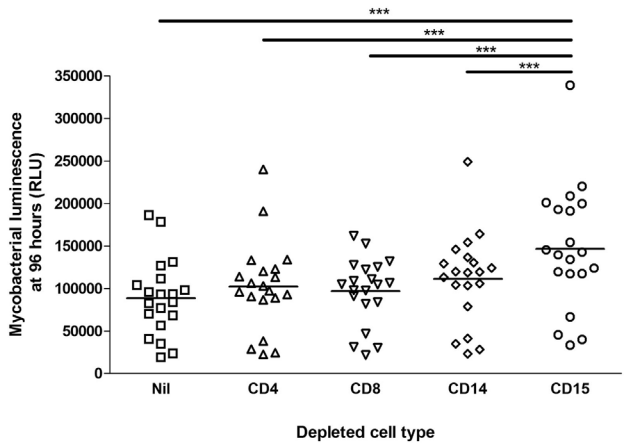


**FIGURE 1** | Impact of cell depletions on control of mycobacterial luminescence in whole blood. Blood was taken from 20 donors. 450 mcl of blood depleted of CD4+, CD8+, CD14+, CD15+ or no cells (in triplicate per donor per condition), diluted 1:1 with RPMI-1640, was infected with approximately $5 \times 10^5$ CFU *Mycobacterium tuberculosis*-lux in 100 mcl PBS. Samples were incubated on a rocking plate (20 rpm) at 37°C for 96 h before removal of supernatants, lysis of red blood cells with water, resuspension in 1 ml PBS and measurement of mycobacterial luminescence on at least two 100 mcl aliquots. Results presented are the mean of all measurements across triplicate samples for the relevant condition. ***$p < 0.001$ (one-way ANOVA).

CD15-depleted versus any other condition). We note that by pairwise comparison versus undepleted blood, both CD4 depletion ($p = 0.016$) and CD14 depletion ($p = 0.004$) also impaired control of mycobacterial luminescence, although the effects were more modest than with CD15 depletion.

## CD15+ Cell Depletion Has Relatively Little Effect on the Cytokine and Chemokine Concentrations in Supernatants of *M. tuberculosis*-Infected Blood

Luminex™ analyses of cytokines and chemokines in the supernatants from depletion experiments (see above) are summarized in **Figure 2** and **Table 1**, which indicate those analytes significantly affected by cell depletion after correction for multiple pairwise comparisons by the Bonferroni methodology. Uninfected control samples showed low or undetectable levels of most analytes, although when they were detected there was evidence that cell depletion by itself had a significant effect on cytokine and chemokine responses; subtracting baseline values from uninfected controls thus removes some of the biological effect of the depletion and we, therefore, present analysis on raw data.

Depletion of CD8+ cells had no effect on the cytokine profile of 96-h blood supernatants, while the effect of depletion of CD4+ cells significantly reduced supernatant concentrations of 16 predominantly Th1-associated cytokines, innate inflammatory cytokines, and chemokines. CD14+ cell depletion markedly reduced the concentration of 16 similar molecules. CD15+ cell depletion had less effect on the supernatant analyte profile than CD4+ or CD14+ cell depletion, but after correction for multiple comparisons there was significantly ($p < 0.05$) reduced CCL2 concentration versus undepleted blood [median (IQR) concentration 1,420 (807–1,684) ng/ml in CD15-depleted blood supernatants versus 1,903 (1,084–3,386) ng/ml in mock-depleted blood supernatants].

## Addition of Viable Neutrophils to Human Whole Blood Improves Mycobacterial Control at 96 h, While Addition of Necrotic Neutrophils Has the Opposite Effect

We proceeded to investigate the effect of augmenting blood with either viable or necrotic neutrophils on mycobacterial restriction and cytokine release. Neutrophils from nine donors were isolated by discontinuous Percoll gradient and divided into two equal aliquots; one half was then heat-shocked at 60°C (and subsequently cooled) and either viable neutrophils, necrotic neutrophils, or medium only were added to aliquots of the same donor's blood (kept aside at the time of venepuncture, for a maximum of 2 h). The whole blood lux assay was conducted as previously described (17, 18).

Overall neutrophil purity assessed by Coulter counting was 97.79 ± 1.16%. The non-augmented mean ± SD neutrophil count was $2.10 \pm 0.75 \times 10^9$/l versus $9.00 \pm 3.30 \times 10^9$/l in those samples augmented with viable neutrophils ($p < 0.0001$). Lymphocyte counts were not significantly different: $1.18 \pm 0.53 \times 10^9$/l in non-augmented versus $1.30 \pm 0.59 \times 10^9$/l ($p = 0.09$) in neutrophil-augmented blood. Luminescence readings at 96 h post-infection (performed in at least duplicate per donor per condition) were 9,379 ± 4,888 RLU per 100 mcl for non-augmented blood. Luminescence was significantly decreased in samples augmented with viable neutrophils (2,766 ± 1,672 RLU per 100 mcl, $p < 0.001$), and 96-h luminescence correlated inversely with the number of neutrophils using all data from non-augmented or viable-augmented samples ($p < 0.001$). Conversely, augmentation with necrotic neutrophils increased mean luminescence at 96 h post-infection to 11,722 ± 6,132 RLU per 100 mcl ($p = 0.013$ versus no augmentation, **Figure 3A**).

## Addition of Viable or Necrotic Neutrophils to Human Whole Blood Results in a Significant Alteration of Cytokine and Chemokine Response

Supernatants from these experiments were assayed by Luminex™ 30-plex technology. In total, 26 cytokines or chemokines were detectable with a median value greater than the lower limit of detection. To identify differences in inflammatory profile between the three conditions (non-augmented, viable neutrophil-augmented, or necrotic neutrophil-augmented) we employed Qlucore™ Omics Explorer v2.3 to perform PCA. Initial exploration of data suggested that neutrophil manipulation by itself had a significant effect on cytokine and chemokine responses; subtracting uninfected control values, therefore, removes some of the biological effect and we present analysis on raw data.

Principal component analysis showed clear differences in inflammatory profiles between the non-augmented, viable neutrophil-augmented, and necrotic neutrophil-augmented conditions

(**Figure 3B**; Table S1 in Supplementary Material details *p*- and *q*-values). **Figure 4** presents analyte concentrations and displays results from one-way ANOVA for each cytokine or chemokine found to contribute to the separation of groups in PCA. Maximal cytokine responses were seen in the non-augmented blood. Viable neutrophil augmentation was broadly anti-inflammatory,

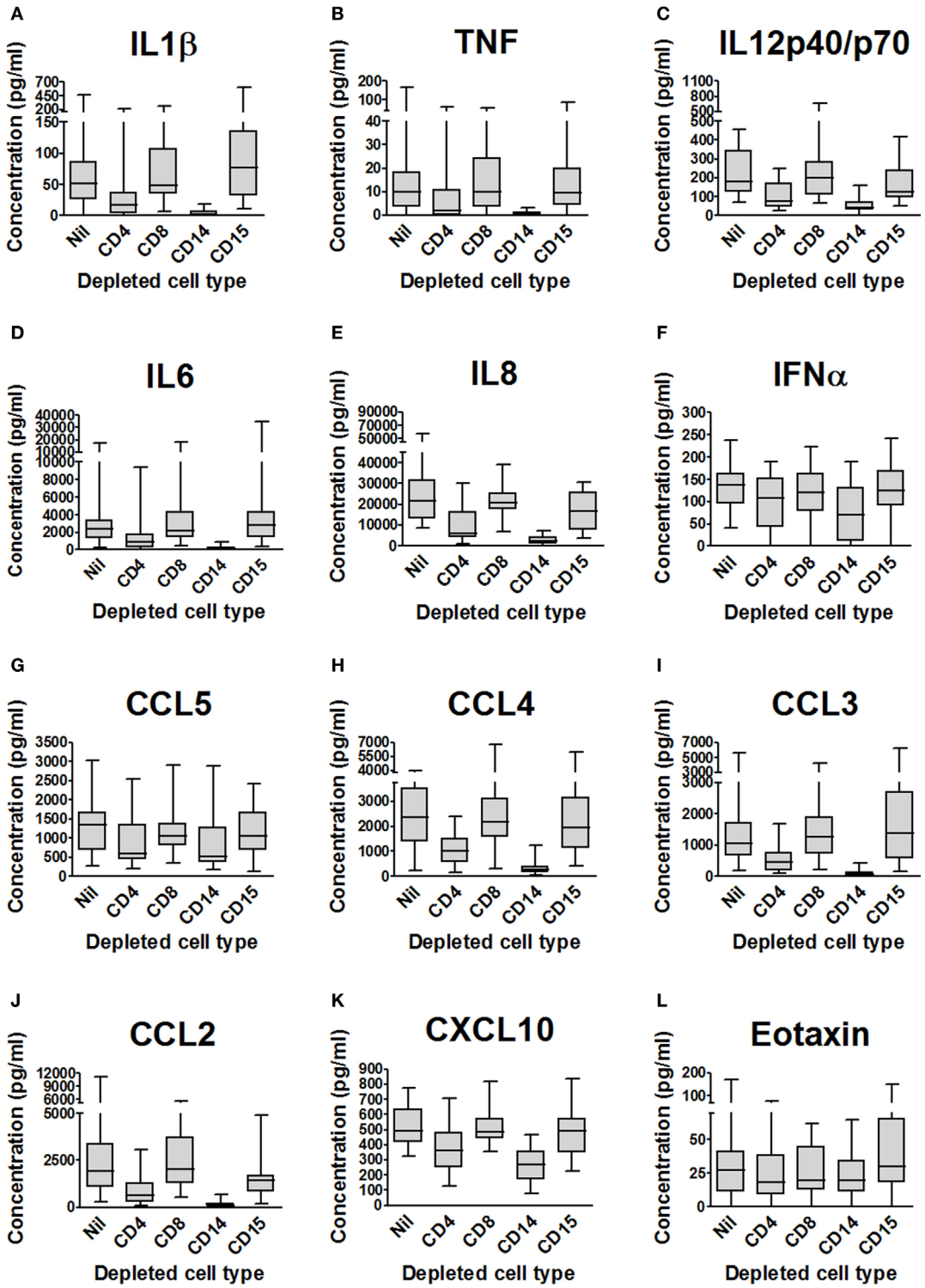


**FIGURE 2** | Continued

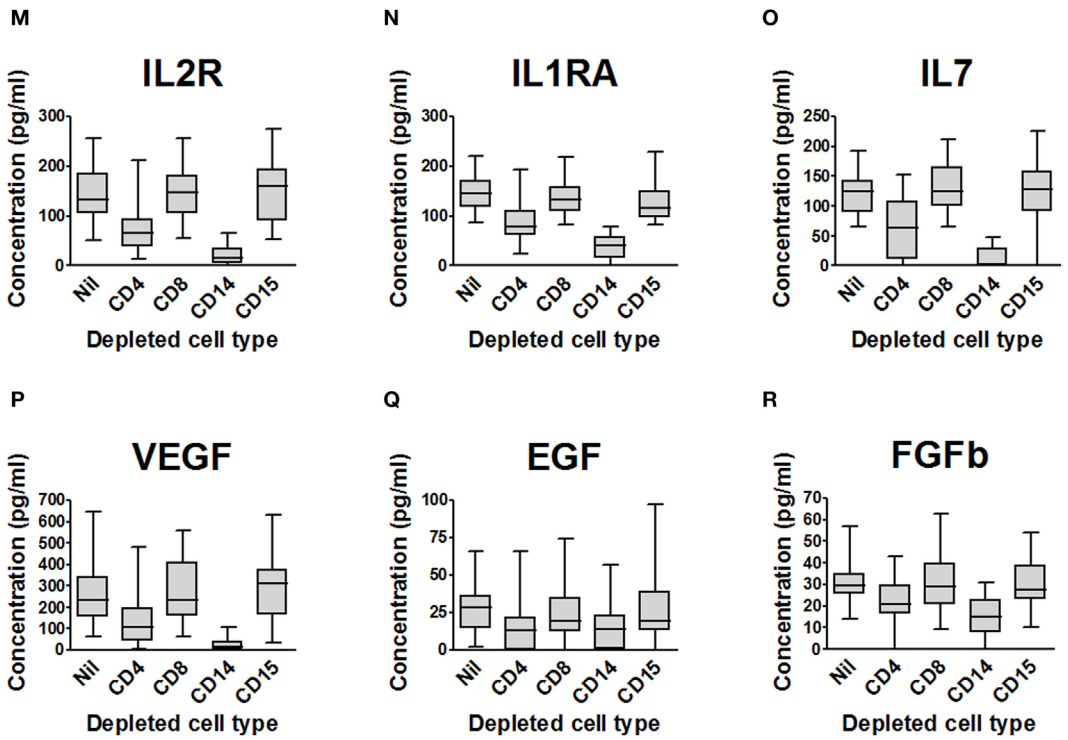


**FIGURE 2** | Impact of cell depletions on supernatant cytokines and chemokines from *Mycobacterium tuberculosis*-infected blood. **(A–R)**. Supernatants from the experiments presented in **Figure 1** (blood from 20 donors depleted of CD4+, CD8+, CD14+, CD15+ or no cells and infected with *M. tuberculosis*-lux for 96 h) were aspirated and stored at −80°C until analysis by Luminex™ technology. Box and whisker plots represent median, interquartile range, minimum and maximum values for each analyte. Abbreviations: VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; IL-2R, interleukin-2 receptor; IL-1RA, interleukin-1 receptor antagonist; FGFb, fibroblast growth factor basic; EGF, epidermal growth factor; CXCL, C-X-C motif ligand; CCL, C-C chemokine ligand; CD, cluster of differentiation.

reducing supernatant concentrations of IL-1β, TNF, IL-12p40/p70, CCL3, CCL5, interleukin-2 receptor (IL-2R), and fibroblast growth factor basic (FGFb); only hepatocyte growth factor (HGF) was increased. The addition of necrotic neutrophils to blood was also associated with lower supernatant concentrations of IL-1β, TNF, IL-12p40/p70, and FGFb, but simultaneously provoked an increase in IL-10, G-CSF, GM-CSF, and CCL2.

## Addition of Necrotic Neutrophils to Whole Blood Reduces Phagocytosis Overall, While Addition of Viable Neutrophils Reduces Phagocytosis of Mycobacteria Specifically by CD14+ Cells

To investigate the mechanism by which neutrophils elicited an anti-inflammatory response to mycobacterial infection, we measured phagocytosis of FITC-labeled *M. bovis* BCG by CD14+ cells (shown earlier to be the major source of cytokines and chemokines in the whole blood model, **Figure 2**).

The addition of necrotic neutrophils reduced the overall percentage of events associating with BCG-FITC+ (mean ± SD percentage 4.51 ± 1.15 versus 6.82 ± 2.21 with no augmentation, $p = 0.047$, and 6.11 ± 0.88 with viable neutrophil augmentation, $p = 0.027$; **Figure 5A**). However, among BCG-FITC+ events, viable—but not necrotic—neutrophils reduced relative phagocytosis of BCG by CD14+ cells after 1 h of incubation (mean ± SD percentage of BCG-FITC+ events associating with CD14+ monocytes: in non-augmented blood 3.62 ± 0.98, in viable neutrophil-augmented blood 1.24 ± 0.38, $p < 0.001$ versus non-augmented, in necrotic neutrophil-augmented blood 2.89 ± 075, $p < 0.01$ versus viable-augmented; **Figure 5B**). Correspondingly, the percentage of BCG-FITC+ associating with neutrophils significantly increased with the addition of viable neutrophils (mean ± SD percentage: in non-augmented blood 69.72 ± 8.90, in viable neutrophil-augmented blood 82.95 ± 3.74, $p < 0.001$ versus non-augmented, in necrotic neutrophil-augmented blood 74.09 ± 7.72, $p < 0.01$ versus viable-augmented; **Figure 5C**).

## The Immunosuppressive Effect of Necrotic Neutrophils Requires Direct Contact With Cellular Debris

We hypothesized that some of the immunosuppressive effect of necrotic neutrophils might be mediated *via* a soluble factor, as has previously been described with other organisms (20). We therefore added necrotic neutrophils, their supernatant, or medium

**TABLE 1** | Summary of impact of cell depletions on supernatant cytokines and chemokines from *Mycobacterium tuberculosis*-infected blood.

| | Depleted cell | | | |
|---|---|---|---|---|
| | **CD4** | **CD8** | **CD14** | **CD15** |
| VEGF | ↓ | | ↓ | |
| TNF | ↓ | | ↓ | |
| IFN-α | ↓ | | ↓ | |
| IL-8 | ↓ | | ↓ | |
| IL-7 | ↓ | | ↓ | |
| IL-6 | ↓ | | ↓ | |
| IL-2R | ↓ | | ↓ | |
| IL-1RA | ↓ | | ↓ | |
| IL-1β | ↓ | | ↓ | |
| IL-12p40p70 | ↓ | | ↓ | |
| FGFb | ↓ | | ↓ | |
| Eotaxin | | | | |
| EQF | | | ↓ | |
| CXCL10 | ↓ | | ↓ | |
| CCL5 | ↓ | | | |
| CCL4 | ↓ | | ↓ | |
| CCL3 | ↓ | | ↓ | |
| CCL2 | ↓ | | ↓ | ↓ |

*Down arrows indicate those analytes which remain significantly decreased in the depleted condition compared to mock-depleted ($p < 0.05$, Wilcoxon matched pairs) after adjustment for multiple comparisons via the Bonferroni methodology.*
*VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; IL-2R, interleukin-2 receptor; IL-1RA, interleukin-1 receptor antagonist; FGFb, fibroblast growth factor basic; EGF, epidermal growth factor; CXCL, C-X-C motif ligand; CCL, C-C chemokine ligand; CD, cluster of differentiation.*

alone to aliquots of blood in 17 donors before incubation with *M. bovis* BCG for 96 h. Supernatants were then aspirated and assayed for TNF and IL-10 using ELISA.

As shown in **Figures 5D,E**, the addition of necrotic neutrophils to blood resulted in reduced TNF and increased IL-10 similar to previous results (mean ± SD TNF concentration in non-augmented blood 1,069 ± 1,351 pg/ml, in necrotic neutrophil-augmented blood 613 ± 814 pg/ml, $p < 0.05$; mean ± SD IL-10 concentration in non-augmented blood 899 ± 478 pg/ml, in necrotic neutrophil-augmented blood 1,236 ± 870 pg/ml, $p < 0.05$). However, addition of the supernatants of necrotic neutrophils alone did not have a significant impact on cytokine levels, albeit trends were evident (mean ± SD TNF concentration 713 ± 680 pg/ml, mean ± SD IL-10 concentration 1,040 ± 582 pg/ml).

## DISCUSSION

Both viable (1) and necrotic (16) neutrophils are found in large numbers at the sites of human tuberculosis disease, where they will interact with mycobacteria and other cells of the immune system. We therefore investigated the impact of these cells on the control of mycobacteria and associated immune responses in a human blood model.

We have previously reported that neutrophils are crucial for the control of mycobacterial growth in human blood (21), and we here confirm and extend that finding *via* both depletion and augmentation experiments. Depletion of CD15+ granulocytes from blood has a greater adverse effect on mycobacterial control in whole blood than depletion of CD4+, CD8+, or CD14+ cells. It was interesting to note that depletion of CD15+ cells has relatively little effect on the cytokine and chemokine profile of *M. tuberculosis*-infected blood, suggesting that the mechanism of restriction of mycobacterial growth by neutrophils is likely to be directly mycobactericidal (or perhaps relates to cytokines and chemokines not measured here). Depletion of CD14+ cells and CD4+ cells did significantly impair the release of cytokines and chemokines, but this apparently had less effect on bacillary metabolism. These results raise the possibility that neutrophils, as the dominant cell type in human peripheral blood, are of importance for preventing hematogenous dissemination of bacilli. We note that, especially in areas of high endemicity, tuberculosis is noted not uncommonly as a cause of neutropenic sepsis (22, 23), while neutropenia is also associated with disseminated non-tuberculous mycobacterial infection and poor outcome from such infection (24).

Consistent with these findings, the addition of viable neutrophils to blood significantly improved control of mycobacteria. However, this intervention suppressed inflammatory responses and was associated with reduced phagocytosis of mycobacteria by CD14+ cells (organisms were instead taken up by neutrophils, which is likely to be simply a consequence of the relative numbers of each cell type). We propose that early intracellular killing by neutrophils results in less subsequent activation of innate and acquired immune responses. There may also be a contribution from the immunosuppressive effects of programmed apoptosis, although we have not specifically assessed this. It is interesting to note that *in vivo* data demonstrate poorer local IFN-γ response from CD4+ T cells in bronchoalveolar lavage samples from cavities, where neutrophils dominate (8). We note that the neutrophil augmentation experiments impacted on cytokine responses far more than neutrophil depletion experiments, which might reflect the greater absolute change in cell count of this manipulation.

We have observed (12) and others have also reported (13, 25) accelerated neutrophil necrosis in HIV-infected persons, and have hypothesized that this may be important in the aberrant host response to pathogens. Animal models also link neutrophil influx and associated necrosis to poor outcome (11), while neutrophilia in active tuberculosis correlates with poor outcome (4). We show here that the addition of necrotic neutrophils to blood, even in healthy donors, enhances the metabolism of *M. tuberculosis* while supressing protective cytokine responses believed to contribute to protection and increasing the release of immunosuppressive IL-10. We have previously demonstrated that the addition of necrotic neutrophils directly to mycobacteria does not restrict their growth by 1 or 24 h (2), and thus the effects seen here in the whole blood model are likely to be mediated *via* an impact on the responses of other immune cells to *M. tuberculosis* infection.

Simultaneously, growth factors (G-CSF and GM-CSF) and the chemokine CCL2 are also increased. Although favorable roles have been suggested for some of these molecules in mycobacterial infection, for example GM-CSF (26), their predominant role at sites of inflammation is to attract more cells—including more neutrophils.

The addition of necrotic neutrophils resulted in a global decrease in phagocytosis, with less cell events associating with organisms than with either viable neutrophil or no augmentation. However,

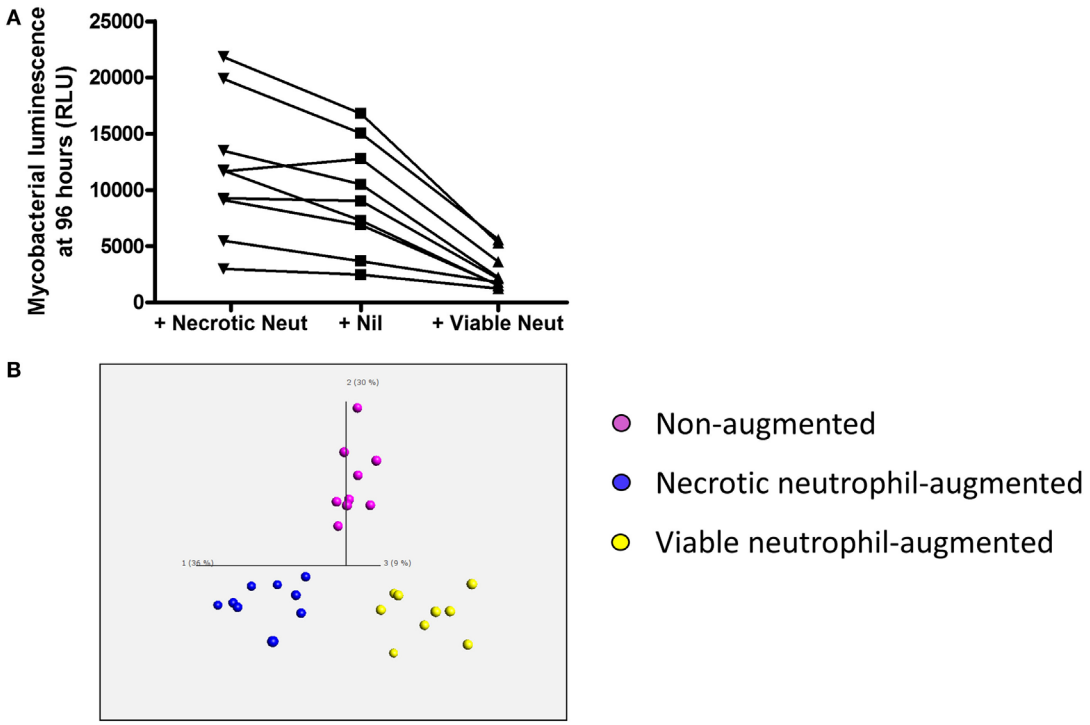


**FIGURE 3** | Impact of viable and necrotic neutrophil augmentation on control of mycobacterial luminescence by whole blood and on supernatant cytokines and chemokines from *Mycobacterium tuberculosis*-infected blood. **(A)** 100,000 RLU of *M. tuberculosis*-lux in 100 mcl PBS was inoculated into samples of 450 mcl whole blood plus either 450 mcl Percoll-isolated autologous neutrophils in RPMI-1640 heat-shocked at 60°C for 20 min and allowed to cool ("+Necrotic Neut"), 450 mcl RPMI-1640 only ("+Nil"), or 450 mcl room temperature Percoll-isolated autologous neutrophils in RPMI-1640 ("+Viable Neut"). After 96-h incubation, red blood cells were lysed and luminescence was measured on at least two aliquots of 100 mcl. Results are shown from nine independent donors. **(B)** Three-dimensional principal component analysis (PCA) plot generated using cytokine/chemokines that significantly contribute to differentiation between supernatants of augmentation conditions (calculated using multi-group comparison; purple = non-augmented, blue = necrotic neutrophil-augmented, yellow = viable neutrophil-augmented). PCA is a technique to reduce the dimensionality of complex datasets by transforming the data to a coordinate system. The first three coordinates (principal components) are represented as a 3D plot. The first principal component accounts for as much variability as possible within the data, and each succeeding component accounts for the next highest proportion of the variability possible, but under the constraint that it is not correlated with preceding components. This allows visualization of the differences between patient samples and analytes within complex datasets. Individual points represent one donor in one augmentation condition and their position in the plot is determined by the combined effects of all parameters measured for the sample that significantly contribute to the overall between-group difference. Component vectors for the three main components are displayed, along with a percentage figure signifying the proportion of the variability in the data that each component accounts for. Analysis is presented using raw values from infected supernatants.

there was not a specific effect on CD14+ cells. We therefore suggest that necrotic neutrophils reduce phagocytosis both by viable neutrophils (hence reducing early killing) and by mononuclear phagocytes (impairing protective cytokine responses), presumably because phagocytes internalize neutrophil debris instead. Indeed, the immunosuppressive process (reduction in TNF production and increase in IL-10), at least with BCG, required direct contact with cellular debris. We had hypothesized a directly suppressive role of neutrophil contents such as HNP 1–3, as previously described in other systems (20), but this does not appear to be a dominant mechanism in our BCG experiments (unfortunately we were unable to perform these same experiments with *M. tuberculosis*). Recent data have suggested an additional mechanism wherein necrotic neutrophils interfere with the phagosomal processing of *M. tuberculosis* by macrophages and thereby subsequent control of the mycobacteria (27).

We thus propose that in the context of active tuberculosis with necrotic neutrophilic inflammation, as may be seen severe tuberculosis or certain pathological states, there will be impaired mycobacterial control, reduction of protective but enhancement of immunosuppressive cytokine responses, and an ongoing influx of neutrophils to perpetuate this pathological cycle. This correlates with *post mortem* descriptions of tuberculous granulomas in HIV-1 co-infected patients which demonstrate extensive necrosis, marked neutrophil infiltration, and reduced TNF staining (13). Similarly, recent findings suggest that the CD15 neutrophil marker co-localizes with areas of necrosis and with IL-10 in tuberculous granulomas, and that the extent of this response correlates with *M. tuberculosis* burden (16). The results presented here suggest that the necrotic neutrophils may directly drive the IL-10 response and increased bacillary numbers rather than being simply epiphenomena.

This work has limitations, in addition to those already discussed above. Human hosts encounter *M. tuberculosis* at mucosal membranes rather than blood, although blood does represent a useful medium for study since it contains both innate

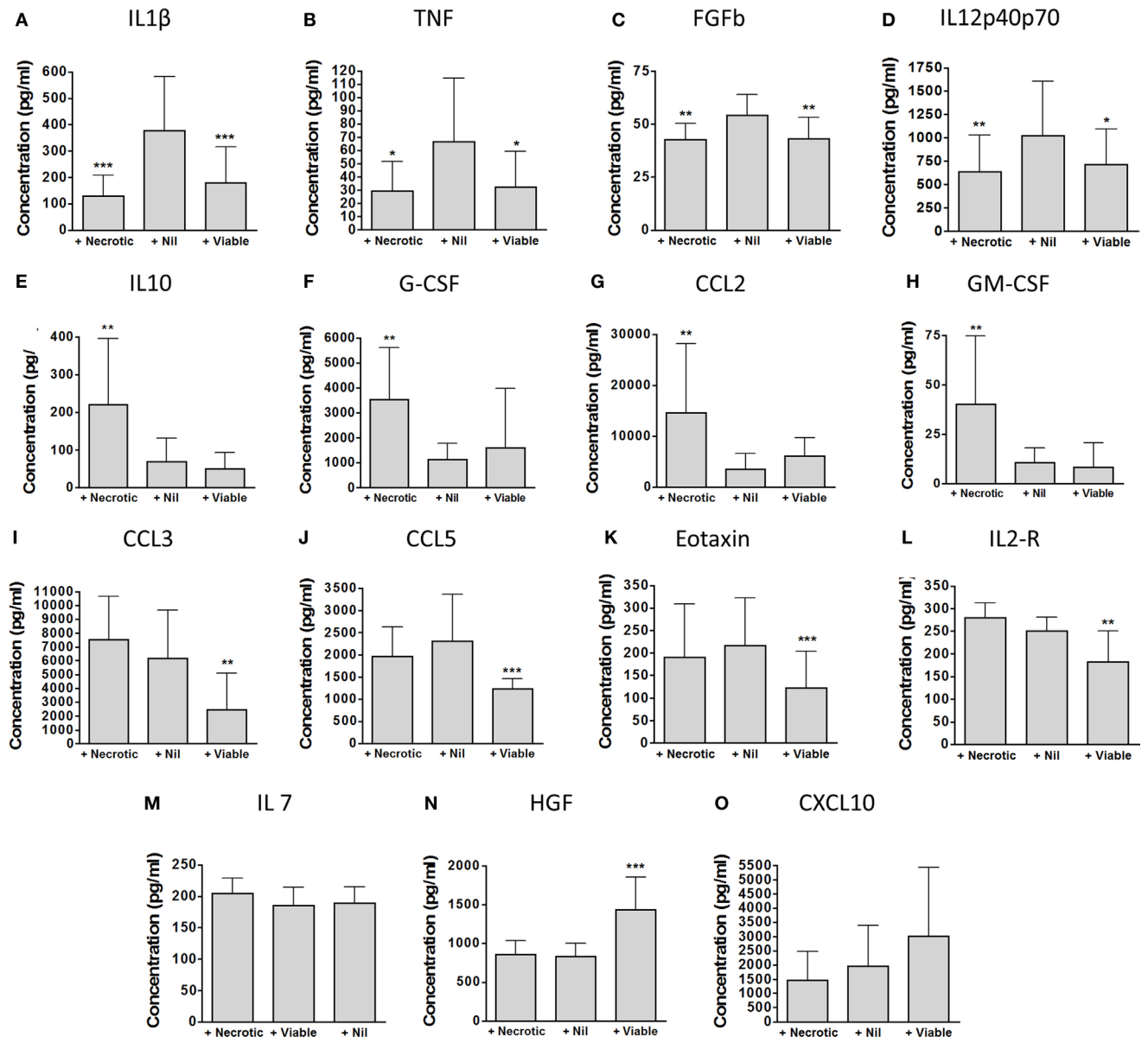


**FIGURE 4** | Detailed impact of viable and necrotic neutrophil augmentation on supernatant cytokines and chemokines from *Mycobacterium tuberculosis*-infected blood. **(A–O)** Cytokines and chemokines were measured in 96-h supernatants of *M. tuberculosis*-infected blood which had been augmented with necrotic autologous neutrophils ("+Necrotic"), with medium only ("+Nil"), or with viable autologous neutrophils ("+Viable"). Bars represent mean ± SD for each analyte found to be significant in principal component analysis. Presented are *p*-values from one-way ANOVA with Bonferroni correction comparing both augmented conditions with the medium-only control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data are from nine separate donors in four independent experiments. Abbreviations: IL, interleukin; TNF, tumor necrosis factor; FGFb, fibroblast growth factor basic; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; CCL, C-C chemokine ligand; CXCL, C-X-C motif ligand; IL-2R, interleukin-2 receptor; HGF, hepatocyte growth factor.

and acquired immune system components. Heat shock is not the mechanism by which neutrophils die *in vivo*, but it does reliably induce necrosis within a short time: this method thus intended that autologous aliquots of viable neutrophils and whole blood should not experience significant cell death or other changes in the interim while necrotic cells were prepared. Depletion was not absolute for the target cells, but we felt that an approximately 90% reduction in cell count was likely to be biologically significant.

As mentioned above, certain experiments were performed with *M. bovis* BCG rather than *M. tuberculosis* for reasons of biosecurity, and although we have previously seen similar results between these organisms in terms of neutrophil phagocytosis (2) and neutrophil control of mycobacterial luminescence (21), care should still be taken in extrapolating between organisms.

In summary, our data support the importance of neutrophils in the host response to tuberculosis both *via* a direct antimycobacterial

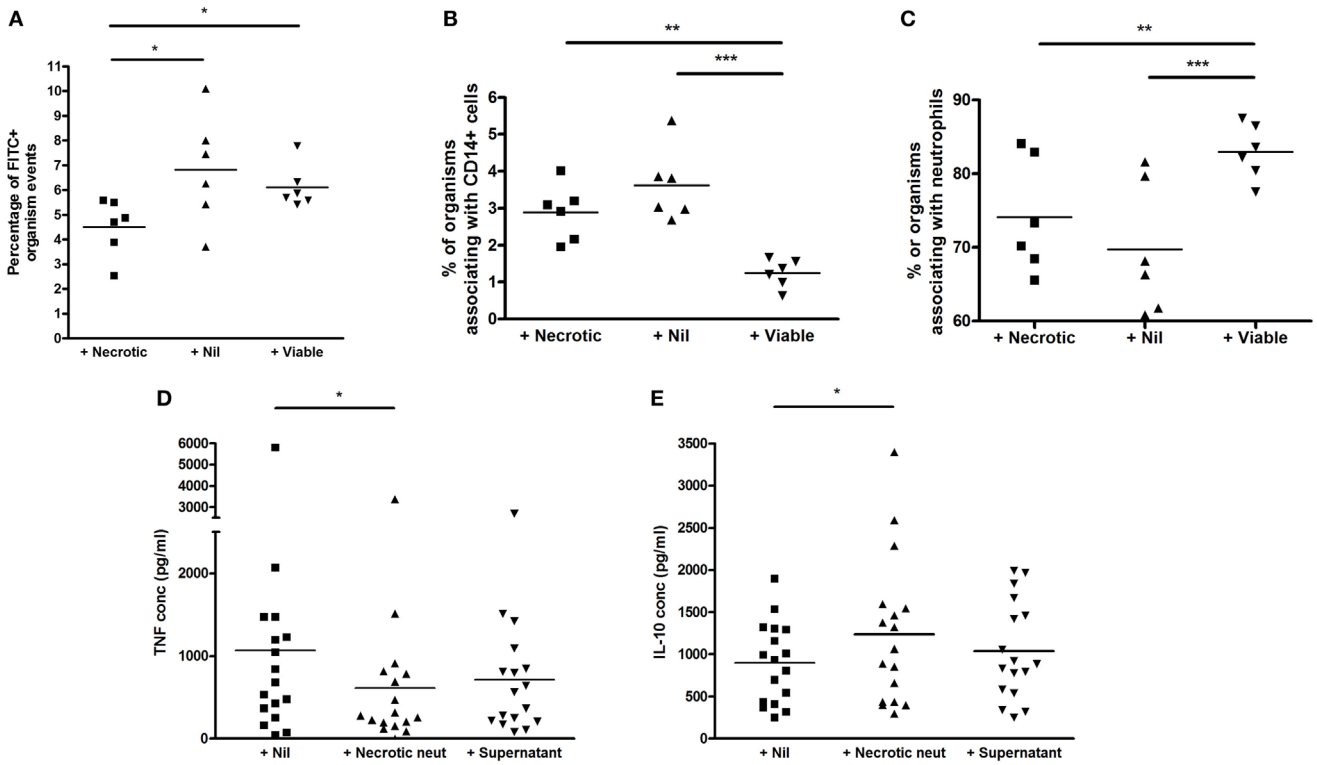


**FIGURE 5** | Effect of viable and necrotic neutrophil augmentation on phagocytosis of FITC-labeled *M. bovis* BCG, and differential effect of necrotic neutrophil augmentation versus supernatant of necrotic neutrophils on cytokine release in blood. **(A–C)** Blood from six donors (four independent experiments) was augmented with viable neutrophils, necrotic neutrophils, or medium alone, infected with $2 \times 10^5$ CFU FITC-labeled *M. bovis* BCG and incubated for 1 h at 37°C. 2 × 100 mcl aliquots were taken from each sample, incubated with CD14-PE and Viability Dye for 20 min, red blood cells were lysed, trypan blue was added to quench extracellular fluorescence and samples fixed in 2% paraformaldehyde before acquisition on a BD Fortessa flow cytometer. Results are presented as the percentage of BCG-FITC+ events **(A)**, the percentage of BCG-FITC+ events associated with CD14+ cells **(B)**, and the percentage of BCG-FITC+ events associated with neutrophils [as defined by forward and side scatter among CD14 negative events **(C)**]. **(D,E)** Blood from 17 donors (4 independent experiments) was augmented with necrotic neutrophils, the supernatant of necrotic neutrophils, or medium alone, infected with $2 \times 10^5$ CFU *M. bovis* BCG and incubated for 96 h at 37°C. Samples were centrifuged and supernatants stored at −80°C until analysis by ELISA for tumor necrosis factor (TNF) **(A)** or interleukin-10 **(B)**. Lines represent means, $^*p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$.

activity and *via* an effect on the development of acquired immune responses. In particular, necrotic neutrophils impair mycobacterial control, reduce protective cytokine responses, and drive IL-10 and growth factor release: this pathological cycle may contribute to the negative association between neutrophilia and host prognosis (4).

## ETHICS STATEMENT

The studies were approved by the University of Cape Town Research Ethics Committee (HREC 545/2010) in South Africa and NHS Research Ethics Committee (REC 08-H0720-46) in the UK and performed in accordance with the Declaration of Helsinki. Participants provided written informed consent.

## AUTHOR CONTRIBUTIONS

DL, JD, KW, RW, and AM conceived the experiments. DL, JD, and RG recruited participants and obtained samples. DL, JD, NB, and JN performed experiments. DL and JD performed data analysis. DL and AM wrote the draft manuscript and all authors contributed to manuscript revision, read and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00903/full#supplementary-material.

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# The Synergistic Effects of the Glutathione Precursor, NAC and First-Line Antibiotics in the Granulomatous Response Against *Mycobacterium tuberculosis*

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*Mycobacterium tuberculosis* (*M. tb*), the causative bacterial agent responsible for tuberculosis (TB) continues to afflict millions of people worldwide. Although the human immune system plays a critical role in containing *M. tb* infection, elimination proves immensely more challenging. Consequently, there has been a worldwide effort to eradicate, and limit the spread of *M. tb* through the conventional use of first-line antibiotics. Unfortunately, with the emergence of drug resistant and multi-drug resistant strains of *M. tb* the archetypical antibiotics no longer provide the same ascendancy as they once did. Furthermore, when administered, these first-line antibiotics commonly present severe complications and side effects. The biological antioxidant glutathione (GSH) however, has been demonstrated to have a profound mycobactericidal effect with no reported adverse consequences. Therefore, we examined if N-Acetyl Cysteine (NAC), the molecular precursor to GSH, when supplemented in combination with suboptimal levels of standalone first-line antibiotics would be sufficient to completely clear *M. tb* infection within *in vitro* derived granulomas from healthy subjects and individuals with type 2 diabetes (T2DM). Our results revealed that by virtue of immune modulation, the addition of NAC to subprime levels of isoniazid (INH) and rifampicin (RIF) was indeed capable of inducing complete clearance of *M. tb* among healthy individuals.

**Keywords: tuberculosis, cytokines, antibiotics, antitubercular, *Mycobacterium tuberculosis*, type 2 diabetes**

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## INTRODUCTION

*Mycobacterium tuberculosis* (*M. tb*), the infectious pathogen responsible for tuberculosis (TB), caused 10.4 million new cases of active disease and 1.8 million deaths worldwide in 2016 alone (1). Additionally, it is estimated that currently one third of the world's population is latently infected with *M. tb* (1). *M. tb*'s mode of transmission is mitigated via aerosol droplets typically by means of coughing, which if inhaled leads to bacterial seeding within the host's lower respiratory tract. Although TB primarily affects the lung parenchyma, it may also impact bone, the central nervous system, and other organ systems (2). Upon infection, a competent immune system governs the integral processes of host defense against the contagion (3, 4).

Archetypically, early innate defenses fail to completely eradicate the invading *M. tb* (5). Consequently, macrophages commit to the cytokine mediated recruitment of additional immune cells, which form an early infectious lesion and beginnings of a granuloma (6, 7). Upon suitable cell signaling, monocytes in the proximity of the infectious region will differentiate into macrophages, a crucial step in granuloma formation and *M. tb* containment (8). A granuloma is a host defense mechanism orchestrated by various immune cells including: macrophages, dendritic cells, T cells, fibroblasts, epithelioid histiocytes, giant cells, and natural killer cells to control and contain a *M. tb* infection by encapsulating the bacteria in order to limit its spread and obstruct it from nutriment; thus, rendering it latent. This granulomatous containment of *M. tb* within the lungs is commonly referred as latent tuberculosis (LTBI), as an individual is no longer infectious at this stage. However, if an individual with LTBI becomes immunodeficient or immunocompromised the granuloma can undergo liquefaction and the latent bacteria undergo reactivation to cause an active TB disease (9).

Individuals with Type 2 diabetes mellitus (T2DM) are typically more vulnerable to bacterial infections, especially *M. tb* as a result of compromised cell mediated immunity (10, 11). T2DM is characterized by hyperglycemia caused by insulin resistance, and accounts for 90–95% of the total prevalence of diabetes (12). Glycated hemoglobin (HbA1c) is indicative of the levels of plasma glucose over a period of several weeks and is correspondingly used as a diagnostic for T2DM. Roughly 10% of TB cases are linked to diabetes, and it is estimated that patients with T2DM are nearly three times more likely to develop an active TB compared to healthy individuals. Conjointly, patients with both T2DM and TB habitually have worse outcomes, thus the risk of mortality is approximately double for those with the comorbidity (13, 14).

Although *M. tb* proliferates within alveolar macrophages, its presence within an adept immune system will induce the release of beneficial cytokines, which in turn activate these macrophages to stimulate their mycobactericidal faculties (15). The cytokines released by macrophages and the other aforementioned cell types possess essential regulatory effects and participate in the host defense against *M. tb* throughout the formation of the granuloma and the subsequent containment and extermination of the mycobacteria (15, 16). The interaction between *M. tb* and granulomatous cells of both the innate and adaptive immune system result in the constant secretion of cytokines; most notably, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin-10 (IL-10), IL-6, IL-2, IL-12, and interferon gamma (IFN-$\gamma$) (15–17). Additionally, the functionality of polarized CD4+ T cells are largely based on these cytokine secretions. Type-1 T helper (Th1) cells produce IFN-γ, IL-2, and TNF-α, a mechanism thought to be a pivotal in the suppression of *M. tb* growth and replication (18). IFN-γ stimulates dendritic and macrophage differentiation and activation, as well as cell-mediated immunity in the interest of control over intracellular infections (19). TNF-α is predominantly responsible for the cellular recruitment and aggregation necessary for granuloma formation as well as the production of other cytokines, such as IL-1, IL-6, and the indirect stimulation of IFN-γ and IL-2, which can then amplify the TNF-α production (20–23). These cytokines serve in the immunomodulatory role that helps establish granuloma formation, macrophage differentiation, and mediate mycobactericidal potentiality in conjunction with their inflammatory functionality.

The Center for Disease Control and Prevention (CDC) recommends that the preferred treatment regimen for active TB includes the four first-line antibiotics: Isoniazid (INH), Rifampin (RIF), Ethambutol (EMB), and Pyrazinamide (PZA) (12). However, there are a myriad severe complications and side effects which can come from consuming these aforementioned antibiotics. For example, patients taking INH often experience elevated levels of liver enzymes, supplementary to a black box warning for severe and sometimes fatal hepatitis (24, 25). Individuals taking RIF are known to experience: fever, gastrointestinal disturbances, rashes, and immunological reactions. More severe side effects, such as peripheral neuropathy, joint pain, and visual impairment, are also possible when these antibiotics are administered (24–26). However, the most adverse reaction a patient may experience is hepatotoxicity; therefore, patients often undergo frequent liver functionality tests to detect early liver damage (24, 25).

To investigate a novel therapeutic agent to augment the treatment of TB we examined the prophylactic effects of N-acetyl cysteine (NAC), the precursor molecule to the synthesis of the biologic antioxidant glutathione (GSH), when supplemented with individual first-line antibiotics. GSH is a tripeptide comprised of glutamate, cysteine and glycine, which primarily functions in the protection of cells and tissues by reducing oxidative damage thus establishing redox homeostasis within the body (27). Our laboratory has previously demonstrated that GSH has direct antimycobacterial effects (28–30). The primary mechanism of NAC/GSH anti-mycobacterial effects could be due to a shift in redox balance. Mycobacteria lack GSH and possess an alternative thiol, mycothiol to regulate redox homeostasis. Therefore, presence of millimolar concentration of GSH (physiological concentrations) inside the macrophages can cause reductive stress in mycobacteria leading to inhibition in the growth of *M. tb* (28–30). Both GSH and NAC have been previously shown to diminish TB pathology and inflammation by immune-modulation, as well as possess antimycobacterial potentiality (31–35). GSH and NAC's potent anti-inflammatory

effects are thought to be by means of inhibiting the activation of nuclear factor-κB (NF-κB) as well as the specific inhibition of other proinflammatory cytokine synthesis (36–38). In both experimental animal models as well as clinical studies, NAC has been shown to have a protective effect against liver damage from anti-tuberculosis medications (39–42). Additionally, Amaral et al. reported that NAC exhibits potent anti-mycobacterial effects and may limit *M. tb* infection and disease both through suppression of the host oxidative response and through direct antimicrobial activity (35). Furthermore, Vilcheze et al. recently demonstrated that the combination of cysteine or other small thiols with either isoniazid or rifampicin prevents the formation of drug-tolerant and drug-resistant *M. tb* cultures by shifting the menaquinol/menaquinone balance toward a reduced state which stimulates *M. tb* respiration and converts persister cells to metabolically active cells. This prevention of both persister cell formation and drug resistance ultimately leads to mycobacterial cell death (43).

Therefore, using *in vitro* generated granulomas from both healthy and T2DM individuals, we tested the synergistic effects of NAC (GSH) as an immunoadjuvant administered in supplementation with either INH, RIF, or EMB in opposition to the highly virulent Erdman strain of *M. tb*. Our findings indicate that there was not only a significant reduction in the intracellular viability of *M. tb*, but an increase in beneficial immunomodulatory effects was also observed when the first-line antibiotics INH, RIF, or EMB were supplemented with NAC, in comparison to the treatment of the standalone antibiotics.

# MATERIALS AND METHODS

## Statement of Ethics

This study was approved by both the Institutional Review Board (IRB) and the Institutional Biosafety Committee (IBC) of the Western University of Health Sciences. All study participants were above the legal age of consent at the time of participation and written informed consent was obtained from the volunteers prior to their participation in the study.

## Subject Recruitment

Thirteen subjects (8 healthy individuals and 5 participants with T2DM) were recruited for this study. Participants belonging to the healthy group were in the age group between 20 and 65 years and had no history of HIV infection or TB. Additionally, the healthy individuals presented a glycated hemoglobin (HbA1c) of less than 5.7%. Individuals with T2DM who participated in this study were recruited without any preference for age, gender, or ethnicity. The inclusion criteria for T2DM group required that the participants have documentation of a T2DM diagnosis through Dr. Airani Sathananthan at the Patient Care Center of Western University of Health Sciences, and a recorded HbA1c above 8%. Exclusion criteria for the T2DM patients stipulated that they presented no history of autoimmune disease, HIV infection, hepatitis, or TB and were not currently being treated with metformin, as previous studies have indicated that metformin has protective effects against *M. tb* (44, 45). After obtaining signed informed consent, forty milliliters (mL) of blood was drawn for research laboratory tests from the volunteers.

## Preparation of Bacteria for Infection Assays

An Erdman strain of *M. tb* was used for all our infection studies, which expresses green fluorescent protein (GFP). This Erdman strain of *M. tb* (henceforth referred to as *M. tb*) was provided by Dr. Selvakumar Subbian at Rutgers New Jersey Medical School. Erdman strain of *M. tb* has slightly faster doubling time and therefore is considered more virulent compared to the standard laboratory H37Rv strain (46). *M. tb* was cultured in 7H9 media medium (Hi Media, Santa Maria, CA, USA) supplemented with albumin dextrose complex (ADC) (GEMINI, USA) and incubated at 37°C until the bacteria reached logarithmic phase of growth. Bacterial cultures were processed for infection once the static cultures reached peak logarithmic growth phase (indicated by an optical density between 0.5 and 0.8 at A600). The processing steps involve washing *M. tb* cultures with PBS (Sigma, St Louis, MO, USA), and subsequently disaggregating the bacterial clumps by vortexing five times with 3 mm sterile glass beads at 3 min intervals. *M. tb* suspension was then filtered using a 5 μm syringe filter to remove any remaining bacterial aggregations. The single cell suspension of now processed *M. tb* was then serially diluted and plated on 7H11 agar (Hi Media, Santa Maria, CA, USA) to determine the bacterial numbers present in the processed stock. Aliquots of processed bacterial stocks were then frozen and stored in cryogenic freezer at −80°C. At the time of the experimental trial, the frozen-processed stocks of *M. tb* were thawed and used for the infection. All infection studies and handling of the *M. tb* was done inside a certified biosafety level 3 facility (BSL-3).

## To Determine the Direct Antimycobacterial Effects of NAC and Synergistic Effects of NAC Added in Conjunction With an Antibiotic

To determine the direct effects of NAC and synergistic effects of NAC+antibiotic in altering the survival of *M. tb*, bacteria ($6 \times 10^4$ /well) were grown in 7H9 media in 24 well tissue culture plates (Corning, NY, USA) in the presence and absence of NAC and NAC+antibiotic for 15 days. *M. tb* cultures were then either sham-treated (control) or treated with the minimum inhibitory concentration (MIC) of various first-line antibiotics with or without NAC supplementation, comprising of: INH (0.125 micrograms/ml), INH (0.125 micrograms/ml) + NAC (10 mM), RIF (0.125 micrograms/ml), RIF (0.125 micrograms/ml) + NAC (10 mM), EMB (8.0 micrograms/ml) or EMB (8.0 micrograms/ml) + NAC (10 mM). With the exception of control category, all treatment groups received their preexisting additives every 4 days with the same aforestated concentration until their termination. *M. tb* cultures were maintained at 37°C, with 5% $CO_2$ until they were terminated at 8 and 15 days to determine the viability of *M. tb*. *M. tb* viability was ascertained by plating the diluted samples on 7H11

agar medium (Hi Media, Santa Maria, CA, USA) enriched with albumin dextrose complex (ADC) (Gemini, USA).

## Isolating Peripheral Blood Mononuclear Cells From Human Blood

Peripheral blood mononuclear cells (PBMC) were isolated from the whole blood of both healthy individuals and T2DM patients by density gradient centrifugation using ficoll histopaque (Sigma, St Louis, MO, USA), a high density-pH neutral polysaccharide solution. PBMCs were then suspended in RPMI (Sigma, St Louis, MO, USA) containing 5% human AB Serum (Sigma, St Louis, MO, USA) and distributed at $6 \times 10^5$ cells/well into 0.001% poly-lysine (Sigma, St Louis, MO, USA) coated 24-well plates (Corning, NY, USA) for further studies.

## PBMC Infection and Treatment of Granulomas

PBMCs were infected with processed virulent *M. tb* at a multiplicity of infection (MOI) of 0.1:1 cell ratio (approximately $6 \times 10^4$ bacteria were added to $6 \times 10^5$ PBMCs). Infected PBMCs were then either sham-treated or treated with the minimum inhibitory concentration (MIC) of various first-line antibiotics with or without NAC supplementation, comprising of: INH (0.125 micrograms/ml), INH (0.125 micrograms/ml) + NAC (10 mM), RIF (0.125 micrograms/ml), RIF (0.125 micrograms/ml) + NAC (10 mM), EMB (8.0 micrograms/ml) or EMB (8.0 micrograms/ml) + NAC (10 mM). With the exception of control category, all treatment groups received their preexisting additives every 4 days with the same aforestated concentration until their termination. Infected PBMCs were maintained at 37°C, with 5% $CO_2$ until they were terminated at 8 and 15 days post-infection, the time determined for sufficient granuloma formation.

## Termination of Granulomas to Determine the Survival of *M. tb*

Previous research by Kapoor et al. demonstrated that granuloma formation occurs approximately seven days post PBMC-infection with *M. tb* (47). Therefore, we terminated and harvested our *in vitro* granulomas at 8 and 15 days post infection to determine the intracellular survival of *M. tb* inside the untreated, antibiotic-treated and antibiotic + NAC-treated granulomas. During termination, cell free supernatants from each well were collected and stored, and granulomas were harvested by adding 250 µl of ice-cold, sterile 1X PBS (Sigma, St, Louis, MO, USA) followed by gentle scraping to achieve maximum recovery of granuloma lysates from the wells. Lysates were then efficaciously vortexed followed by a freeze/thaw cycle in order to ensure that there is sufficient rupture of cells and release of intracellular *M. tb*. The collected lysates and supernatants were then diluted as necessary in sterile 1X PBS (Sigma, St, Louis, MO, USA) and plated on 7H11 agar medium (Hi Media, Santa Maria, CA, USA) enriched with ADC (GEMINI, USA) and incubated at 37°C for 3 weeks, in order to evaluate the mycobacterial growth or survival under different treatment conditions by counting the colony forming units (CFUs).

## Cytokines Measurements of Granuloma Culture Medium

Levels of cytokines such as IFN-γ, TNF-α, IL-12, IL-10, and IL-6, were measured in the granuloma supernatants at 8 and 15 days post-infection to determine the effects of antibiotic and antibiotic + NAC treatments in altering the production of cytokines. Cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA). The assay was performed as per the manufacturer's protocol (Affymetrix, San Diego, CA, USA).

## Assessment of GSH Levels in the Cellular Lysates

The effects of antibiotic and antibiotic + NAC treatments in altering the levels of GSH in *M. tb*-granulomas was determined by measuring the levels of GSH in the granuloma lysates by colorimetric method using an assay kit from Arbor Assay (K006-H1). Granuloma lysates were first comprehensively mixed with an equal volume of cold 5% sulfosalicylic acid (SSA), followed by incubation for 10 min at 4°C, and subsequently centrifuged at 14,000 rpm for 10 min. The GSH was thereupon measured in the supernatants following the manufacturer's instructions. The rGSH (reduced GSH) was then calculated by subtracting GSSG (oxidized glutathione) from the total GSH. All measurements were accordingly normalized by the total protein levels and the results were reported in moles of GSH per gram of protein.

## Quantifying Levels of Malondialdehyde

Malondialdehyde (MDA), a byproduct of lipid peroxidation serves as an important marker for oxidative stress. MDA levels were measured calorimetrically using an assay kit (TBARS assay kit) from Cayman Chemicals. The assay principle is based on reaction between MDA and thiobarbituric acid at 100°C leading to a color change, which can be measured calorimetrically at 535 nm. All measurements were corrected for total protein levels.

## Staining and Imaging of Granulomas

The experimental setup in each trial includes setting aside granulomas for microscopic studies (light and fluorescent). This was accomplished by generating granulomas on cover glass positioned in wells of tissue culture plates. The granulomas on cover glasses were dedicated for cellular imaging. Granulomas terminated at 15 days post-infection were fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature and then washed with 1X PBS for 5 min to remove any cell debris. Fixed granulomas on the cover glasses were then stained with Hematoxylin and Eosin (H&E) (Poly Scientific, Bay Shore, NY, USA) for 2 min at room temperature and destained with tap water. The coverglass with the stained granulomas were mounted onto glass slides with mounting media (HistoChoice).

The alternative cover glasses with granulomas were used to determine the extent of acidification of the *M. tb*-containing phagosomes within the macrophages present.in the granulomatous. LysoTracker red DND99 (Introgen, Eugene, Oregon, USA), an acidotropic dye and a weak base conjugated to a red fluorophore, was added to the cells on the designated coverglass wells on the day of infection. LysoTracker freely

permeates the cell compartment and get trapped inside the acidified compartments such as lysosomes. Acidified compartments were therefore labeled with LysoTracker. Since the *in vitro* granulomas were generated using *M. tb* that express GFP, LysoTracker staining will help determine the whether or not the pathogen is inside the acidified compartment inside the cells. Granulomas terminated at 15 days post-infection were fixed with 4% PFA and cover glasses were mounted on glass slides with 4.5 μL of mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA). The stained slides were observed under an inverted fluorescent microscope to quantify the extent of co-localization of GFP-expressing *M. tb* with LysoTracker. Images were obtained using an integrated digital camera and analyzed by counting the green, red and yellow bacterial cells. Fixed granulomas on cover glasses were also stained for necrosis, reactive oxygen intermediate (ROI) production, and for the expressions of CD86 (marker on macrophages and dendritic cells), CD4 and CD8. ROI production in the granulomas was determined by cellROX staining. Untreated and NAC-treated granulomas from healthy subjects and individuals with T2DM were treated with 5 μM cellROX green reagent (Life Technologies, C10444) and incubated at room temperature for 30 min in dark. Stained slides were observed under an inverted fluorescent microscope to evaluate the extent of ROI production. Untreated and NAC-treated granulomas from healthy subjects and individuals with T2DM were also treated with 0.1 μg/ml of Propidium Iodide (eBioscience) and incubated at room temperature for 10 min in dark. The stained coverglasses were mounted on glass slides and observed under an inverted fluorescent microscope to determine the extent of cell death due to necrosis. To determine the types of immune cells, present in the *in vitro* granulomas, fixed samples from healthy subjects and individuals with T2DM were stained with antibodies conjugated to the fluorescent markers such as CD86-PE (eBioscience, 12–0149), CD4-Cy5 (eBioscience, 15–0049), and CD8-Cy5 (eBioscience, 15–0088), and incubated in the dark for 30 min. Slides were observed under an inverted fluorescent microscope to determine the cell types present in the granulomas.

## Statistical Analysis

Statistical data analysis was performed using GraphPad Prism Software version 7. Levels of cytokines, GSH, MDA and CFUs were compared between untreated control, antibiotic-treatment and treatment with antibiotics in conjunction with NAC using the unpaired *t*-test with Welch correction. Reported values are in means. $p < 0.05$ was considered significant ($^{*}p < 0.05$, $^{**}p < 0.005$).

# RESULTS

## Survival of Erdman Strain of *M. tb* in Growth Media

We first tested the direct effects of NAC and synergistic NAC + antibiotic combination in altering the growth of *M. tb* grown in 7H9 media. Approximately, $6 \times 10^4$ *M. tb* were maintained in 7H9 media in 24-well plates and incubated for 15 days in the presence and absence of additives. *M. tb* cultures were terminated at 8-day and 15-day time points. In sham-treated control group, there was a significant increase in the bacterial survival at the 15-day time point (**Figure 1A**). To identify whether NAC has direct killing effects on *M. tb*, we treated the Erdman strain with 10 mM NAC four times during the study, and this resulted in a significant inhibition in the growth of *M. tb* at both 8 and 15 days time points (**Figure 1B**). There was a significant decrease at 15 days (**Figure 1B**). The synergistic effects of NAC with front line antibiotics against *M. tb* was also tested in the absence of host cells. The trends for all the 3 antibiotics, INH, RIF and EMB, were the same, i.e. there was a statistically significant decrease in the viability of *M. tb i*n single antibiotic categories (**Figures 1C–E**). Administration with the combination of antibiotics and NAC had a completely clearance of bacteria (**Figures 1C–E**).

## *M. tb* Survival and Effector Responses Against the Pathogen Inside NAC-Treated Granulomas From Healthy Subjects

To build an *in vitro* model for human granulomas, we first infected PBMCs isolated from peripheral blood of healthy individuals with *M. tb* at MOI 0.1:1 and incubated for 15 days. PBMCs tended to form microscopic granulomas at 7-day time point and this was confirmed by staining the *in vitro* granulomas with Hematoxylin-Eosin. We observed more solidified and robust aggregation of cells in the granulomas treated with NAC compared to the untreated control group from healthy individuals (**Figures 2C,D**). NAC-treatment also resulted in a significant three-fold decrease in the intracellular survival of *M. tb* inside the granulomas compared to the untreated control group (**Figure 2B**).

To determine the underlying effector mechanisms against *M. tb* infection in NAC-treated granulomas, we quantified the levels of GSH, cytokines and the extent of phagosome-acidification. There was more than a two-fold increase in the levels of total GSH in NAC-treated granulomas compared to the untreated control group from healthy individuals (**Figure 2E**). Enhancement in the levels of GSH in NAC-treated granulomas was accompanied by a significant three-fold increase in the production of IFN-γ (**Figure 2F**). Phagosome acidification is considered to be one of the important effector mechanism that is responsible for intracellular killing of *M. tb*. NAC-treatment of granulomas also resulted in downregulation in the production of TNF-α. Measurement of TNF-α levels in the supernatants recovered from NAC-treated granulomas at 8 and 15 days indicated a two and three-fold decrease, respectively in the production of this cytokine (**Figures 2G,H**). In comparison to the untreated control group there was a statistically significant increase in the number of *M. tb* inside the acidified compartments in NAC-treated granulomas (**Figures 2I,J**). However, in untreated granulomas, bacteria were mostly observed inside the non-acidified compartments (**Figure 2I**). To examine the production of ROI in the granulomas cellROX staining was performed. Intense cellROX staining was observed in untreated granulomas (**Figure 2K**). NAC-treatment resulted in a decrease in the levels of ROI as evident from the diminished uptake of cellROX (**Figure 2L**).

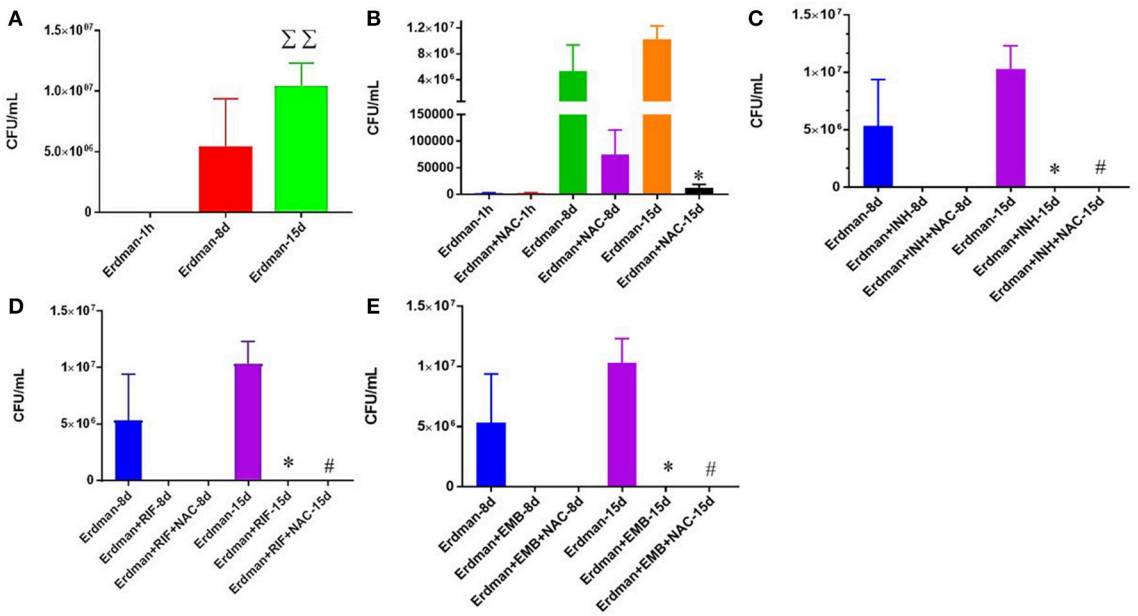


**FIGURE 1 |** Survival of Erdman strain of *M. tb* in 7H9 media**.** *M. tb* grown in 7H9 media containing no additives **(A)**, *M. tb* grown in 7H9 media containing NAC **(B)**, *M. tb* grown in 7H9 media containing INH and INH+ NAC **(C)**, *M. tb* grown in 7H9 media containing RIF and RIF + NAC **(D)**, and *M. tb* grown in 7H9 media containing EMB and EMB+ NAC **(E)**. There was a significant increase in bacterial numbers at 15 days when *M. tb* was grown in 7H9 in the absence of any additives **(A)**. There was a significant reduction in the bacterial numbers at 15 days when *M. tb* was grown in 7H9 in the presence of NAC **(B)**, INH and INH+NAC **(C)**, RIF and RIF+NAC **(D)** and EMB and EMB+NAC **(E)**. Data represent means ±SE from experiments performed in triplicate. $*p < 0.05$ when comparing samples to their respective controls. $\#p < 0.05$ when comparing samples to their respective antibiotic only treatments. $\Sigma\Sigma p < 0.005$ when comparing Erdman only samples at 15 days to Erdman only samples at 1 h.

Propidium iodide staining was performed to determine the extent of necrosis in the granulomas. Increased necrosis was observed amongst the untreated granulomas (**Figure 2M**). NAC-treatment resulted in a decrease in the extent of necrosis (**Figure 2N**). Microscopic observation of the immunofluorescent staining indicate that the *in vitro* granulomas constitute cell types such as: macrophages, monocytes, dendritic cells CD4 and CD8 T cells all of which contribute to the innate and adaptive immune responses against *M. tb* infection (**Figures 2O–T**). An intense staining for CD4, CD8, and CD86 molecules was observed in NAC-treated granulomas suggesting that reduction in the extent of necrosis and ROI production will maintain the viability of myeloid and lymphoid cells in the granulomas (2L, N, P, R, T).

## *M. tb* Survival And Effector Responses Against the Pathogen Inside INH and INH+NAC-Treated Granulomas From Healthy Subjects

INH is one of the most important first line antibiotic used in conjunction with other antibiotics to treat active TB. Treatment of *in vitro* granulomas with combination of NAC and INH resulted in total clearance of *M. tb* when compared to granulomas solely treated with INH alone (**Figure 3A**). INH+NAC treatment resulted in more numbers of solid and stable granulomas compared to granulomas treated only with INH (**Figure 3B**).

We then determined the effector responses against *M. tb* inside INH and INH+NAC treated granulomas from healthy individuals. There was a significant two-fold increase in the levels of total GSH in INH+NAC treated granulomas similar to the NAC treated category (**Figure 3C**). Increased levels of GSH in INH+NAC treated granulomas was accompanied by a significant three-fold increase in the production of IFN-γ (**Figure 3D**). Excessive production of pro-inflammatory cytokines, IL-6 and TNF-α is associated with oxidative stress and inflammation. INH+NAC treatment resulted in a significant decrease in the levels of IL-6 (**Figure 3E**). INH+NAC treatment also resulted in a significant decrease in the levels of TNF-α at both 8 days and 15 days (three-fold decrease) post-infection compared to untreated control category (**Figures 3F,G**). Importantly, INH+NAC treatment resulted in a five-fold increase in the number of *M. tb* inside the acidified compartments (**Figure 3I**). However, there was no notable increase in the number of bacteria in the acidified compartments in granulomas solely treated with INH (**Figure 3H**).

## *M. tb* Survival and Effector Responses Against the Pathogen Inside RIF and RIF+NAC-Treated Granulomas From Healthy Subjects

RIF is another important first line antibiotic generally used with INH to treat TB clinically. We observed a total clearance of *M. tb* in the granulomas treated with a combination of NAC and RIF when compared to sole treatment with RIF (**Figure 4A**). RIF+NAC treatment resulted in solid and dense aggregation of

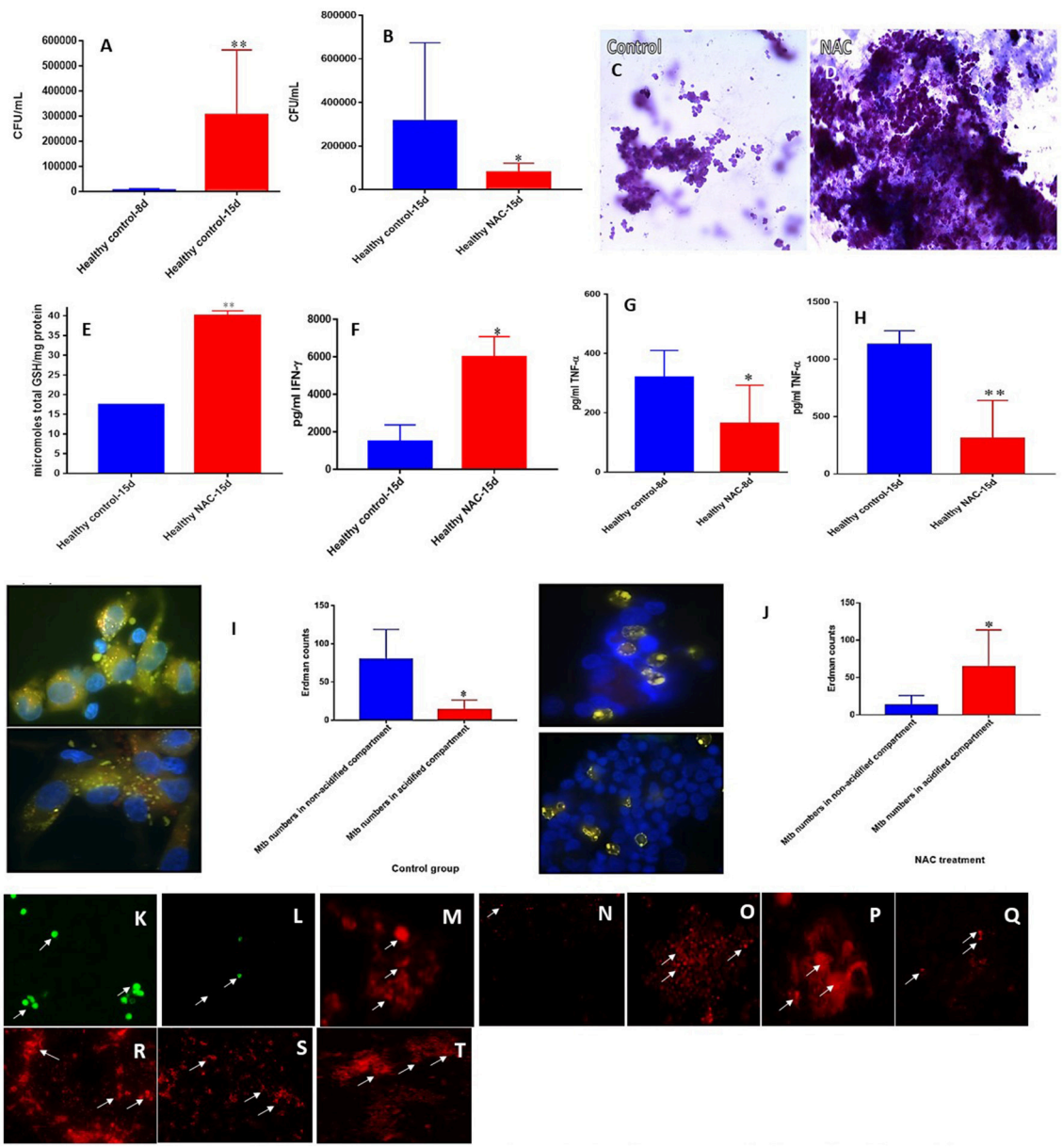


**FIGURE 2 |** NAC effects on *in vitro* granulomas developed using immune cells from healthy subjects. Survival of *M. tb* inside untreated granulomas **(A)**, Survival of *M. tb* inside NAC-treated granulomas **(B)**, Hematoxylin and Eosin staining of untreated **(C)** and NAC-treated granulomas **(D)** from healthy individuals, determination of GSH levels at 15 days post-infection **(E)**, IFN-γ levels at 15 days post-infection **(F)**, TNF-α levels at 8 days **(G)** and 15 days **(H)** post-infection, phagosome acidification at 15 days post-infection **(I,J)**, cellROX staining of untreated **(K)** and NAC-treated granulomas **(L)** from healthy individuals. Propidium iodide staining of untreated **(M)** and NAC-treated granulomas **(N)** from healthy individuals. CD4 staining of untreated **(O)** and NAC-treated granulomas **(P)**. CD8 staining of untreated **(Q)** and NAC-treated granulomas **(R)**. CD86 staining of untreated **(S)** and NAC-treated granulomas from healthy individuals **(T)**. Data represent means ±SE from 8 healthy individuals. $^{**}p < 0.005$ when comparing samples at 8 and 15 days. $^{*}p < 0.05$ when comparing samples at 15 days with or without NAC treatment. $^{**}p < 0.005$ when comparing samples treated with NAC to the controls. $^{*}p < 0.05$ when comparing samples treated with NAC to the controls.

immune cells when compared to granulomas treated with RIF alone (**Figure 4B**).

We then determined the underlying mechanisms by which RIF+NAC-treatment augmented the effector responses against *M. tb* inside the granulomas from healthy individuals. We assayed the levels of total GSH in both RIF and RIF+NAC treated granuloma lysates. There was a significant three-fold increase in the levels of total form of GSH in both RIF and RIF+NAC treated granulomas compared to the untreated group (**Figure 4C**). Increased levels of GSH in both RIF and RIF+NAC treated granulomas were accompanied by a notable increase in the production of IFN-γ and decreased levels of IL-6 (**Figures 4D,E**). RIF+NAC treatment also resulted in a significant decrease in the levels of TNF-α at both 8 days and 15

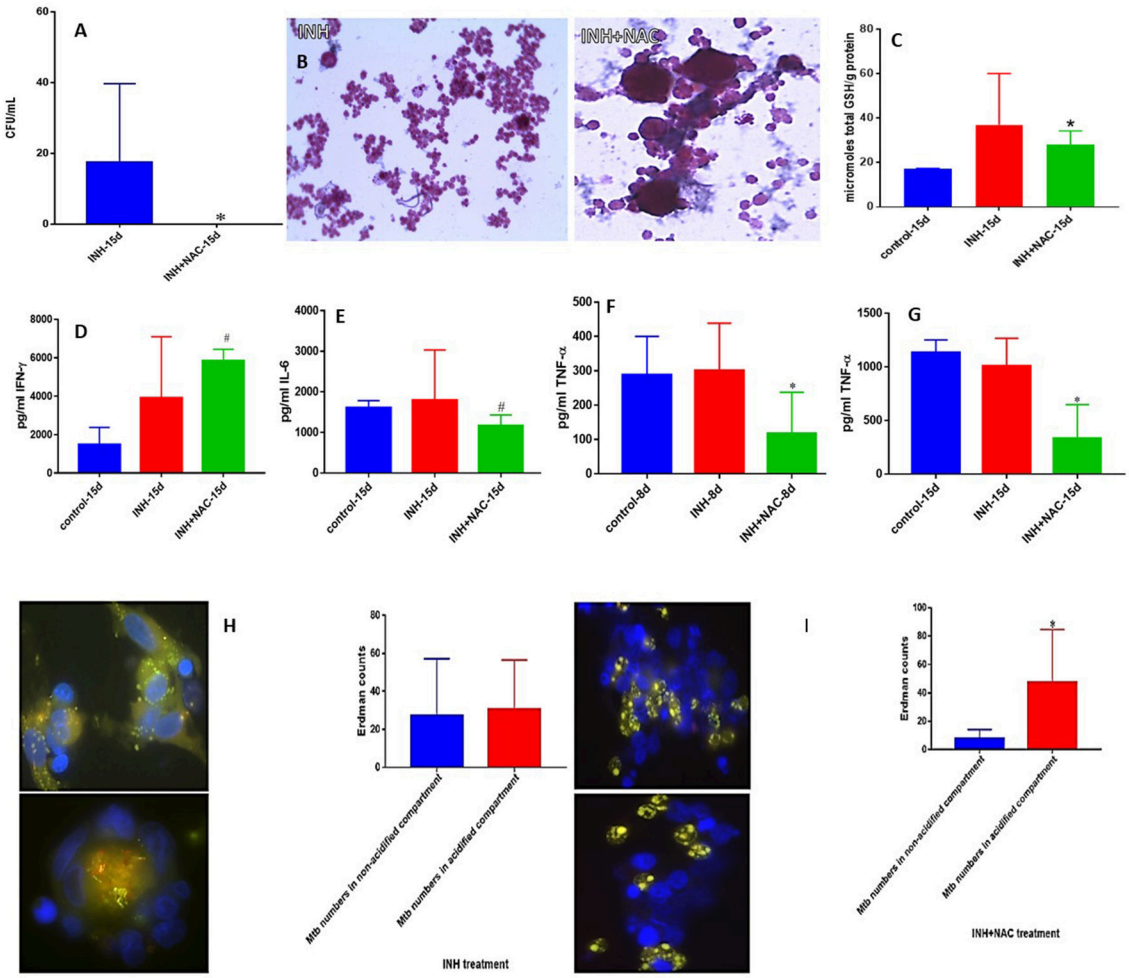


**FIGURE 3 |** INH and INH+NAC effects on *in vitro* granulomas developed using immune cells from healthy subjects. Survival of *M. tb* inside INH and INH+NAC-treated granulomas **(A)**, Hematoxylin and Eosin staining of INH and INH+NAC-treated granulomas from healthy individuals **(B)**, determination of GSH levels at 15 days post-infection **(C)**, IFN-γ levels at 15 days post-infection **(D)**, IL-6 levels at 15 days post-infection **(E)**, TNF-α levels at 8 days **(F)** and 15 days **(G)** post-infection, and phagosome acidification at 15 days post-infection **(H,I)**. INH+NAC treatment resulted in a significant and complete clearance of *M. tb* infection inside the granulomas from healthy subjects compared to treatment with INH alone **(A)**. INH+NAC treatment also induced formation of solid and stable granulomas **(B)**. $^{*}p < 0.05$ when comparing samples treated with INH only to those treated with INH and NAC. $^{\#}p < 0.05$ when comparing samples treated with INH and NAC to their controls.

days (three-fold decrease) post-infection compared to untreated control category (**Figures 4F,G**). Treatment of granulomas with RIF+NAC resulted in three-fold increase in the number of *M. tb* inside the acidified compartments (**Figure 4I**).

## *M. tb* Survival and Effector Responses Against the Pathogen Inside EMB and EMB+NAC-Treated Granulomas From Healthy Subjects

EMB is also a first line antibiotic administered during the initial 2 months of TB treatment in combination with INH, RIF, and PZA. Although there was no complete clearance of bacterial infection, Treatment of granulomas with EMB+NAC resulted in a significant decrease in the number of *M. tb* when compared to granulomas solely treated with EMB (**Figure 5A**). EMB+NAC treatment also resulted in dense aggregates of immune cells forming stable granulomas (**Figure 5B**).

Consistent with our previous findings, EMB+NAC treatment resulted in a significant decrease in the levels of IL-6 (**Figure 5E**). We observed a significant decrease in the levels of TNF-α in EMB-treated granulomas at both 8 and 15 days post-infection (**Figures 3G**, **5F**). Furthermore, EMB +NAC treatment resulted in further downregulation (three-fold decrease) and a significant decrease in the levels of TNF-α at both 8 and 15 days post-infection compared to untreated control category (**Figures 3G**, **5F**). EMB+NAC treatment also resulted in a four-fold significant increase in the number of *M. tb* inside the acidified compartments (**Figure 5I**). We did not observe any increase in the number of *M. tb* inside the acidified compartments in granulomas solely treated with EMB (**Figure 5H**).

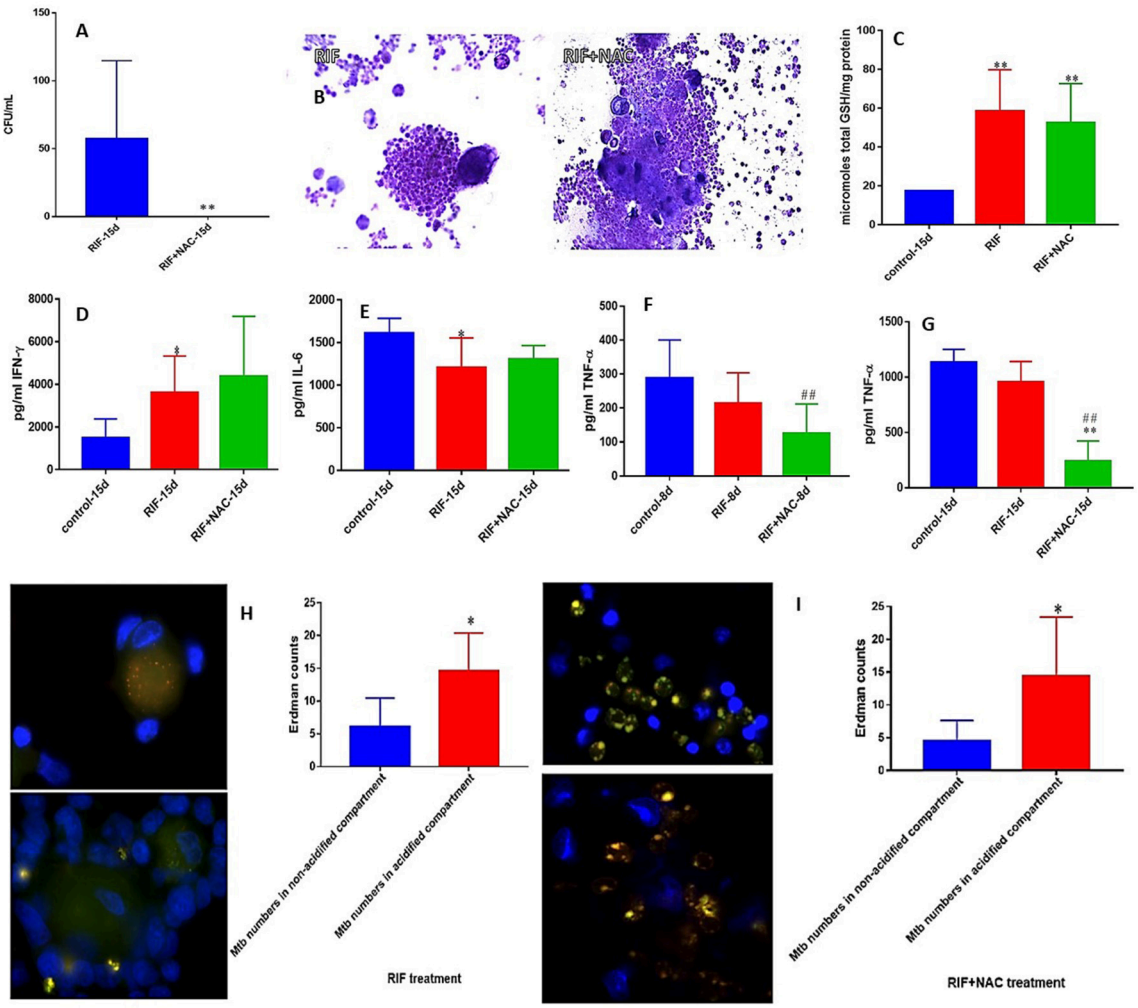


**FIGURE 4 |** RIF and RIF+NAC effects on *in vitro* granulomas developed using immune cells from healthy subjects. Survival of *M. tb* inside RIF and RIF+NAC-treated granulomas **(A)**, Hematoxylin and Eosin staining of RIF and RIF+NAC-treated granulomas from healthy individuals **(B)**, determination of GSH levels at 15 days post-infection **(C)**, IFN-γ levels at 15 days post-infection **(D)**, IL-6 levels at 15d post-infection **(E)**, TNF-α levels at 8 days **(F)** and 15 days **(G)** post-infection, and phagosome acidification at 15 days post-infection **(H,I)**. $^{*}p < 0.05$ when comparing samples treated with RIF only to those treated with RIF and NAC. $^{\#}p < 0.05$ when comparing samples treated with RIF and NAC to their controls. $^{**}p < 0.005$ when comparing samples treated with RIF only to those treated with RIF and NAC. $^{\#\#}p < 0.005$ when comparing samples treated with RIF and NAC to their controls.

## Granulomas From Individuals With T2DM, Structure, the Levels of Cytokines and Oxidative Stress Markers, and Survival of *M. tb* Inside Untreated and NAC-Treated Granulomas

We also attempted to characterize the effector immune responses inside the *in vitro* granulomas from individuals with T2DM. We observed a significant decrease in the levels of TNF-α, IFN-γ, IL-12, and IL-6 in the baseline plasma samples from individuals with T2DM indicating a systemic downregulation in the cytokine levels among uncontrolled diabetes (**Figures 6A–D**). Decreased levels of GSH along with increased levels of malondialdehyde (MDA) in the red blood cells (RBCs) serve as an important marker for systemic oxidative stress. GSH levels in the RBCs isolated from individuals with T2DM were significantly diminished compared to the healthy subjects (**Figure 6E**). Decreased levels of GSH in the RBCs isolated from individuals with T2DM correlated with increased levels of MDA (**Figure 6F**). *In vitro* granulomas generated from PBMCs isolated from individuals with T2DM were not as robust and solid as the healthy individuals and this observation correlated with impaired ability of granulomas to control *M. tb* infection. Granulomas from individuals with T2DM were found to harbor significantly more intracellular *M. tb* than their healthy counterparts (**Figure 6G**). Increased survival of *M. tb* also correlated with a significant increase in the levels of MDA and IL-6 in granulomas from individuals with T2DM individuals (**Figures 6G–I**) as well as a decrease in TNF-α levels (**Figure 6K**). NAC-treatment resulted in a significant four-fold decrease in the intracellular

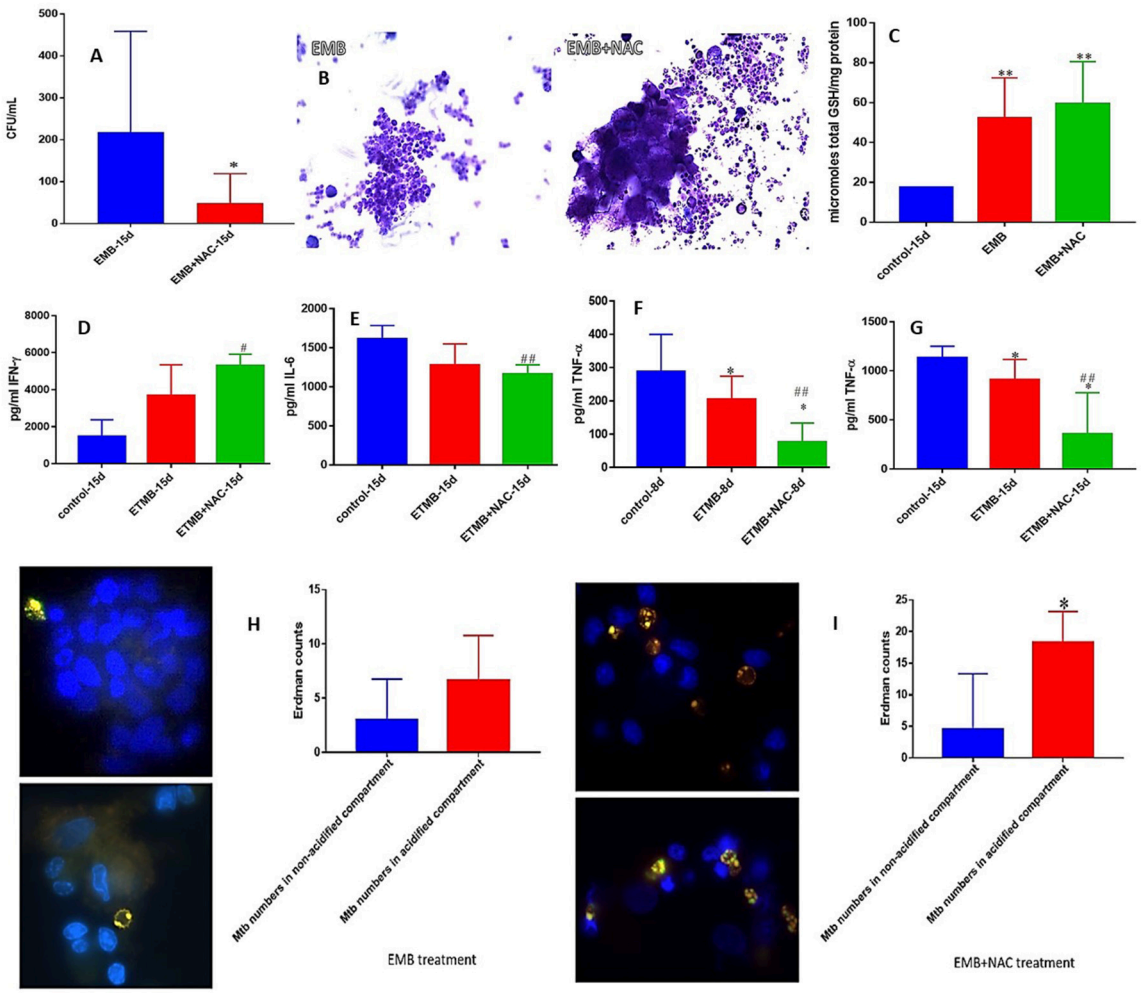


**FIGURE 5 |** EMB and EMB+NAC effects on *in vitro* granulomas developed using immune cells from healthy subjects. Survival of *M. tb* inside EMB and EMB+NAC-treated granulomas **(A)**, Hematoxylin and Eosin staining of EMB and EMB+NAC-treated granulomas from healthy individuals **(B)**, determination of GSH levels at 15 days post-infection **(C)**, IFN-γ levels at 15 days post-infection **(D)**, IL-6 levels at 15 days post-infection **(E)**, TNF-α levels at 8 days **(F)** and 15 days **(G)** post-infection, and phagosome acidification at 15 days post-infection **(H,I)**. $^{*}p < 0.05$ when comparing samples treated with INH only to those treated with EMB and NAC. $^{\#}p < 0.05$ when comparing samples treated with EMB and NAC to their controls. $^{**}p < 0.005$ when comparing samples treated with INH only to those treated with EMB and NAC. $^{\#\#}p < 0.005$ when comparing samples treated with EMB and NAC to their controls.

survival of *M. tb* inside the granulomas from individuals with T2DM (**Figure 7A**). Although NAC-treatment improved cell aggregation, nonetheless, granulomas from individuals with T2DM were not as solid as those from healthy subjects (**Figure 7B**). NAC-treatment of granulomas from individuals with T2DM also resulted in a significant decrease in the levels of TNF-α (**Figure 7C**). There was a significant increase in the number of *M. tb* in the non-acidified compartments in untreated granulomas from individuals with T2DM (**Figure 7D**). In contrast to the untreated-treated granulomas there was a significant increase in the number of *M. tb* inside the acidified compartments of NAC-treated granulomas from individuals with T2DM (**Figure 7E**). An intense cellROX staining was observed in untreated granulomas from T2DM group, indicative of oxidative stress (**Figure 7F**). Treatment of granulomas from T2DM group with NAC resulted in a decrease in the levels of ROI as evident from the diminished uptake of cellROX (**Figure 7G**). Increased uptake of Propidium iodide was also observed in the untreated granulomas from T2DM group (**Figure 7H**). NAC-treatment of granulomas from T2DM group resulted in a decrease in the extent of necrosis (**Figure 7I**). The cell types that constituted *in vitro* granulomas from T2DM group were macrophages, monocytes, dendritic cells CD4 and CD8 T cells (**Figures 7J–L**).

## *M. tb* Survival and Effector Responses Against the Pathogen Inside INH and INH+NAC-Treated Granulomas From Subjects With T2DM

There was a significant ten-fold reduction in the survival of *M. tb* inside INH+NAC treated granulomas from subjects with T2DM compared to granulomas treated with INH alone (**Figure 8A**).

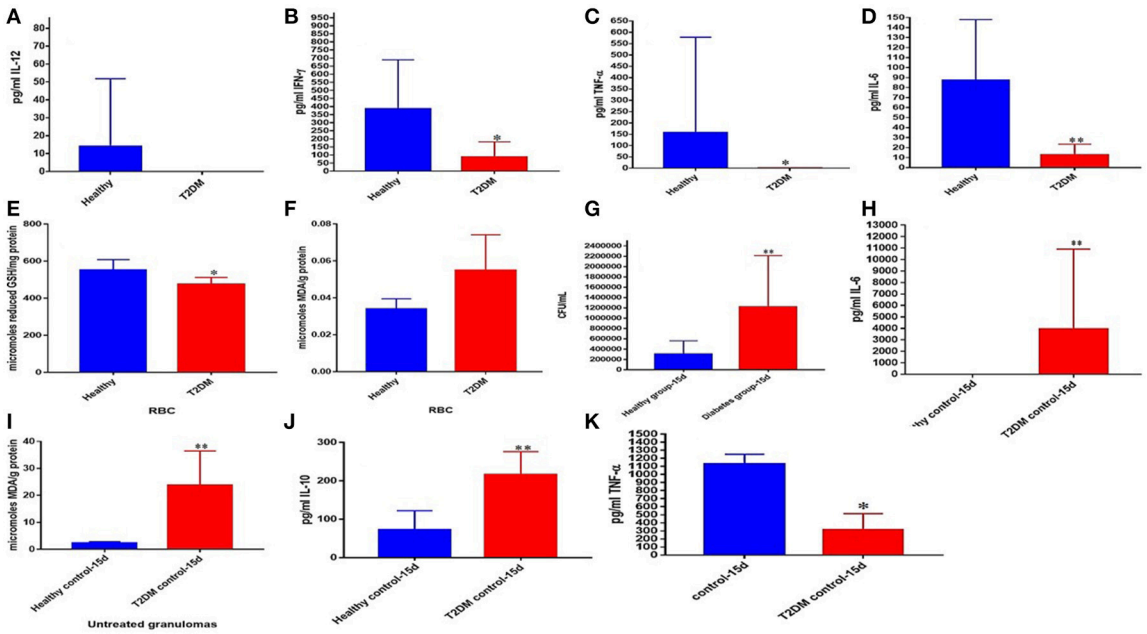


**FIGURE 6 |** Comparison of systemic markers and granulomas from healthy individuals and T2DM individuals. Levels of IL-12 **(A)**, IFN-γ **(B)**, TNF-α **(C)**, and IL-6 **(D)** in the plasma samples of healthy and T2DM individuals. Levels of GSH **(E)** and MDA **(F)** in red blood cells of healthy and T2DM individuals. Comparison of *M. tb* survival in healthy individuals and participants with T2DM **(G)**. Levels of IL-6 **(H)**, MDA **(I)**, IL-10 **(J)** and TNF-α **(K)** in granuloma supernatants from healthy subjects and individuals with T2DM. *$p < 0.05$ when comparing samples of healthy individuals to those with T2DM. **$p < 0.005$ when comparing samples of healthy individuals to those with T2DM.

However, in contrast to healthy subjects, INH+NAC treatment of granulomas from T2DM group did not result in complete clearance of *M. tb* (**Figures 3A**, **8A**). In contrast to the healthy group, INH+NAC treated granulomas from the T2DM group did not form solid and stable granulomas (**Figure 8B**).

In order to test whether the trend in the effector responses against *M. tb* in INH+NAC granulomas from T2DM group is similar to the healthy counterpart, we measured the levels of cytokines and phagosome acidification. A distinct and significant decrease in the levels of TNF-α was observed in the INH+NAC treated granulomas compared to both untreated and INH treated counterparts in granulomas from subjects with T2DM (**Figure 8D**). NAC-treatment of granulomas from T2DM group did not result in the restoration in the levels of GSH (data not shown). In contrast to healthy subjects, treatment of granulomas from subjects with T2DM with INH+NAC did not result in an increase in the levels of IFN-γ. On the contrary, there was an observable decrease in the levels of IFN-γ in the INH and INH+NAC treated granulomas from subjects with T2DM compared to that in the untreated counterpart (**Figure 8C**). INH+NAC treatment of granulomas from subjects with T2DM also resulted in a significant increase in the production of IL-10 (**Figure 8E**). Treatment of granulomas from individuals with T2DM with INH and INH+NAC resulted in significant number of *M. tb* numbers inside the acidified compartments (**Figures 8F,G**). Our results signify that NAC-treatment has direct antimycobacterial effects, can reduce the levels of TNF-α, and can synergistically enhance the effects of INH in reducing the bacterial burden. However, INH+NAC treatment of granulomas from subjects with T2DM resulted in decrease in the levels of IFN-γ and increase in the production of IL-10.

## *M. tb* Survival and Effector Responses Against the Pathogen Inside RIF and RIF+NAC-Treated Granulomas From Subjects With T2DM

There was a significant hundred-fold decrease in the number of *M. tb* inside the RIF+NAC treated granulomas from subjects with T2DM, compared to treatment with RIF alone (**Figure 9A**). Consistent with INH+NAC treatment, treatment of granulomas from T2DM group with RIF+NAC did not result in the formation of robust granulomas (**Figure 9B**).

Treatment of granulomas from T2DM subjects with RIF+NAC resulted in a significant decrease in the levels of IFN-γ (**Figure 9C**) and TNF-α (**Figure 9D**), and a significant increase in the levels of IL-10 (**Figure 9E**). These results are similar to the findings from INH+NAC treatment in the diabetic group. Treatment of T2DM granulomas with RIF alone resulted in a significant and six-fold increase in the number of *M. tb* in the acidified compartments (**Figure 9F**). Furthermore, treatment with RIF+NAC resulted in a significant and ten-fold increase in the number of *M. tb* in the acidified compartments (**Figure 9G**) indicating that NAC has direct effects in promoting phagosome-lysosome fusion. Inability of NAC to enhance the levels of IFN-γ and decrease the levels of IL-10 in granulomas from individuals with T2DM could directly be linked to GSH insufficiency since NAC cannot effectively restore the levels of

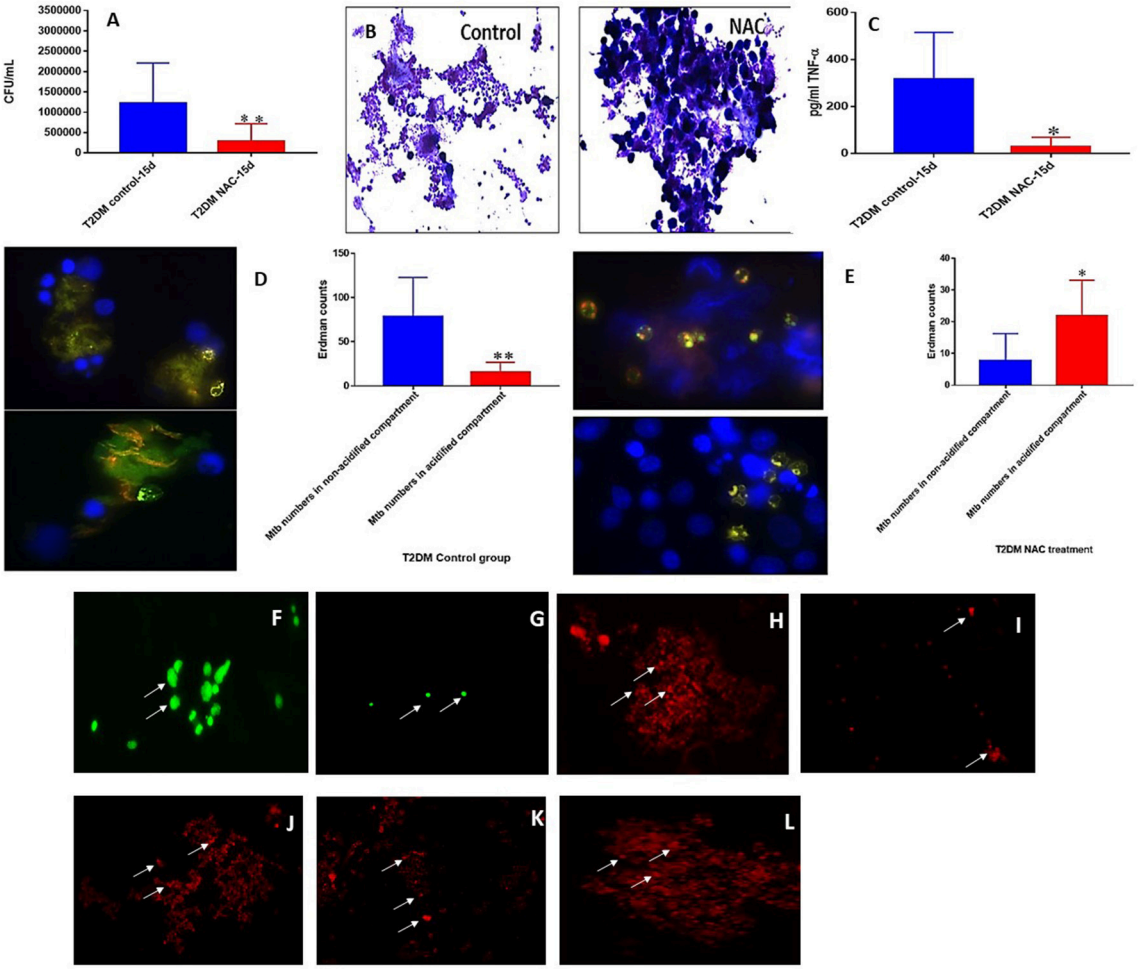


**FIGURE 7 |** NAC effects on *in vitro* granulomas developed using immune cells from T2DM subjects. *M. tb* survival in untreated and NAC treated granulomas from individuals with T2DM **(A)**. Hematoxylin and eosin staining of untreated and NAC treated granulomas from T2DM individuals **(B)**. Assay of in TNF-α in supernatants form untreated and NAC treated granulomas from individuals with T2DM **(C)**. Quantification of phagosome acidification in untreated **(D)** and NAC **(E)** treated granulomas from individuals with T2DM. cellROX staining of untreated **(F)** and NAC-treated granulomas **(G)** from individuals with T2DM. Propidium iodide staining of untreated **(H)** and NAC-treated granulomas **(I)** from individuals with T2DM. CD4 staining of untreated granulomas **(J)** from individuals with T2DM. CD8 staining of untreated granulomas **(K)** from individuals with T2DM. CD86 staining of untreated granulomas **(L)** from individuals with T2DM. *$p < 0.05$ when comparing healthy to T2DM. **$p < 0.005$ when comparing healthy to T2DM.Microscopy work was done with a light microscope at 1000x magnification under oil immersion.

GSH due to diminished levels of GSH *de novo* synthesis enzymes in the diabetic group.

## *M. tb* Survival and Effector Responses Against the Pathogen Inside EMB and EMB+NAC-Treated Granulomas From Subjects With T2DM

EMB+NAC treatment resulted in a significant and approximately three-fold reduction in the viability of *M. tb* inside the granulomas from subjects with T2DM (**Figure 10A**). EMB+NAC-treatment did not enhance the formation of solid and stable aggregation of immune cells (**Figure 10B**).

Treatment of granulomas from individuals with T2DM with EMB or EMB+NAC resulted in a significant reduction in the levels of IFN-γ (**Figure 10C**). EMB+NAC treatment of granulomas from individuals with T2DM also resulted in a significant reduction in the levels of TNF-α (**Figure 10D**). Treatment of T2DM granulomas with EMB alone resulted in a significant and six-fold increase in the number of *M. tb* in the acidified compartments (**Figure 10E**). Importantly, treatment with EMB+NAC resulted in a significant and approximately twelve-fold increase in the number of *M. tb* in the acidified compartments (**Figure 10F**).

## DISCUSSION

Worldwide, TB is considered to be the leading cause of mortality due to a single infectious agent (1). Furthermore, immunocompromised individuals, such as those with

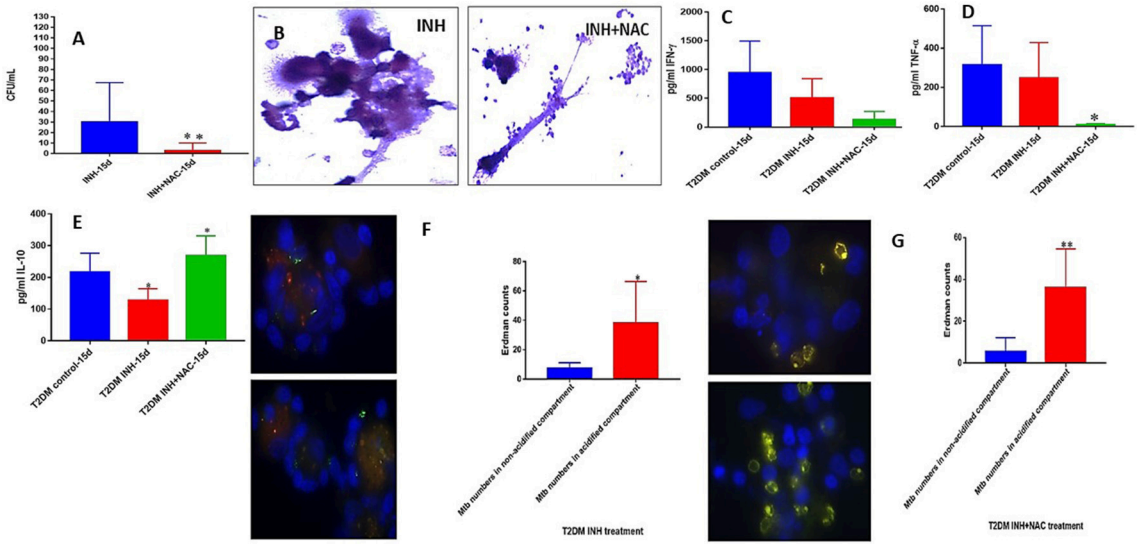


**FIGURE 8 |** INH and INH+NAC effects on *in vitro* granulomas developed using immune cells from individuals with T2DM. Survival of *M. tb* inside INH and INH+NAC-treated granulomas **(A)**, Hematoxylin and Eosin staining of INH and INH+NAC-treated granulomas from T2DM individuals **(B)**, IFN-g levels at 15 days post-infection **(C)**, TNF-a levels at 15 days post-infection **(D)**, IL-10 levels at 15 days **(E)** post-infection, and phagosome acidification at 15 days post-infection **(F,G)**. $^{*}p < 0.05$ when comparing samples treated with INH+NAC to controls. $^{**}p < 0.005$ when comparing samples treated with INH+NAC to controls.

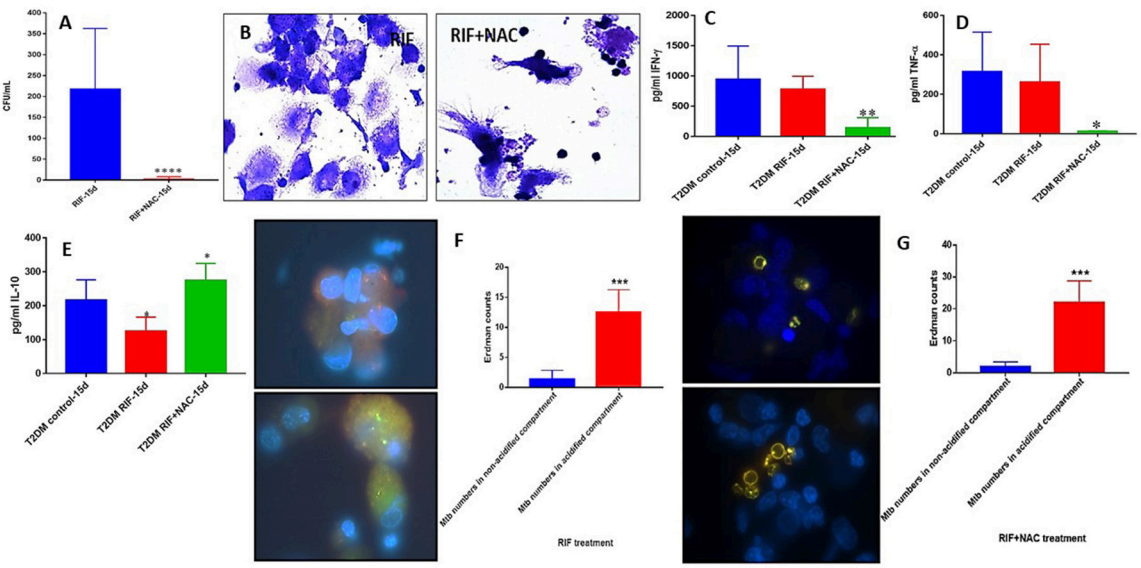


**FIGURE 9 |** RIF and RIF+NAC effects on *in vitro* granulomas developed using immune cells from individuals with T2DM. Survival of *M. tb* inside RIF and RIF+NAC-treated granulomas **(A)**, Hematoxylin and Eosin staining of RIF and RIF+NAC-treated granulomas from T2DM individuals **(B)**, IFN-g levels at 15d post-infection **(C)**, TNF-a levels at 15 days post-infection **(D)**, IL-10 levels at 15 days **(E)** post-infection, and phagosome acidification at 15 days post-infection **(F,G)**. $^{****}p < 0.00005$ when comparing samples treated with RIF only to those treated with RIF and NAC. $^{*}p < 0.05$ when comparing samples treated with RIF+NAC to controls. $^{**}p < 0.005$ when comparing samples treated with RIF+NAC to controls. $^{***}p < 0.0005$ when comparing samples treated with RIF+NAC to controls.

uncontrolled diabetes, are increasingly susceptible to *M. tb* infection (9). There exists abundant evidence which affirms that patients with diabetes are at a higher risk for developing an active TB infection, which is commonly believed to be by means of a diminished cell-mediated immunity (10, 11, 48–50). The conventional method for *M. tb* abolition is with the standard 6-month antibiotic course of treatment (51).

Our lab has formerly reported that H37Rv, the virulent laboratory-strain of *M. tb*, is vulnerable to GSH when supplemented i*n vitro* (28, 52). Likewise, we have established

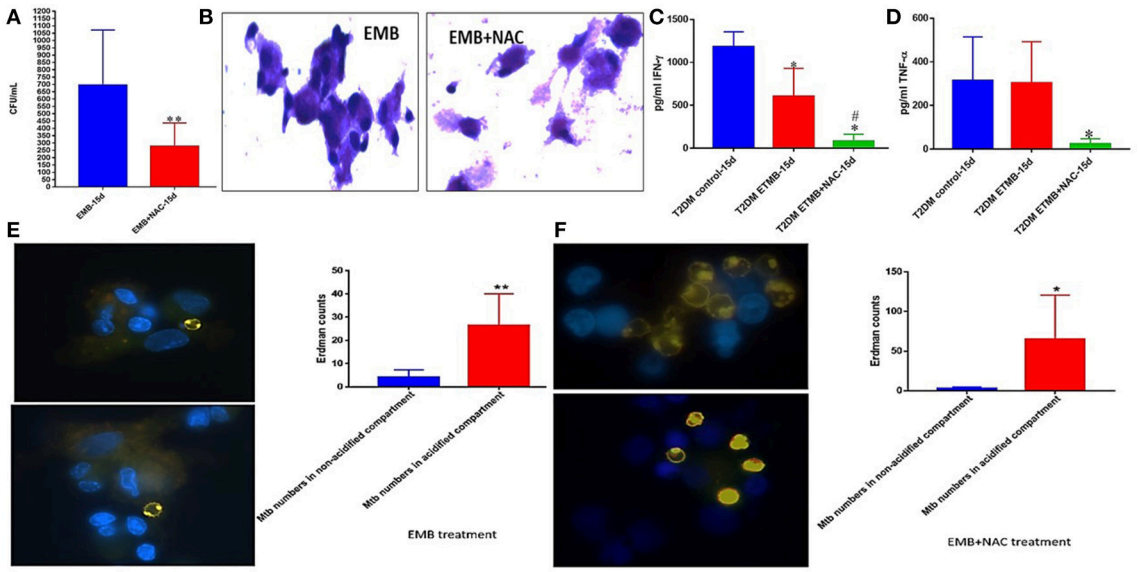


**FIGURE 10 |** EMB and EMB+NAC effects on *in vitro* granulomas developed using immune cells from individuals with T2DM. Survival of *M. tb* inside EMB and EMB+NAC-treated granulomas **(A)** Hematoxylin and Eosin staining of RIF and RIF+NAC-treated granulomas from T2DM individuals **(B)**, IFN-g levels at 15d post-infection **(C)**, TNF-a levels at 15 days post-infection **(D)**, and phagosome acidification at 15 days post-infection **(E,F)**. $^{**}p < 0.005$ when comparing samples treated with EMB only to those treated with EMB and NAC. $^{*}p < 0.05$ when comparing samples treated with EMB+NAC to controls. $^{**}p < 0.005$ when comparing samples treated with EMB+NAC to controls. $^{\#}p < 0.05$ when comparing samples treated with EMB+NAC to controls.

that enhancing the levels of GSH through treatment with NAC inhibits the intracellular survival of H37Rv (28, 29, 36, 53). Therefore, GSH possess direct antimycobacterial potentiality, which assists human macrophages in the innate defense against *M. tb* infection. Furthermore, our lab has previously revealed that the levels of intracellular GSH are significantly abridged among individuals with T2DM, in consequence to compromised levels of the enzymes involved in GSH synthesis (33, 34, 54–57).

Accordingly, we investigated the synergistic efficacy of the first-line antibiotics INH, RIF, and EMB administered at sub-optimal levels in conjunction with NAC, the GSH precursor, on PBMC-derived *in vitro* granulomas; processed from the blood of both healthy and T2DM individuals and infected with the Erdman strain of *M. tb*. This study focuses on the mechanistic actions of immune cells during an *M. tb* infection within granulomas, to illuminate their subsequent immune activity in the uniquely regulated environment. Our *in vitro* granulomas constitute cell types such as: macrophages, monocytes, dendritic cells, CD4 and CD8T cells all of which contribute to the immune responses necessary for granuloma formation in consequence to *M. tb* infection.

We first examined the rate of growth or mortality of *M. tb* in 7H9 media, in the absence immune cells, when sham treated, supplemented with the various standalone antibiotics or in combination with lone antibiotics and the GSH antecedent NAC. We observed a roughly 1-log growth of the mycobacteria when it was left untreated (**Figure 1A**). This confirms the ability of Erdman strain of *M. tb* to actively replicate in the absence of therapeutics. Significantly, when the immunoadjuvant NAC was administered, over a 5-log reduction in the viability of *M. tb* was observed (**Figure 1B**). These results support previous findings which indicate that NAC possess mycobactericidal capacity (35, 39, 43). Furthermore, all three first line antibiotics investigated showed a drastic bacterial reduction of statistical significance compared to the controls, and when NAC was co-administered with the lone antibiotics a further statistically significant diminution was detected (**Figures 1C–E**).

We next reproduced the aforestated experimental applications on *in vitro* derived granulomas emanated from the PBMCs of healthy individuals. Similar to the previous experiment without immune cells present, we observed a statistically significant increase in *M. tb* abundance, when the cells were not accompanied by treatment additives (**Figure 2A**). Enhancing the levels of GSH in healthy PBMCs, by NAC application, induced significant inhibition in the intracellular growth of *M. tb* (**Figure 2B**). This data further demonstrates NAC's (GSH's) antimycobacterial activity, and functionality in the immune defense against *M. tb*. A vehement decrease in mycobacterial survival was again observed after each respective antibiotic was administered to the cells (**Figures 3A**, **4A**, **5A**). As expected, each antibiotic delivered at its MIC caused a drastic reduction of viable *M. tb*, and outright mycobacterial elimination was not observed. However, when each individual antibiotic was administered in combination with NAC, a statistically significant decrease in mycobacterial quantity was observed in comparison to the treatment of unaccompanied antibiotics (**Figures 3A**, **4A**, **5A**). Importantly, complete *M. tb* clearance was detected following the combined treatment of both INH and NAC, as well as RIF and NAC (**Figures 3A**, **4A**). This evidence suggests that the combined treatment of various first-line

antibiotics supplemented with NAC (GSH) encompasses greater prophylactic efficacy than either of the treatments given unassisted. Therefore, the synergistic effects of NAC (GSH) and antibiotics grant improved innate immune control over intracellular *M. tb* and advocates GSHs therapeutic capability as an adjunct with first-line antibiotics in clearing a *M. tb* infection.

We have previously reported that *M. tb* infection can cause considerable depletion of intracellular GSH compared to uninfected cells, which in turn can promote *M. tb* survival (34, 54–57). When NAC was administered to the granulomas of healthy individuals, a statistically significant increase of GSH was witnessed (**Figure 2E**). These results affirm that the addition of NAC, GSH's forerunner, will indeed cause the levels of GSH to become subsequently elevated in response. Enhancing the levels of GSH with NAC treatment, in *M. tb* infected cells appropriately displays an inverse correlation to the significant reduction of *M. tb* intracellular survivability (**Figures 2A,E**). The treatment of *M. tb* granulomas with each of the first-line antibiotics resulted in a significant upsurge of GSH levels (**Figures 3C**, **4C**, **5C**). We believe that the increase in GSH observed after the treatment with lone antibiotics is due to a restorative effect, which is most likely as a result of the antibiotics diminishing the *M. tb* bio-burden thus enabling the host cells to restore the levels of GSH which further improves their ability to combat the infection. Likewise, treatment of the granulomas with each of the antibiotics in concurrence with NAC resulted in the elevation of GSH for all three categories, all of which presented a statistically significant increase in relation to the control group (**Figures 3C**, **4C**, **5C**).

An effective innate immune response against *M. tb* infection is established by virtue of the efficient regulation of immune cells, mediated by the release of cytokines (58, 59). The cytokine IFN-γ is crucial in both innate and adaptive immunity, serving as a catalyst for macrophage activation, and MHC molecule expression (60). Additionally, IFN-γ possesses immunostimulatory properties which induce effector mechanisms pivotal not only toward the activation of macrophages, but aid in granuloma formation, and promote macrophage phagosome-lysosome fusion, thus provoking enhanced *M. tb* infection management (61, 62). The successive supplementation of NAC to the granulomas from healthy subjects resulted in the statistically significant increase in the levels IFN-γ (**Figure 2F**). Likewise, each standalone antibiotic induced an IFN-γ release (**Figures 3D**, **4D**, **5D**). Importantly, a marked increase in IFN-γ was also detected for all of the antibiotic categories co-supplemented with NACcompared to their standalone counterparts (**Figures 3D**, **4D**, **5D**). This data reveals that again a restorative effect is displayed once the antibiotics have been administered to *M. tb* granulomas as distinguished by the elevation in IFN-γ released from the cells. Similarly, the profound amplification of IFN-γ after lone NAC supplementation and the co-supplementation of antibiotics administered with NAC, reveals the mechanistic potentiality for which these additives exert their prophylactic measures.

It has previously been shown that a decrease in the levels of proinflammatory cytokines such as IL-6, and an increase in immunosuppressive cytokines such as IL-10 can be immensely beneficial in limiting the pathology of a *M. tb* infection once the appropriate granulomatous response has taken place (30). IL-6 is a proinflammatory cytokine generated in response to factors which create cellular damage such as infections and injured tissues (63). However, persistent IL-6 production or overexpression can lead to the development of various diseases particularly those related to chronic inflammation and autoimmunity (64). Furthermore, it has been shown that IL-6 can inhibit the cytokine IFN-γ during a *M. tb* infection (65). Therefore, it has been suggested that virulent mycobacteria purposefully upregulate IL-6 production to combat innate immunity (65). A reduction in IL-6 was detected among all the antibiotic categories when supplemented in conjunction with NAC; furthermore, statistical significance was recognized for the addition of NAC to INH and EMB. This reduction in IL-6 illustrates how NAC administration with first-line antibiotics can impede *M. tb*'s targets of immune suppression, as well as aid in the downregulation of pathological nonspecific damage to host cells due to overactive proinflammatory defenses during the *M. tb* infection.

Antibiotic treatment given in conjunction with NAC also resulted in decreased production of TNF-α. TNF-α, another proinflammatory cytokine, is primarily produced by macrophages, and is essential in fostering the formation and maintenance of a granuloma in both acute and chronic phases of infection (66–69). However, the overexpression of TNF-α also been connected to many inflammatory and autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, ankylosing spondylitis, and psoriasis (70, 71). Additionally, elevated levels of TNF-α have been shown to incite pulmonary tissue damage during host defense against *M. tb* infection (71–73). The TNF-α levels for each first-line antibiotic category given in combination with NAC was significantly reduced (**Figures 3F,G**, **4F,G**, **5F,G**). Likewise, when NAC was administered alone, a significant reduction in the levels of TNF-α was observed (**Figures 2G,H**). Interestingly, the levels of TNF-α detected from the lone NAC category directly corresponds with the relative levels of TNF-α observed among the antibiotic categories given in combination with NAC (**Figures 2G,H**, **3F,G**, **4F,G**, **5F,G**). This suggests the reduction in TNF-α observed is primarily due to NAC's incorporation. Therefore, our results reveal that NAC treatment modulates the levels of TNF-α, which we believe to be sufficient enough to arrest hyperactive cellular inflation thus limiting tissue damage, yet potent enough to maintain a healthy granuloma. Altogether, the aforestated cytokine data advocates that NAC/GSH supplementation can assist the innate and adaptive immune responses against *M. tb* by means of cytokine modulation, which ultimately translates to increased mycobactericidal ability and enhanced control over the pathogen (**Supplemental Figure 1**).

We then performed microscopic analysis of the stained granulomas to visualize how the modified effector mechanisms alter the granulomatous structure in response to the various treatment categories. When NAC was administered to the infected cells, we observed more solid and stable granulomas compared to the control categories (**Figures 2C,D**). Similarly, in relation to lone antibiotic treatment, the antibiotic categories which were supplemented with NAC displayed not only a

marked increase in cellular size, but the cellular density appears increased as well (**Figures 3B**, **4B**, **5B**). These observations indicate that NAC supplementation promotes formation of more substantial and concentrated granulomas. This advancement in granulomatous composition inversely correlates with extent of bacterial survival discussed previously. This signifies that the cellular enhancement observed after NAC treatment advocates comprehensive control over *M. tb* infection.

An important effector mechanism involved in the control of *M. tb* infection is phagolysosomal fusion within macrophages, wherein phagosomes fuse with an acidic lysosome causing the breakdown of the contents inside the compartment. We therefore quantified this mycobactericidal mechanism with fluorescent imaging by staining for acidified cellular compartments and examining where the GFP labeled bacteria reside. In the absence of NAC, *M. tb* tend to be loosely sequestered around the nuclei of the granulomatous cell aggregations, and acidification is relatively low due to the inexistent of red and the overlapping yellow color detected (**Figures 2I**, **3H**, **4H**, **5H**). Conversely, the addition of lone NAC, or NAC to the antibiotic categories causes a drastic alteration in intracellular morphology and bacterial locality (**Figures 2I**, **3I**, **4I**, **5I**). The inclusion of NAC causes the bacteria to become exceedingly more compartmentalized within the granulomas, and the magnitude of phagolysosomal acidification is augmented as well, as illustrated by the portion of red, and yellow colored bacteria (**Figures 2**, **3I**, **4I**, **5I**). These assays further demonstrate that the addition of NAC promotes intracellular killing of mycobacteria, by depicting an increased quantity of phagolysosomal fusion within the *M. tb* occupied macrophages.

Before we performed the same experimental method on individuals with T2DM, we first examined the RBCs and plasma samples of the healthy and T2DM participants for baseline comparison. We observed that prior to *M. tb* infection, the plasma cytokines IL-12, IFN-γ, TNF-α, and IL-6 were all drastically reduced among the T2DM group compared to the healthy individuals (**Figures 6A–D**). These results are consistent with previous findings which ascertain that individuals with T2DM present dysregulation of normal cytokine production (54, 74). Therefore, this dysregulation may impede host defense mechanisms and result in the altered immune responses observed during *M. tb* infection. Next, we tested the RBCs, and found that the individuals with T2DM displayed significantly compromised levels of GSH as well as elevated levels of malondialdehyde (MDA) (**Figures 6E,F**). MDA is a byproduct of lipid peroxidation and is therefore used as a descriptive measurement of oxidative stress. This inverse relationship between the levels of GSH and MDA is to be expected, which reveals that as the levels of GSH dwindle among the diabetic individuals, their redox homeostasis becomes subverted as well, resulting in increased systemic oxidative stress.

Compared to the healthy controls there was a statistically significant increase in the total *M. tb* numbers and the levels of IL-6, IL-10 and MDA, in granulomas from the T2DM group (**Figures 6G–J**). This data demonstrates superior bacterial viability emanating from diabetic cells, which is indicative of a worse prognosis; and further advocated by increased inflammation due to elevated IL-6, and greater oxidative damage represented by inflated MDA levels. However, the supplementation of NAC to T2DM-granulomas resulted in a statistically significant reduction of nearly two-thirds the bacterial load (**Figure 7A**). This advancement as a result of NAC supplementation is accompanied by a moderate increase in granulomatous size and density, as well as augmented phagolysosomal fusion as observed from the microscopic imaging, when compared to the sham-treated controls (**Figures 7B,D,E**). This data suggests that similar to the healthy individuals, treatment of T2DM samples with NAC results in enhanced prophylactic *M. tb* control at the cellular level. When the samples from T2DM subjects were treated with antibiotics, a significant reduction was revealed similar to that of the healthy individuals, although the bacterial quantity, and thus viability was elevated in comparison to the treatment of healthy subjects (**Figures 8A**, **9A**, **10A**). Likewise, the co-administration of NAC and antibiotics resulted in a statistically significant reduction compared to the lone antibiotics, though the bacterial abundance was greater than the healthy subjects, and complete clearance was no longer observed for the INH or RIF categories co-treated with NAC (**Figures 8A**, **9A**, **10A**). The fluorescent imaging of the antibiotic medicant categories illustrates that the incorporation of NAC into treatment significantly enhances the phagolysosomal fusion (**Figures 8F,G**, **9F,G**, **10E,F**). This data affirms that NAC supplementation improves mycobactericidal proficiency, and aids in infection ascendancy for both healthy and T2DM individuals. However, the increased cytokine production of IFN-γ observed among the healthy individuals subsequent to the administration NAC was not detected amongst the T2DM subjects (**Figures 8C**, **9C**, **10C**). Additionally, the levels of total GSH did not increase due to NAC supplementation among the T2DM subjects either (data not shown). Similarly, a preceding study reported that after oral NAC supplementation, the levels of *in vivo* GSH did not improve among T2DM patients (75). We suspect that this is due to the fact that T2DM individuals have attenuated levels of GSH synthesizing enzymes, and therefore despite elevating NAC (the GSH precursor), they are not capable of upregulating the extent of GSH produced (54). Additionally, the H&E imaging of the T2DM samples reveal that unlike the former experimental findings, the addition of NAC to the antibiotic treatment categories does not result in more solid stable granuloma formation (**Figures 8B**, **9B**, **10B**). We suspect that the reason the antibiotics co-administered with NAC do not form stable granulomas is due to the insufficient quantity of TNF-α present after NAC supplementation (**Figures 8D**, **9D**, **10D**). Unlike the aforementioned experimental conditions, the levels of TNF-α are already depressed amongst the T2DM subjects, comparable in measure to the healthy individuals after NAC supplementation. Therefore, the additional evanescence of TNF-α in consequence to NAC administration results in an inadequate supply for maintaining appropriate granuloma formation.

These novel findings illustrate that adding NAC to antibiotic treatment is capable of eliciting significant improvement in the granulomatous and mycobactericidal responses against a *M. tb*

infection (**Supplemental Figures 1**, **2**). Our results demonstrate that the addition of NAC results in significant reduction of *M. tb* burden in both healthy and diabetic individuals. Furthermore, in healthy individuals, NAC promotes the formation more solid and stable granulomas, as well as increased acidification of *M. tb* inhabited phagosomes. Accordingly, our results indicate that NAC can be advantageous as a prophylactic adjunct to first-line antibiotics, bolstering cytokine modulation, as well as the reduction and clearance of *M. tb* infection. Therefore, we believe that enhancing GSH by means of NAC supplementation in the antibiotic treatment of TB would not only reduce the toxicity of anti-TB medications through GSH's redox potentiality, but permit lessening the required antibiotic dosages to confer mycobacterial clearance, which could promote enhanced treatment compliance and circumvent the emergence of additional strains of DR-TB.

## AUTHOR CONTRIBUTIONS

The studies were conceived by the corresponding author, VV. VV designed the studies and mentored his lab members to conduct the studies and draft the manuscript. VV analyzed the data. GT and RC equally contributed to the work by performing all the experiments. GT was actively involved in drafting the manuscript. HI conducted the experiments along with GT and RC, AM, and AS recruited healthy subjects and participants with type 2 diabetes for this study. RP and CP provided technical support for microscopy. MF and LZ provided partial funding support and guidance to the students, respectively. SS provided Erdman strain of M.tb and expertise advise on granuloma imaging.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02069/full#supplementary-material

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REVIEW
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# The Pathogenesis of Tuberculosis: The Early Infiltrate of Post-primary (Adult Pulmonary) Tuberculosis: A Distinct Disease Entity

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It has long been recognized that tuberculosis (TB) induces both protective and tissue damaging immune responses. This paper reviews nearly two centuries of evidence that protection and tissue damage are mediated by separate disease entities in humans. Primary TB mediates protective immunity to disseminated infection while post-primary TB causes tissue damage that results in formation of cavities. Both are necessary for continued survival of Mycobacterium tuberculosis (MTB). Primary TB has been extensively studied in humans and animals. Post-primary TB, in contrast, is seldom recognized or studied. It begins as an asymptomatic early infiltrate that may resolve or progress by bronchogenic spread to caseous pneumonia that either fragments to produce cavities or is retained to produce post-primary granulomas and fibrocaseous disease. Primary and post-primary TB differ in typical age of onset, histopathology, organ distribution, x-ray appearance, genetic predisposition, immune status of the host, clinical course and susceptibility to protection by BCG. MTB is a highly successful human parasite because it produces both primary and post-primary TB as distinct disease entities in humans. No animal reproduces this sequence of lesions. Recognition of these facts immediately suggests plausible solutions, animal models and testable hypotheses to otherwise inaccessible questions of the immunity and pathogenesis of TB.

**Keywords: tuberculosis, pathogenesis, post-primary, human, x-ray, pathology, lung**

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## INTRODUCTION

It the past 200 years tuberculosis (TB) has killed more people than all other epidemic infections combined and it still kills around 5,000 people per day, more than any other infection (1). Mycobacterium tuberculosis (MTB) is an extremely well adapted human parasite (2). While MTB can infect many animals, they cannot transmit the infection to others. The continued survival of MTB, therefore, depends upon transmission among humans. This is best accomplished by producing a cavity in the lung for proliferation of massive numbers of MTB to be coughed into the environment over a period of decades while the host remains healthy enough to circulate in the community. This requires that the host maintains effective systemic immunity to prevent disseminated infection by the masses of organisms being produced and released from the cavity.

While we are making previously unimaginable progress in defining the cells, molecules and pathways of TB, we are making little discernable progress in putting the pieces together to

understand how the organisms accomplish the two functions of systemic immunity to protect the host and local susceptibility to produce and maintain a cavity. Our lack of understanding of these functions is still a major impediment to development of vaccines and new therapies (3–6). Dr. Anthony Fauci, Director of NIAID, expressed a nearly universal opinion with the statement: "We need to better understand the delicate balance between the host and pathogen in the context of the entire biological system and this requires a 'radical and transformational approach.' "Our goal should be to transform the entire field." (7, 8).

## SHIFTING LIMITATIONS OF TB RESEARCH

Inability to explain host resistance/susceptibility to TB is not a new concern, but the specifics have reversed. Fifty years ago Georges Canetti wrote "so much knowledge on TB mingles with so much obscurity on certain essential aspects of its pathogenesis … the obscurities are not due to lack of knowledge about the characteristic features of TB in man … The persistence of much ignorance in the pathogenesis of TB originates probably from lack of … experimental approaches to the disease" (9). Pinner expressed the same sentiment. "We understand the sequence of morbid changes that leads from infection to established pulmonary TB." However, understanding the biologic processes "is a task for the years to come" (10). Canetti, Pinner and others knew the clinical presentation, pathology, and x-ray appearance of each of the stages of TB very well, but the basic science of their day was unable to address the biologic questions effectively. Today, the situation is reversed. We have the means to investigate disease in ways Canetti and Pinner could not have imagined, but we have forgotten the characteristic features of TB in man. Knowledge of the pathology of human pulmonary TB gained by 150 years of study by many investigators has been replaced by the fantasy that granulomas are the key lesion of all TB. Pinner wrote, "Progressive primary TB does not play a role in the development of phthisis in the adult" (10). Modern science is trying to understand the pathogenesis of TB in animals that don't develop the human disease guided by a badly flawed paradigm. Observations that do not fit with the prevailing paradigm are usually ignored.

The problem is that research on TB began around 1800, stopped in the 1950's and began again in the 1990s with little carry forward of information. The first phase in the preantibiotic era studied humans with untreated TB. Many detailed descriptions of the pathology, radiology and clinical course of untreated TB were published. The second phase continuing today uses advanced technologies to study animal tissues, human peripheral blood and BAL, occasional lymph node or other biopsies and lung resections of treated lesions, but not lungs with untreated TB. Unfortunately, none of these tissues contain the early infiltrate of post-primary disease. The called for "radical and transformational" approach may be simply to put the two phases together: to regain understanding of the pathology and clinical course of untreated human pulmonary TB and to use modern technologies to do coordinated studies of human and validated animal models to address the disease as it occurs in human lungs.

## FOCUSED REVIEW OF THE LITERATURE ON PATHOLOGY OF TB

Reviews have accused us of bias in review of the literature of the preantibiotic era. Indeed, the literature on TB has always been replete with disagreements, contradictions, and alternative hypotheses. Many studies focused on issues that are no longer pertinent. From the beginning, we focused on learning what investigators saw clinically and pathologically, not on how they thought it worked. Even so, finding understandable and consistent information in the vast literature on human pulmonary TB was a formidable task. The nomenclature had changed and the older papers contained few pictures. Little progress was made until we obtained histologic lung sections of acute post-primary TB and were able to see for ourselves the relevant lesions and understand the nomenclature. We have now collected histologic sections from over 100 autopsies of people with untreated pulmonary TB. This has produced a remarkable degree of consistency. In time, we focused largely on two questions: First, is there any support for the modern idea that cavities form by erosion of granulomas into bronchi? Second, does post-primary TB begin as an obstructive lobular pneumonia that spreads asymptomatically through bronchi before undergoing necrosis to become caseous pneumonia that fragments to form cavities? We were unable to find even a single article written by a person who studied the pathology of human TB that supports the former, but scores of publications that support the latter in whole or in part and none that contradict it.

## THE FIRST ERA OF TB RESEARCH (1800-1950)

This era began when Laennec, using clinical observations, auscultation, and gross pathology, reported that 16 different conditions were all manifestations of one disease, TB (11). Microscopic observations begun in the mid nineteenth century demonstrated that granulomas were the characteristic lesion of primary TB, while obstructive lobular pneumonia was the characteristic lesion of developing post-primary disease (12). Pulmonary TB in the adult is different from primary TB from its inception (10). Rich wrote, "It has been found by all who have studied early human pulmonary lesions that they represent areas of caseous pneumonia rather than nodular tubercles" (13). The first demonstrable lesion of post-primary TB is a small focus of macrophages in alveoli, the early infiltrate, **Figure 1** (10). This is a lobular pneumonia that spreads through bronchi, not the blood or lymphatics as does primary TB. It may regress or undergo necrosis to become caseous pneumonia that softens and fragments to produce cavities or is retained to become the focus of fibrocaseous disease (consumption or phthisis). Granulomas in post-primary TB form as a reaction to retained caseous pneumonia (15). Fibrocaseous disease begins as a post-primary granuloma that is easily distinguished from primary granulomas because its cores are composed of the ghosts of alveoli rather than being a homogeneous mass of caseous debris. Cavities arise from dissolution of caseous pneumonia, not from erosion of

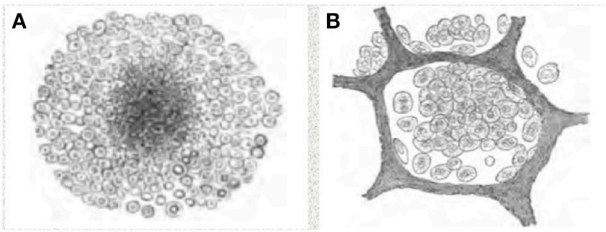


**FIGURE 1 |** Characteristics of primary and post-primary TB. **(A)** Granuloma of primary TB, **(B)** Early alveolar infiltrate, Assmann's focus, of post-primary TB. Primary and post primary TB are distinct disease entities caused by the same organism, just as chicken pox and shingles are distinct disease entities caused by varicella zoster virus. Primary TB produces primary granulomas while post-primary TB begins with the early infiltrate. Over a period of months, most early infiltrates regress asymptomatically, but some spread via bronchi as obstructive lobular pneumonia, develop into caseous pneumonia that fragments to produce cavities or to become the focus of post-primary granulomas and fibrocaseous disease. The pathology of these lesions has been described since the 1850's and they have been monitored by x-ray since the 1920's. These drawings are from Cornil and Ranvier (14).

granulomas into bronchi as is commonly believed today. This is not a hypothesis or speculation, but is a scientific fact supported by dozens of publications by investigators who studied hundreds or thousands of cases of untreated TB over a period exceeding 150 years and it has been confirmed by modern investigations. (9–32).

Development of x-rays facilitated monitoring disease over time. In 1925, Assmann drew attention to the early infiltrate, a solitary infraclavicular opacity which he had observed by x-rays in young adults with slight symptoms, no physical signs and a definite history of contact with TB. He suggested that this opacity might represent the early tuberculous focus of adults (33, 34). The early infiltrate was considered important because it was the onset of TB in the age groups in which the disease did then, and still does, cause the greatest devastation (34). This was rapidly confirmed by other investigators (10, 35–37). One could frequently demonstrate that the early infiltrates were TB by culture of gastric aspirates. Worldwide interest in the significance of this early infiltrate, the Assmann's focus, stimulated numerous studies and publications through the 1940's (33–44) Investigators were able to longitudinally observe the progression and/or regression of subclinical post-primary TB for months before the onset of symptoms.

Studies of correlation of x-rays of the early infiltrate with pathological changes in the lung were conducted by multiple investigators (37). Typically, lungs were removed from the body, inflated to their normal size with formalin vapor and examined with stereoscopic x-ray plates. (43). The early infiltrates were shown to be small areas of exudative bronchopneumonic TB typically near the pleural surface in the upper posterior part of the lung. Few tubercle bacilli were seen by AFB staining. Using serial x-rays, it was noted that such lesions frequently resorbed as completely as pneumococcal pneumonias although much more slowly (10). The lesions pathologically were shown to be fan shaped with centered on small bronchi and extending to the pleura. The discharge of bacilli into the sputum might be only intermittent because of the semisolid caseous material produced bronchial obstruction that trapped the organisms (34).

Developing early infiltrates of TB seen on an x-ray plates were easily recognized as a diffuse heavy mottling, frequently seen scattered along the main trunks suggesting "raisins upon a stem" (43). In favorable cases, the opacity became less well defined, less homogeneous and less dense, and eventually completely disappeared (33). Such a focus did not give rise to abnormal physical signs, was often associated with no symptoms, either local or constitutional, but could be detected in a radiograph of good quality when it is less than a quarter of an inch in diameter (37). In unfavorable cases, the lesions became confluent and produced pseudo-lobar caseous pneumonia. Tuberculous broncho pneumonia could exist in a large area without causing any signs or symptoms. Its recognition suggests a grave prognosis. However, spontaneous regression with evident absorption of the exudate and clinical cure were well documented. In one report, noticeable absorption of tuberculous exudate occurred in 59 cases of a series of 489 or more than 12 percent. In 7 cases the change was slight, in 24 the change was moderate, in 18 the change was marked. There was clinical improvement all of the patients. By use of stereoscopic X-rays, the a most vital developmental phase of the disease can readily be followed and studied more fully in the living human. The observations were startling because the changes noted were neither slight nor infrequent (43).

A thorough study of x-ray chest plates made it possible read the changes in terms of actual pathology (43). This required coordination of the pathologic and x-ray examinations. The localized fans, cone-shaped lesions, walled in by the septa were never seen in post mortem studies if the pathologist's usual sweeping cut from apex to base was made. But careful dissection along the bronchi revealed these lesions immediately under the pleura as seen on x-ray. So, too, reading chest x-rays without respect to trunks also failed to see the characteristic fan shaped lesions. But again, by following different trunks, it was possible to interpret the images in terms of the actual progressive pathology (43).

The major x-ray findings of developing pulmonary TB were confirmed and extended with high resolution CT in 1993. Im reported that post-primary or reactivated pulmonary TB begins with an acute necrotizing pneumonia in the subapical lung, followed by transbronchial spread (38, 39). They also studied CT findings of pulmonary TB before and after treatment and correlated the results with isolated cadaveric lungs of patients who died of TB (38). The presence of multiple fluffy nodules approximately 5 mm in diameter, described as acinar nodules, was described as the classical radiographic pattern of bronchogenic dissemination of TB (38). Centrilobular branching linear structures, the tree-in-bud sign, were seen in all patients with newly diagnosed pulmonary TB or reactivation TB, except in a patient with miliary TB. These lesions disappeared after 5–9 months of treatment in all 40 patients. (38). The CT findings were superior to bacterial isolation from sputum in assessing response to treatment.

Several recent publications have confirmed and extended these findings and suggested that the two-state paradigm of active and latent TB is an oversimplification and that TB has as a spectrum of infection states, with a subclinical phase of disease during which pathology evolves before symptomatic presentation (45–49). The tree-in-bud sign is now recognized by some as a characteristic CT sign of developing post-primary TB. It is probably the same as the "raisins on a stem" pattern published in 1924, but with a much less well developed conception of the underlying pathology. New technologies have greatly expanded studies. For example, whole-blood transcriptional signatures have identified adolescents who were at risk of developing active TB up to 12 months before clinical diagnosis (22, 50). In addition, positron emission tomography and computed tomography (PET–CT) have been used to study both human and a animal TB with unprecedented precision (51). However, leading publications still state that "the pathological hallmark of human TB is the granuloma, which is an organized and localized aggregate of immune cells that consists of macrophages, lymphocytes and other host immune cells" (51). In our opinion, failure to recognize the true nature of the pathology of developing human pulmonary TB is a major implement to research.

The first period of research ended rather abruptly in the 1950's with the introduction of antibiotics and decline in availability of human tissues from autopsies. Many people thought that TB was no longer a problem. In addition, basic sciences of immunology and inflammation were diverging from morphologic pathology. As a result, much information was purposely ignored and rapidly forgotten.

It seems hard to believe that the scientific community could have forgotten key facts about the pathology, x-ray appearance and clinical presentation of TB that had been learned by over a century of research. However, when one considers the environment of the 1950', it becomes not only understandable, but arguably inevitable. Seaview Hospital in Staten Island NY is an instructive example (52). In 1940, Seaview had 1400 beds for TB patients and was the largest healthcare facility in New York. Physicians were very good at physical and X-ray examinations with pathologic correlations. According to their archives, "A 'cure' was discovered here in 1957." The number of beds dropped to 26 and the hospital ceased operations (34). Most of the employees lost their jobs. In addition, the nation was recovering from two world wars and a depression. People wanted change. The "cure" was drugs that resulted from research in pharmacology, not pathology, or x-rays. Further, pathology and x-rays had little to contribute to the new sciences of genetics, cellular immunology, and macrophage biology that dominated the late twentieth century. Pathologists of the time were known to state that there was little as dull as a lung with a cavity (16).

## THE SECOND ERA OF RESEARCH ON TB (1990-PRESENT)

Research on MTB never stopped, but the introduction of antibiotics shifted the focus away from the disease in humans to basic science topics. MTB was largely considered to be a model for studying basic aspects of immunology and inflammation. The threat of the disease, after all, had ended. Once a leading cause of death, TB was now widely considered relegated to history. Early studies focused on lymphocytes and macrophages and expanded to a broad spectrum of cellular and molecular sciences (53). Much basic immunology was learned from studies of purified components of MTB in animal models. This included the activation of macrophages in granulomas, new vaccine adjuvants, and immunomodulating agents. BCG and its components were intensively investigated as immunotherapy for cancers. Very few investigators had access to BSL3 facilities for animal infections.

The emphasis on human TB revived in the 1990's when increasing drug resistance and HIV stimulated renewed interest in the disease. By this time, investigators of the earlier period had retired, animal models for studies of basic immunology of granulomas were well established and most investigators, myself included, had never seen a case of TB. Since, MTB produced granulomas in animals, people studied granulomas (51, 54–56). Because of the decades long gap, established insights into the clinical course, pathology and x-rays of human pulmonary TB were forgotten and many misconceptions became dogma. There were multiple contributing factors. With the decline in autopsies and interested pathologists, investigators did not have either the knowledge or resources to challenge the emerging dogma. Our priority was to use new technologies to study basic biologic phenomena. The internet did not yet exist so it could not help with literature searches. Finally, driven by opinions of peer reviewers for journals and funding agencies, virtually the entire scientific world accepted the dogma that granulomas are the important lesion of all TB.

## CURRENT PARADIGM OF THE PATHOGENESIS OF TB

Most recent research was guided by the paradigm that granulomas are the important lesion of both primary and post-primary TB. However, in over a decade of searching, we have not found a single article written by an investigator who personally studied the pathology of developing human post-primary pulmonary TB that supports the paradigm that granulomas are the characteristic lesion of both primary and post-primary TB and that cavities arise by erosion of granulomas in to bronchi. In other words, there is a disconnect between the clinical, x-ray and pathologic studies of the preantibiotic era and the basic understanding of the disease that guides today's research on TB. Some people dismiss the older reports as being "old," not recognizing that they studied the actual human disease in ways that are no longer ethically possible. Furthermore, a paradigm is "a framework containing the basic assumptions, ways of thinking, and methodology that are commonly accepted by members of a scientific community." (Dictionary.com). There is little chance of answering relevant questions if the assumptions, ways of thinking, and methodology discourage the most informative studies. Such paradigms are extremely difficult to dislodge (57).

The idea that cavities arise from erosion of granulomas into bronchi is a relatively recent concept. It became accepted as dogma during a period when cutting edge science used animal models and cell culture in the emerging fields of genetics, cellular immunology, and molecular microbiology. Morphologic pathology had little to contribute at this stage and was purposely ignored. TB produced granulomas in animal models and much excellent research was done to understand their biology. When interest was rekindled by the rise of drug resistance and HIV, investigators sought to justify their studies as models of human TB, but they had little knowledge of or access to untreated human tuberculous lung tissue. It had been reported that cavities in rabbits caused by *M. Bovis* form by erosion of granulomas into bronchi (58). It was, and is still, not generally recognized that MTB and *M. Bovis* produce cavities via different lesions (28). In the absence of contrary opinions, the observations using *M. Bovis* led to near universal acceptance of the paradigm that granulomas are the important lesion of both primary and post-primary TB. Guinea pigs are considered a good model because they produce "human like granulomas" without recognition that primary granulomas are only one type of human lesion. Similarly, mice are criticized because they do not produce caseating granulomas without recognition of the fact that their lesions resemble other phases of human TB (59, 60). Available evidence suggests that most, if not all, of the commonly used animal models develop lesions that are a mixture of elements of both primary and post-primary TB (60, 61). Most studies have focused on early events that are characteristic of primary TB. Enhanced recognition of the pathology of post-primary TB should enable design of animal experiments focused on that part of the human disease.

## FUNCTIONS OF PRIMARY AND POST-PRIMARY TB

The life cycle of MTB is to infect a person and induce systemic immunity that causes the lesions to heal. Then after a period that may extend for decades, develop an active pulmonary infection through either reactivation or reinfection that progresses to produce a cavity in the lung capable of producing masses MTB to be coughed into the environment. The organisms benefit most when large numbers of MTB pass through the airways and are expelled for decades from a healthy host, **Figures 2**, **3**. Osler reported measurements of MTB in sputum of a man with moderately advanced pulmonary tuberculosis 16 times in a 3 month period. He found 1.5 to 4 billion organisms per 24 h (18). Illness or death of the host diminishes the chance of transmission of the parasite. Laennec reported people with large thin walled cavities lined by a gray membrane (pellicle of masses of MTB), but no other lesions of TB who had survived in good health for many years and died of unrelated causes (11). Such largely asymptomatic carriers of TB are well known to be especially dangerous for the transmission of infection (13, 18, 37). Accordingly, the survival of MTB depends on producing a cavity in which the organisms can divide in vast numbers in a host who is otherwise highly resistant to infection. These are the functions of post-primary and primary TB respectively. Primary TB protects the host by producing effective systemic immunity that prevents disseminated infection. Post-primary TB, in contrast, somehow evades and distorts systemic immunity to produce cavities in which the organism can multiply and escape into the environment. Primary and post-primary TB accomplish their functions through distinct pathologic entities.

Primary TB is the infection that occurs in a person with insufficient immunity to localize and control MTB in granulomas. It can produce a spectrum of clinical disease states ranging from disseminated TB in persons with AIDS, meningitis, miliary tuberculosis and probably extra pulmonary granulomas. It typically occurs in the very young, immunologically naive, very old, or immunosuppressed persons. The infection can disseminate via lymphatics or the blood stream to lymph nodes and diverse other organs. In immunocompetent people, the infection is controlled in weeks and the lesions heal. Primary TB produces systemic immunity that effectively protects the entire body from disseminated infection. This immunity has been extensively studied and has become the "central dogma" of protective immunity mediated by macrophages, granulomas

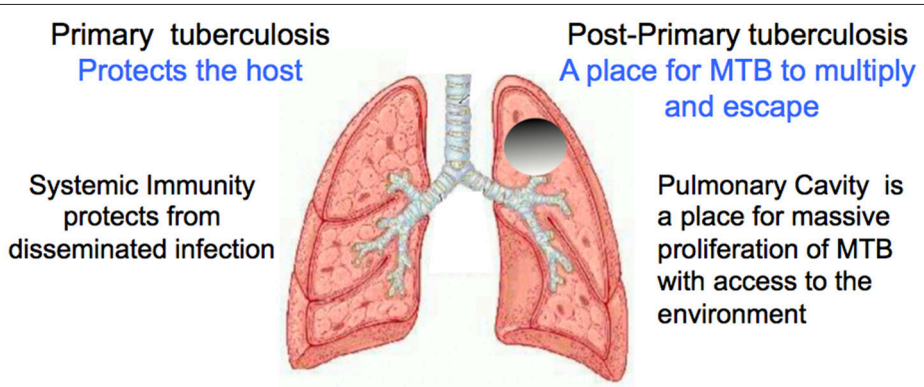


**FIGURE 2 |** Functions of primary and post-primary TB. The survival of this parasite depends on keeping its host alive while large numbers of organisms are produced and transmitted into the environment over a period of decades. Image modified from https://www.slideshare.net/saktivinayaga/trachea-lungs Dr Mohammed Faez, Department of Anatomy IMS/MSU.

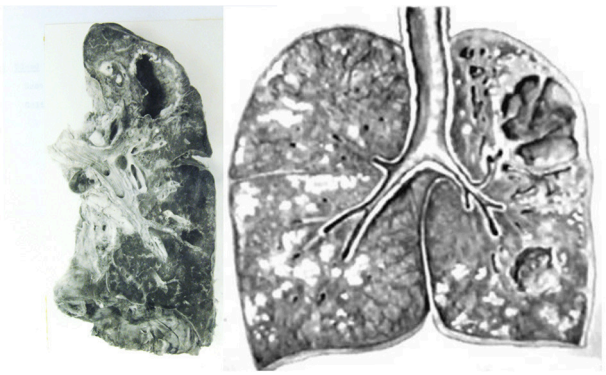

**FIGURE 3 |** Gross images of pulmonary TB. **(Left)** Lung with cavity but no other active TB. This is the type of lesion is most favorable to the parasite because the person had only minor symptoms of TB and spread infection for years before dying of other causes. The entire host was protected except for the cavity. **(Right)** Lung of a person who died of post-primary TB. TB has continued to spread beyond the cavity as obstructive lobular pneumonia, caseous necrosis, and chronic fibrocaseous disease. The necrotic lung that was not coughed out was surrounded by granulomas to become the focus of post-primary granulomas. Photos courtesy of Rich (13).

and production of IFN gamma by $CD4^+$ T cells (6). BCG replicates primary TB sufficiently to protect from disseminated TB and meningitis, but not from post-primary TB. Maintenance of protection depends on remaining immunocompetent. Lesions of primary TB can recur whenever immunosuppression reduces systemic T cell mediated immunity.

Post-primary TB, as its name suggests, typically begins only after primary TB has established systemic immunity. While the pathology and radiology of post-primary TB have been described in detail, very little is known about its mechanisms. The organisms somehow manipulate the host to produce the early infiltration. This isolates parts of the lung and so the organisms can survive in alveolar macrophages as an obstructive lobular pneumonia. Over a period of months secreted mycobacterial antigens and host lipids gradually accumulate in foamy alveolar macrophages that are trapped by obstructed bronchioles (26–30, 62). The early infiltrations slowly spread through bronchi to larger areas of lung. Many regress, but some undergo necrosis to produce caseous pneumonia that softens and is expelled to produce cavities or is retained to produce post-primary granulomas and fibrocaseous disease. Patients with the strongest immune responses measured by skin tests are the most likely to develop clinical disease (63, 64). This process was described by many pathologists in the pre antibiotic era and observed on x-rays by multiple investigators since the 1920's (33, 35). Several investigators reported that they could tell from x-rays who would develop clinical disease months in advance (35, 37). The radiologic appearance of the early infiltrate of subclinical pulmonary TB has been rediscovered several times. In the 1920's it was called Assmann's focus characterized by "raisin on a stem" appearance (33, 36, 43). In the 1990's, CT scans revealed greater detail and coined the term "tree-in-bud" sign as the characteristic feature of advancing pulmonary TB (65–67). In the past few years, this has been confirmed by studies in South Africa and Latvia using CT and PET-CT (22, 40, 45).

## IMPLICATIONS FOR FUTURE RESEARCH

As discussed above, research on TB has already begun twice with little carry forward of information. Investigators in the first period developed detailed description of the pathology, imaging, and clinical presentation of untreated TB, but did not have the scientific tools to advance it. Investigators today have far advanced analytic tools, but are hampered by a flawed paradigm that does not recognize the sequence of lesions of post-primary TB. This is beginning to change with the increased recognition of subclinical TB and relevant animal models. Nevertheless, a third new beginning is now necessary to merge the clinical, pathologic and radiologic insights of the first era with the immunology, cell biology and genetics of the second so that the tools of modern science can finally be used to study the actual human disease. Recognition of primary and post-primary as separate pathologic entities immediately suggests resolution of long standing questions and multiple testable hypotheses as follows (28–30, 62, 68–70).

- **How can MTB be an obligate human parasite when people are more resistant than any of the animals studied?** Humans are more resistant to TB because most develop effective immunity against primary TB in weeks whereas most animals die within months of progressive disease composed of a mixture of primary and post-primary components. MTB is an obligate human parasite because only humans develop post primary TB that progresses to pulmonary cavities from which infection can be transmitted to new hosts.
- **What is the nature of the immunity that protects most people from post-primary TB?** Most current research focuses on control of primary TB by T cells and macrophages. Post-primary TB is different. It does not begin until primary TB has established a degree of systemic immunity. MTB apparently manipulates, strengthens, and uses our strongest immune responses locally in the early infiltration to develop caseous pneumonia and cavities from which it can escape to infect new hosts. Most early infiltrations regress spontaneously. The challenge is to understand why and develop means to make them all regress.
- **How can multiple pulmonary lesions in a single lung act independently as if the others did not exist?** Bronchial obstruction is a local process of the early infiltration that begins in different parts of the lung at different times. This starts the clock for accumulation of mycobacterial antigens, host lipids and cells that drive the disease.

## OBSTACLES TO RESEARCH

Several major obstacles must be overcome to pursue research on post-primary TB. The first is availability of informative tissue for study. Studies using peripheral blood, lymph nodes, and bronchoalveolar lavage cells are unlikely to be able to dissect multiple types of independently developing lesions in a single

lung. Surgical resections and hospital autopsies seldom have the key lesions since nearly all people with TB who reach medical care are treated. As was discovered in the early days of antibiotic therapy, treatment rapidly abolishes the early infiltration of post-primary TB. The only sources are lungs from autopsies or emergency pneumonectomies for hemorrhage in people with untreated pulmonary TB. Patients who die of untreated TB are likely to be autopsied by medical examiners or forensic pathologists. Unfortunately few of these people work on TB or have close relationships with the TB research community. There are also significant legal, cultural and religious objections to research using autopsies. Nevertheless, with 5,000 deaths from TB/day, specimens do exist. Since human lungs frequently contain multiple lesions of TB that behave independently at both early and late stages, the entire range of disease processes could be studied in a small number of cases. The problem is to find them and build relationships with the appropriate people and institutions to access them for research.

Most widely used animal models are focused on early lesions in naïve animals that are largely models of primary TB. MTB is an obligate human parasite because no animal develops the post-primary lesions required for transmission to new hosts as do humans. However, many animal models can be adapted to produce lesions resembling those of particular stages of post-primary TB. For example, mice, rabbits, and guinea pigs all develop a pattern of pulmonary burden of MTB that is consistent with the early infiltrate of developing post-primary TB (60, 71). As stated by Robert North, a central problem in TB research is to explain why immunity to infection does not enable mice, guinea pigs, rabbits, or susceptible humans to resolve this lung infection and thereby stop the development of disease (61). Progressive pulmonary tuberculosis is not due to increasing numbers of viable bacilli in rabbits, mice, guinea pigs, and humans who develop paucibacillary disease, but is due to a continuous host response to mycobacterial products (72). This is reminiscent of the early infiltrate of post-primary TB in each of these species. Various manifestations of the primary and post-primary TB probably occur in animal models as described elsewhere, but they are not separated or coordinated as in humans (60, 71). This provides opportunities to develop animal models of particular components of post-primary TB by reproducing the conditions in animals that exist in humans at particular stages of infection (16, 36, 73).

## POTENTIAL OF ADVANCED TECHNOLOGY

Driven largely by advances in cancer research, the capabilities for study of formalin-fixed, paraffin embedded (FPPE) tissues on slides has expanded enormously. In the preantibiotic era, pathologists could only look at routine H&E stained sections and count acid-fast bacilli. Now, multi-color immunofluorescence with advanced image analysis can be done on slides with preservation of the tissue architecture and intact microenvironment (74). Our preliminary studies illustrate the value of newer technology. Sections of characteristic lesions of human primary TB, caseating granulomas, early infiltrate and cavities of post-primary were selected for quantitative immunohistochemical studies of macrophages, lymphocytes, endothelial cells, and mycobacterial antigens (69). Abundant mycobacterial antigen, but very few AFB, were present in foamy alveolar macrophages of early infiltrates. Primary granulomas contained a preponderance of $CD4^+$ T cells while the early infiltrate lesions contained more $CD8^+$ T cells. More $foxp3^+$ (Treg) cells were found in cavity walls than in other types of lesions. In other studies, we investigated the presence of regulatory markers associated within early infiltrates of post-primary TB (70). We chose three markers of mTOR signaling (pmTOR, insulin-like growth factor-1 receptor and activated Akt) and a second pathway of macrophage activation, COX-2. The results suggested that foamy macrophages in early infiltrate lesions over activate mTORC1, potentially inhibiting autophagy of the infected cell and limiting MTB killing. In addition, programed death-1 ligand (PD-L1) was highly expressed in foamy macrophages, surrounded by PD-1-expressing lymphocytes in the alveolar walls. Thus, in this critical MTB microenvironment of foamy alveolar macrophages, PD1, PD-L1 and two suppressor host response pathways appear active (mTOR and COX-2), possibly facilitating TB disease progression.

It is now possible to assess gene expression of both host cells and bacteria on routinely prepared slides (75). With recent advances in the immunotherapy of cancer, the methods for studying mutations, cell maturation and differentiation, immune parameters, inflammation, and healing on slides have advanced dramatically and will continue to increase (70). Thousands of studies can be done on single paraffin embedded samples over a period of years. Enhanced imaging can monitor the lesions over time. This is providing new opportunities for studying human tissues and for developing animal models to be used in a coordinated fashion with human tissues to successfully address previously inaccessible components of human TB.

In summary, evidence produced over nearly 200 years demonstrates that primary and post-primary TB are distinct disease entities that function to protect the human hosts from disseminated infection and to produce cavities for transmission to new hosts respectively. The existence of these two disease entities suggests plausible answers and testable hypotheses to long standing questions about the pathogenesis of TB and have significant implications for the design of vaccines and host directed therapies. However, post-primary TB is exceedingly difficult to study because it occurs fully developed only in human lungs and there is no ethical reason to do biopsies or resections of developing lesions. However, available evidence suggests that animal models can be constructed to replicate particular stages of the human post-primary TB. There is reason for optimism that coordinated studies of such animal models with available human tissues and advanced imaging will lead to significant advances.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# IL-22: An Underestimated Player in Natural Resistance to Tuberculosis?

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Approximately 10% of individuals latently infected with *Mycobacterium tuberculosis* (Mtb) develop active tuberculosis (TB) during their lifetime. Although it is well recognized that T-helper 1 immune responses are crucial for containing latent TB infection, the full array of host factors conferring protective immunity from TB progression are not completely understood. IL-22 is produced by cells of the innate and adaptive immune system including innate lymphoid cells, and natural killer cells as well as T lymphocytes (Th1, Th17, and Th22) and binds to its cognate receptor, the IL-22R1, which is expressed on non-hematopoietic cells such as lung epithelial cells. However, recent studies suggest that Mtb induces expression of the IL-22R1 on infected macrophages and multiple studies have indicated a protective role of IL-22 in respiratory tract infections. Reduced concentrations of circulating IL-22 in active TB compared to latent TB and decreased percentages of Mtb-specific IL-22 producing T cells in TB patients compared to controls designate this cytokine as a key player in TB immunology. More recently, it has been shown that in type 2 diabetes (T2D) and TB co-morbidity serum IL-22 concentrations are further reduced compared to TB patients without co-morbidities. However, whether a causative link between low IL-22 and increased susceptibility to TB and disease severity of TB exists remains to be established. This review summarizes the contribution of IL-22, a potentially under-appreciated key player in natural resistance to TB, at the interface between the immune response to Mtb and the lung epithelium.

**Keywords: tuberculosis, *Mycobacterium tuberculosis*, interleukin-22, IL-22R1, T lymphocytes, respiratory infections**

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## INTRODUCTION

A quarter of the human population is infected with *M. tuberculosis* (Mtb) (1) of which ∼10% will develop the active and contagious form of tuberculosis (TB) during their lifetime (2). Various intrinsic and extrinsic factors determine the natural course of mycobacterial infection, and resistance vs. susceptibility to disease progression. These factors include host genetic susceptibility (3), virulence of the infecting strain (4) and presence of acquired immune deficiencies such as HIV infection and type 2 diabetes (T2D) (5). The role of IL-22 during the host defense against Mtb is poorly understood. The subsequent sections highlight our current knowledge of the protective function of IL-22 during respiratory tract infections, including TB.

## SOURCE AND TARGETS OF IL-22

IL-22 is produced by cells of the innate as well as the adaptive immune system including tissue resident innate lymphoid cells (ILCs), NK cells, macrophages, NKT cells, activated Th1, Th17, and Th22 cells as well as Tc-cell subsets and γδ T cells (6). Alveolar macrophages from both humans and mice are also able to produce and release IL-22 (7). In mice, antigen-specific IL-22 production is driven by Th1 and Th17 cells, but only a small subset of Th17 cells produce IL-22 in humans. In contrast to mice, humans have a distinct subset of T helper cells, called Th22 cells, which produce IL-22 and TNFα. Unlike Th1 and Th17 subsets, human Th22 cells, which were initially characterized in skin neither produce IL-17 nor IFNγ (8). Apart from secreting IL-22, Th22 cells can also express granzymes, IL-13 and increased levels of Tbet showing a remarkable plasticity to skew the immune response toward pro- or anti-inflammatory depending on the Th1 or Th2 stimulus *in vitro* (9).

IL-22 binds to its heterodimeric receptor complex consisting of the IL-22R1 and the IL-10R2 to activate the JAK-STAT signaling pathways (10). The IL-22R is present on epithelial cells of the lung, gut and skin, the liver, pancreas, and kidneys. It is not expressed on hematopoietic cells, neither in resting/naïve nor activated macrophages, T or B cells, nor the human monocyte THP-1 cell-line (11, 12). However, three independent studies reported that Mtb induces expression of the IL-22R1 in infected macrophages (13–15). The significance of this is discussed in the next section.

A T cell-derived soluble IL-22 binding protein (IL-22BP), which shares sequence homology with the extracellular domain of the membrane bound IL-22R1, acts as endogenous inhibitor of IL-22 by preventing its binding to the IL-22R1. Activation of the IL-22 signaling pathway in epithelial cells results in epithelial tissue proliferation, regeneration, and healing, therefore this cytokine plays an important role in protection from infection-induced tissue damage at mucosal surfaces (10). IL-22 induces expression of the chemokines CXCL1 and CXCL5 in bronchial epithelia in a *Klebsiella pneumoniae* infection model (16), but reduces CXCL8, a neutrophil attracting chemokine, in A459 human lung carcinoma cells (17). Most importantly, IL-22 stimulates the production of antimicrobial peptides such as β-defensins, the S100 family of peptides, Reg3β and γ, lipocalin-2, calprotectin and calgranulin A in various cell types (18–21), thereby controlling bacterial growth and reducing the risk of secondary bacterial infections after viral injury (22).

## IL-22 AS IMMUNE-MODULATOR TO INHIBIT MYCOBACTERIAL GROWTH

In addition to the well-described effect of IL-22 on epithelial cells the recent reports that Mtb induces expression of the IL-22R1 on macrophages, the primary host immune cells targeted by mycobacteria, is particularly intriguing. Treerat and colleagues report IL-22R1 positive macrophages by immunohistochemistry in granulomas of HN878 infected mice, but whether this positive signal is due to HN878 induced IL-22R1 expression on macrophages or through ingestion of IL-22R1 positive epithelial cell debris by the lung macrophages remains to be confirmed (15). Two previous studies report a modest induction of IL-22R1 expression on macrophages after stimulation with Mtb H37Rv and Erdman by flow cytometry (13, 14). Upregulation of the IL-22R1 in infected macrophages may be a host-mechanism to combat the infection, as there is growing evidence that IL-22 can modulate mycobacterial growth within macrophages.

In initial experiments Dhiman et al. observed that Mtb-infected human monocytes induce production of IL-22 by co-cultured autologous NK cells in a IL-15 and IL-23 dependent manner. This NK mediated IL-22 production resulted in reduction of intra-macrophagic bacteria and was reversed through neutralization of IL-22 suggesting that the mycobacterial growth inhibition is at least in part attributable to IL-22 (13). In subsequent experiments by the same group exogenous addition of recombinant IL-22 (rIL-22) to infected macrophages promoted phagolysosomal fusion and reduced bacterial burden (23). The anti-mycobacterial activity of IL-22 was mediated through increased expression of the anti-microbial peptide calgranulin A and siRNA knock down of calgranulin A abrogated the IL-22 dependent mycobacterial containment in monocyte derived macrophages (23). An additional mechanism by which IL-22 may contribute to reduction in mycobacterial burden is the observed increased TNFα production by Mtb-infected bone marrow-derived macrophages when pre-treated with rIL-22 (15), however this mechanism requires confirmation through TNFα neutralization experiments.

An unusual observation that a subset of $CD4^+$ T-cells in Mtb-infected humans and macaques retain IL-22 at the cell membrane instead of secreting it was reported by Zeng et al. who speculated that a membrane-bound IL-22 may enjoy longer half-life. The authors show that $IL\text{-}22^+CD4^+$ T cells reduce intra-macrophagic mycobacteria by direct cell-to-cell contact, however whether the anti-mycobacterial effect is indeed mediated by direct interaction of membrane-bound IL-22 on T cells with the IL-22R1 on macrophages remains to be corroborated with additional data (14).

The responsiveness of macrophages to IL-22 has also been shown in a different context, where IL-22 modulates cholesterol efflux from macrophages (24). This may have implications for control of mycobacteria, which catabolize host sterols to sustain a persistent infection (25). A summary of our current knowledge of the actions of IL-22 is shown in **Figure 1**.

## THE ROLE OF IL-22 IN EXPERIMENTAL ANIMAL MODELS OF LUNG INFECTION

In an experimental murine model of *Streptococcus pneumoniae* rapid accumulation of IL-22 producing ILC3 in the lungs were observed and associated with protection from lethal infection (26). $IL\text{-}22^{(-/-)}$ mice had greater streptococcal burden compared to wild-type mice and administration of rIL-22 reduced bacterial burden (27). Similarly, reduction of IL-22 production by depletion of ILCs in a *Pseudomonas aeruginosa* murine model induced lung injury was associated with reduced survival (28)

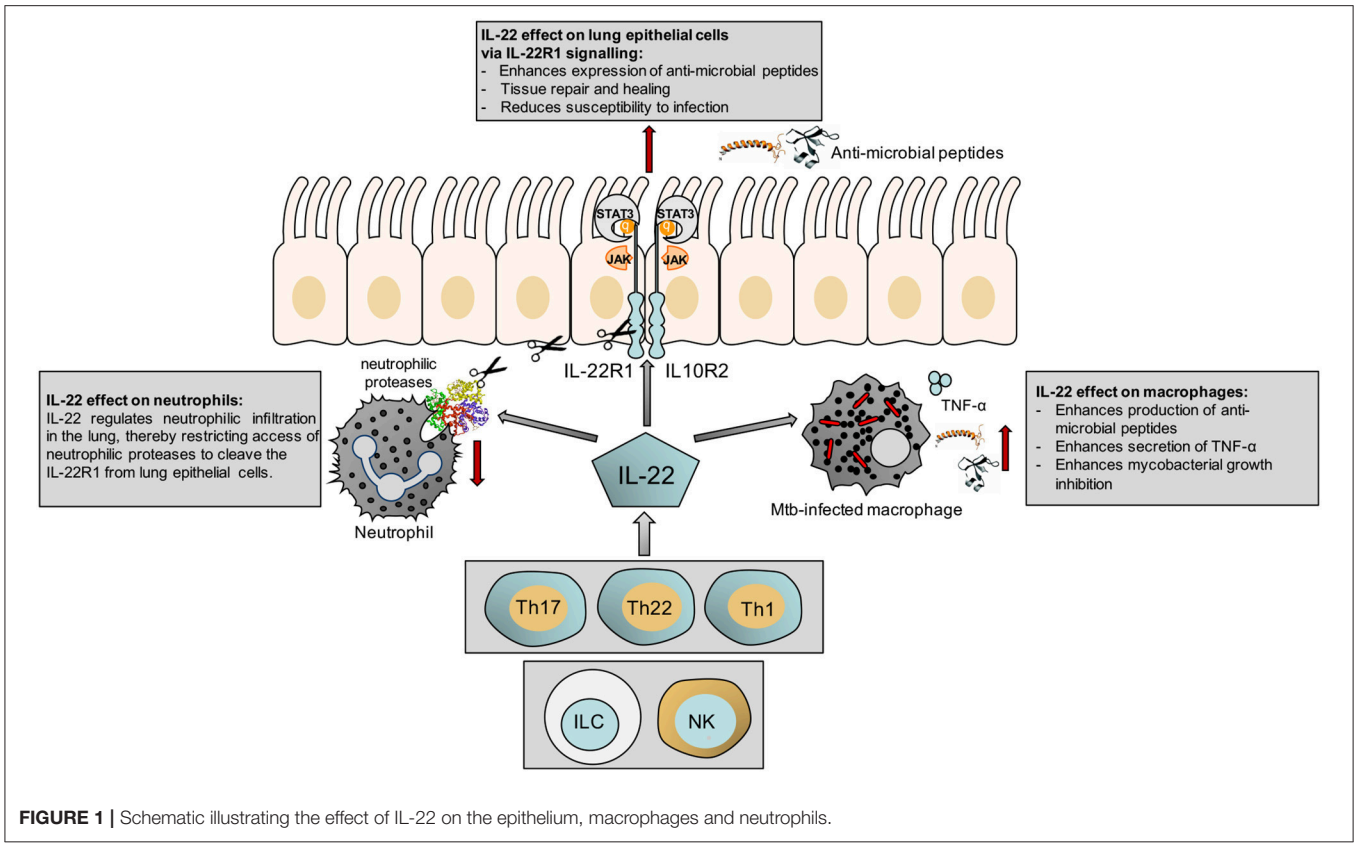


**FIGURE 1 |** Schematic illustrating the effect of IL-22 on the epithelium, macrophages and neutrophils.

pointing toward a host-protective role of IL-22 in both lung infection models. Interestingly, antibody-based neutralization of IL-22 led to increased neutrophilic infiltration and susceptibility to *P. aeruginosa* (29). This observation is consistent with the finding that IL-22 reduces expression of the neutrophil attracting chemokine CXCL8 from lung epithelial cells (17) and therefore a lack of IL-22 likely increases CXCL8, which in turn may drive the neutrophilic infiltration.

Neutrophilic proteases were previously shown to cleave the IL-22R1 on human bronchial epithelial cells and impair IL-22-dependent β-defensin expression, potentially contributing to pathogen replication (30). Administration of rIL-22 or neutralization of IL-22BP resulted in a decrease in lung damage and increased survival (29). Interestingly, *P. aeruginosa* has developed an immune-evasion strategy by secreting a serine protease which cleaves IL-22 resulting in its degradation, thereby weakening tissue repair and the anti-microbial defense (30). It will be interesting to investigate whether Mtb has acquired similar immune-evasion strategies and possesses proteases which cleave IL-22 and thus impair the IL-22R signaling pathway and host tissue repair. A murine model of *Haemophilus influenzae* infection further confirmed the beneficial effects of IL-22 observed in *P. aeruginosa* infected mice, where IL-22$^{(-/-)}$ mice had increased bacterial burden and administration of exogenous IL-22 boosted bacterial clearance and limited lung tissue damage (31). IL-22 secretion by Th17 cells is crucial for control of the Gram-negative pulmonary pathogen *K. pneumoniae* and promotion of lung epithelial cell proliferation (16). IL-22 also reduces lung inflammation during influenza A virus infection and protect against secondary bacterial infection (22). In summary, there is evidence from various lung infection models that IL-22 plays a protective effect on host mucosal surfaces, whereas the effect of IL-22 on bacterial clearance appears to be pathogen-specific.

Mycobacterial infection models using IL-22$^{(-/-)}$ mice have yielded conflicting results. IL-22$^{(-/-)}$ mice infected with both high and low dose H37Rv had comparable pro-inflammatory cytokine profiles in the lung as wild-type C57BL/6 mice with exception of increased IL-6 and reduced MMP-9 and CXCL-10 (32). Recruitment of macrophages and granulocytes to the lung were similar between IL-22$^{(-/-)}$ and wild-type mice and there were no significant differences in bacterial burden and survival. Similar to the studies in IL-22$^{(-/-)}$ mice, administration of an IL-22 neutralizing antibody to wild-type mice 12 weeks post-infection did not compromise survival or alter bacterial burden (33). However, the timing of anti-IL22 administration may be crucial as the *P. aeruginosa* infection model suggests that elevated IL-22 concentrations prior to infection are important for conveying the protective effects (29). The Mtb infection studies in IL-22$^{(-/-)}$ mice by Behrends and colleagues were carried out with H37Rv and different results were obtained when the knock out mice were infected with HN878 (15). Mtb HN878 infection induced IL-22 production *via* a TLR2 and IL-1β-dependent pathway and in this model IL-22 plays

an important role in recruitment of myeloid cells to the lung (15). IL-22$^{(-/-)}$ mice were more susceptible and exhibited higher bacterial burden during the chronic stage of HN878 infection 100 days post-infection, whereas no differences in susceptibility were observed during the acute phase 30 days post-infection. Therefore, these data suggest that susceptibility of IL-22$^{(-/-)}$ mice to mycobacteria is largely driven by the infection stage (acute vs. chronic) and the mycobacterial strain (H37Rv, Erdman vs. HN878). Strain specific differences in eliciting an IL-22 response have also been shown in PBMCs from TB patients stimulated with cell wall extracts from Mtb HN878, which resulted in greater production of IL-22 compared to H37Rv cell wall extracts (15). In addition, as the timing of exogenous administration of IL-22 appears to be important from other infection models, further studies in IL-22$^{(-/-)}$ mice with administration of IL-22 prior to Mtb infection are required. Such studies are however complicated by the short half-life of IL-22 and its off-target effects on mucosal tissues other than the lung.

In a non-human primate model, Mtb infection resulted in reduced IL-22 mRNA expression in peripheral blood but increased expression in the lymphocytes of the lungs, bronchial lymph nodes, and the spleen (34). These observations from primates are consistent with human studies where elevated IL-22 protein was found at the site of disease in human broncho-alveolar lavage fluid (BALF) (35–37) as well as increased percentages of IL-22$^+$ CD4$^+$ T cells in BALF compared to blood (38). Although elevation of IL-22 in primates was associated with severe TB, it is not clear whether IL-22 production is induced as a consequence of enhanced inflammation to counteract immunopathology or directly contributes to pathology itself. IL-22 producing T cells were also observed in BALF from Mtb-infected compared to un-infected primates and were visualized in TB granulomas by immunohistochemistry (39). Furthermore, IL-22 expression was also found in lung and lymph node granulomas of *Mycobacterium bovis* infected cattle (40). Interestingly, in this species IL-22 was shown to be one of the dominant surrogates of protection from bovine TB after *M. bovis Bacille-Calmette-Guerin* (BCG) vaccination (41). Whether IL-22 is a surrogate of protection from human TB remains to be established.

## IL-22 IN HUMAN LATENT AND ACTIVE TB

In humans Mtb induces a distinct antigen specific IL-22$^+$ CD4$^+$ T cell population with central memory phenotype, which was first identified in antigen stimulated whole blood from mycobacteria exposed individuals (35). People with latent TB infection (LTBI), who have not progressed to active TB, have significantly higher frequencies of these Mtb specific IL-22 producing CD4$^+$ cells compared to active TB patients (42), which is consistent with the increased frequencies in IFNγ-producing Th1 cells during LTBI vs. TB. It is likely that both the Th1 and Th22 cell populations in addition to Th17 cells contribute to protection from progression to TB. Furthermore, a single nucleotide polymorphism in the promoter of the IL-22 gene, which is associated with higher Mtb-antigen specific IL-22 production from PBMCs is over-represented in controls compared to TB patients suggesting that it is associated with reduced susceptibility to TB (43). Some studies report higher serum concentrations of IL-22 in individuals with LTBI compared to TB patients (44–46), whereas other studies do not show significant differences in circulating IL-22 concentrations (42). These different observations may be due to the ethnic background and Mtb strains prevalent in the respective study cohorts.

At the site of disease however, several studies consistently report increased concentrations of IL-22 in BALF from TB patients compared to controls and higher IL-22 concentrations at the site of disease vs. peripheral blood (35–37), which may be due to migration of antigen specific IL-22 producing T cells to the site of disease, the lung. In patients with TB pleurisy, IL-22, and IFNγ were also elevated in pleural fluid as were antigen-specific IL-22 producing CD4+ T cells (47). In patients with extra-pulmonary TB-associated pericardial and pleural effusions IL-22 concentrations correlated with MMP-9 expression (36). However, whether IL-22 contributes to immunopathology or is produced to counteract immunopathology was not established in this context, although MMP-9 production has been linked to improved epithelial barrier function in the gut (48) and it is possible that IL-22 and MMP-9 are induced in order to promote healing rather than being drivers of immunopathology. Successful TB treatment restores antigen-specific IL-22 responses by reducing the frequencies of CD19+CD1d+CD5+ regulatory B cell, which were shown to suppress IL-22 production (49).

In patients with *Mycobacterium avium complex (MAC)* infection, low IL-22 concentrations in BALF were associated with a neutrophil dominant inflammatory response, radiological severity and progression to pulmonary MAC disease, whereas individuals high IL-22 concentrations in BALF had greater percentages of lymphocytes and less disease severity (50). This finding is consistent with the observation that IL-22 regulates neutrophilic infiltration as shown in an animal model of lung infection (29). Collectively these data point toward an important role of IL-22 in mycobacterial infection and highlight the need to further define its role in progression from latent to active TB as well as in treatment outcomes.

## IL-22 IN TB-DIABETES CO-MORBIDITY

The threat of TB and diabetes (T2D) comorbidity to TB control programs is well recognized, but the underlying mechanisms contributing to increased susceptibility of T2D patients to TB and the increased risk of poor treatment outcomes in patients with TB-T2D comorbidity are poorly understood (51, 52).

T2D patients with LTBI have lower frequencies of Mtb-specific Th1, Th17, and Th2 responses compared to normoglycemic individuals with LTBI. Once T2D patients develop TB they exhibit higher circulating concentrations of Th1 and Th17 cytokines compared to TB patients without T2D (52). Despite this increased production of Th1 and Th17 cytokines, which are important for protective immune responses to Mtb, TB-T2D patients are more likely to fail treatment and relapse after initial cure (53). Interestingly, IL-22 is the only cytokine

found at lower concentrations in serum of TB-T2D patients compared to TB patients without co-morbidities (44, 46), but a causative link between low IL-22 serum concentrations and risk of poor treatment outcomes is far from established. Interestingly, Kumar et al. reported that T2D patients with LTBI had higher IL-22 serum concentrations compared to individuals with LTBI and no T2D (45). Although this appears puzzling, it is possible that latently infected T2D patients with high basal concentrations of IL-22 are less likely to progress to active disease.

A study based on a high fat diet mouse model of T2D showed that the induction of IL-22 from $CD4^+$ cells is impaired in obese mice in response to challenge with the intestinal pathogen *Citrobacter rodentium*, making them more susceptible to infection. This defect was restricted to IL-22 producing T cells and IL-22 secretion by ILCs was not affected (54). Administration of rIL-22 not only improved mucosal host defense, but also many of the metabolic symptoms including hyperglycemia and insulin resistance in this and another murine T2D model (54, 55). This further raises the question whether IL-22 may be useful as adjunct host-directed therapy in the context of TB-T2D.

## CONCLUSIONS

IL-22 is a key regulator of immunity and inflammation at mucosal surfaces including the lung. Current evidence suggests that an optimal amount of this cytokine prior to infection can contribute to containment of bacteria and to protection from excessive tissue damage. The contribution of IL-22 and Mtb-specific $IL\text{-}22^+$ T cells in protection from progression to TB in presence and absence of T2D co-morbidity in humans and the importance of this cytokine in TB treatment response requires further studies.

## AUTHOR CONTRIBUTIONS

KR, RS, and MC reviewed the current literature. KR and RS wrote the manuscript. All authors critically reviewed the manuscript and MC created **Figure 1**.

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REVIEW
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# Genetic Resistance to *Mycobacterium tuberculosis* Infection and Disease

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Natural history studies of tuberculosis (TB) have revealed a spectrum of clinical outcomes after exposure to *Mycobacterium tuberculosis*, the cause of TB. Not all individuals exposed to the bacterium will become diseased and depending on the infection pressure, many will remain infection-free. Intriguingly, complete resistance to infection is observed in some individuals (termed resisters) after intense, continuing *M. tuberculosis* exposure. After successful infection, the majority of individuals will develop latent TB infection (LTBI). This infection state is currently (and perhaps imperfectly) defined by the presence of a positive tuberculin skin test (TST) and/or interferon gamma release assay (IGRA), but no detectable clinical disease symptoms. The majority of healthy individuals with LTBI are resistant to clinical TB, indicating that infection is remarkably well-contained in these non-progressors. The remaining 5–15% of LTBI positive individuals will progress to active TB. Epidemiological investigations have indicated that the host genetic component contributes to these infection and disease phenotypes, influencing both susceptibility and resistance. Elucidating these genetic correlates is therefore a priority as it may translate to new interventions to prevent, diagnose or treat TB. The most successful approaches in resistance/susceptibility investigation have focused on specific infection and disease phenotypes and the resister phenotype may hold the key to the discovery of actionable genetic variants in TB infection and disease. This review will not only discuss lessons from epidemiological studies, but will also focus on the contribution of epidemiology and functional genetics to human genetic resistance to *M. tuberculosis* infection and disease.

**Keywords: host genetics, resistance, tuberculosis, resisters, susceptibility**

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## INTRODUCTION

Tuberculosis (TB), caused by the human pathogen *Mycobacterium tuberculosis*, was the leading cause of death due to a single infectious agent in 2016, resulting in 1.6 million deaths (1). The bacterium is spread through the air by droplet nuclei containing *M. tuberculosis* from the lungs of individuals with active disease to the respiratory tract of uninfected individuals (2). Infection by *M. tuberculosis* is a complex, multistage process progressing from the first encounter with the bacterium (**Figure 1**). For this reason a multistep course of disease has to be imagined (5).

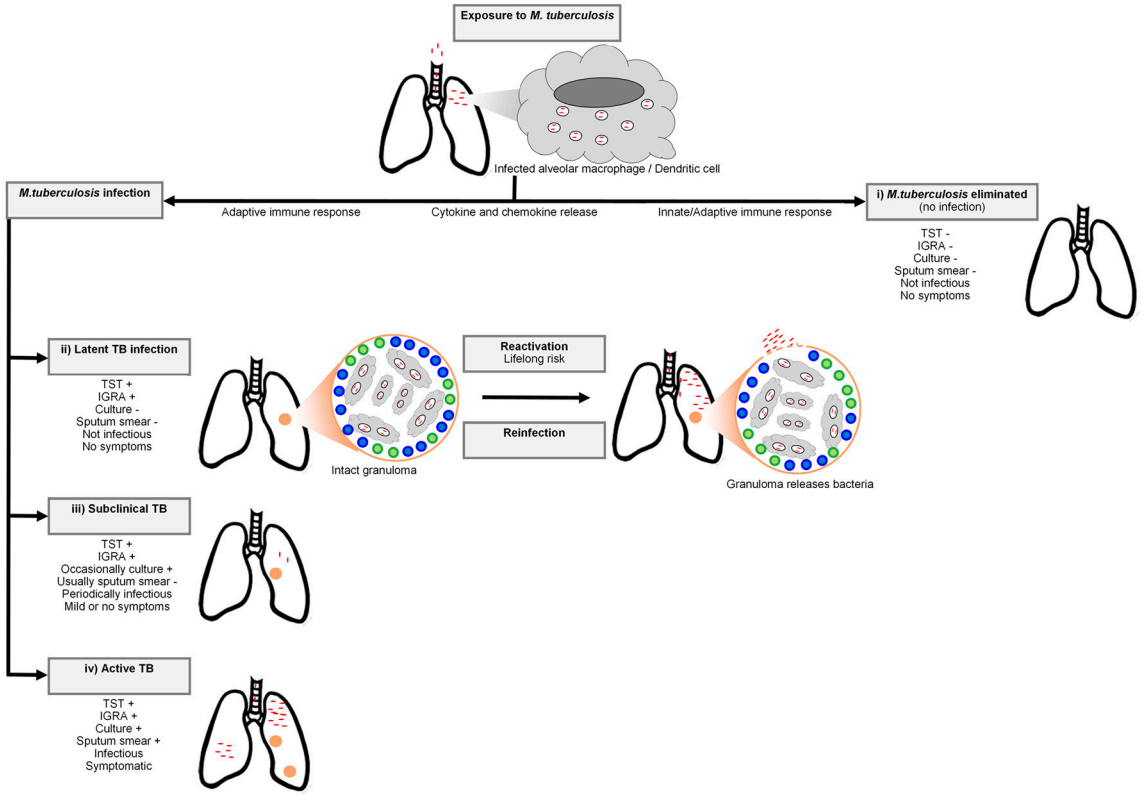


**FIGURE 1 |** A simplified representation of the *Mycobacterium tuberculosis* infection spectrum and outcomes. The bacteria enter the respiratory system of the host via inhaled droplets and are engulfed by macrophages and dendritic cells. There are four potential outcomes after bacterial inhalation: (i) *M. tuberculosis* is immediately eliminated by the pulmonary immune system, (ii) the bacteria are contained in granulomas by recruited adaptive immune cells (including T cells and B cells) and infection does not progress to active TB. Although this containment can last for a lifetime, *M. tuberculosis* can also disseminate from granulomas (reactivation) or reinfection with another mycobacterial strain can occur, resulting in active TB, (iii) sub-clinical disease characterized by intermittent symptoms and periodic infectiousness, or (iv) infection develops into active TB. Adapted from Pai et al. (3) and Möller et al. (4).

After inhalation, the droplet nuclei move to the alveoli where the bacteria are phagocytosed by alveolar macrophages and dendritic cells. The phagocytosis of the bacterium invokes a strong host cellular immune response and a cascade of events is triggered that involves cytokines and chemokines (2). Not all individuals exposed to the bacterium will become infected and depending on the infection pressure, many will remain free of infection. In infected individuals the bacteria will begin to replicate in the intracellular environment and migrate to lymph nodes in the lung through the lymphatic system (6). In the first 2–8 weeks after infection, cell-mediated immunity will develop (7) and conversion to tuberculin reactivity takes place (6). To limit the spread and replication of the bacteria, granulomas are formed by activated T lymphocytes and macrophages. The majority of individuals will remain asymptomatic and contain the bacterium, and enter a stage termed latent TB infection (LTBI). Remarkably, it is estimated that ∼25% of the global population was latently infected with *M. tuberculosis* in 2014 (8). At this stage the immune system can contain the infection, but if it fails, the infection may progress to active disease (7). Only 5-15% of immunocompetent LTBI positive individuals will progress to clinical TB (8). In these cases, the bacteria continue to replicate and disease symptoms will start to appear. Common symptoms of TB include persistent coughing, fever, coughing of blood, night sweats, weight loss, and chest pain. Diagnosis of pulmonary TB is possible through smear microscopy, bacterial culture of sputum or GeneXpert (9).

LTBI is at present inferred from measures of acquired anti-mycobacterial immunity, such as a tuberculin skin test (TST) and/or interferon gamma release assay (IGRA). The TST was the original gold standard for LTBI diagnosis (10). A delayed hypersensitivity reaction to mycobacterial antigens is measured by injecting tuberculin purified protein derivative (PPD) intradermally into the forearm, followed by measuring the induration 48 h later (10). A positive TST in an immunocompetent individual is defined as an induration of 10 or more millimeters in high prevalence countries. The PPD antigens are not specific to *M. tuberculosis* and may result in false positive reactions if individuals were exposed to non-tuberculous mycobacteria or immunized with *M. bovis BCG* (10). In contrast, IGRA is a whole blood assay, which uses the specific *M. tuberculosis* antigens ESAT-6, CFP-10, & TB7.7 to stimulate antigen-specific CD4 T cells to release interferon gamma, which is then measured. Even within LTBI individuals there is a

spectrum of infection states ranging from the early elimination of infection to subclinical TB, which cannot be differentiated by TST or IGRA (11–16).

In the case of immediate bacterial clearance, or complete resistance to infection (observed in a small fraction of the population) the innate immune system will inactivate the bacteria at the site of infection without the stimulation of an acquired immune response. These individuals, recently labeled innate resisters by Simmons et al. will have continued negative TST or IGRA results despite heavy and continued exposure to *M. tuberculosis* and will not be at risk of clinical TB (11, 17). The resister phenotype is likely heterogeneous and could include individuals who mount a protective adaptive immune response (termed adaptive resisters) perhaps involving B cells or unconventional T cell responses during early clearance of the bacterium (17). Also of interest are those LTBI individuals who have no risk of progression to clinical TB, labeled non-progressors, possibly due to an exceptionally well-contained infection or absence of viable bacteria in the granuloma (18). The elucidation of the genetic correlates that contribute to these infection and disease resistance phenotypes is a priority as it may translate to new interventions to prevent, diagnose, or treat TB.

Genetic investigations of TB susceptibility have been ongoing for decades, but gained momentum in recent years due to the availability of improved methodological approaches and technological advances. The majority of studies made use of classical approaches employed by clinical genetics and genetic epidemiology (linkage and association studies), but these have encountered difficulties also faced by genetic investigations of other complex diseases. Three continuing challenges involve polygenicity, the definition of TB phenotypes and the collection of appropriately large study cohorts with carefully defined homogenous phenotypes (11, 19, 20). More recently functional genetic studies, including epigenetics, microRNAs and transcriptomics, have also shed light on the genetic basis of TB susceptibility (21). This review will discuss the early epidemiological evidence of genetic susceptibility to *M. tuberculosis* infection and disease progression, but will also focus on the contribution of genetic epidemiology and functional genetics while highlighting controversies, current research gaps, and future developments.

## EPIDEMIOLOGICAL EVIDENCE OF GENETIC SUSCEPTIBILITY TO *M. TUBERCULOSIS* INFECTION AND CLINICAL TB RESISTANCE

Epidemiological evidence confirms the presence of both resisters and non-progressor phenotypes in high exposure settings. Approximately 50% of close household contacts develop positive TST or IGRA tests (22–26) and there are multiple examples of homogenous high exposure and heterogeneous infection [reviewed by Verrall et al. (27)]. For example the HIV epidemic resulted in TB control failure in South African mines during the 1990s with notification results exceeding 4,000 per 100 000 person-year (28). In the midst of this perfect TB storm, 13% of HIV negative miners had TST = 0 mm responses (28). TB outbreaks onboard ships of the United States Navy yielded similar findings with 5–10% of crew members at highest risk of exposure remaining TST negative (29, 30). Likely resisters were also detected amongst nurses who were exposed to TB patients (31–34). The contribution of hereditary factors to clinical disease susceptibility was recognized even before the discovery of the bacterium, due to the observation that TB often occurred in several individuals from the same family (35). Koch's discovery of the bacterium in 1882 meant that it would be several decades before the host genetic component would again be considered as a contributing factor to disease. In 1943 a seminal study investigating TB concordance found that monozygous twins were significantly more likely to both become diseased than dizygous twins (66.7 vs. 23%) (36). The study also included relatives of the twins and found that the degree of relatedness to the TB index case determined the risk of developing active disease (36). Descriptions of the natural history of clinical TB from the era before antibiotic treatment provide valuable insights into resistance to active disease. For example, pulmonary TB in immunocompetent individuals with no antimicrobial chemotherapy was fatal in ∼50% of cases; 25% of individuals remained ill with chronic TB and the remaining 25% spontaneously achieved cure (37).

In addition to these "experiments of nature," animal models have provided important evidence for a contribution of host genetics to TB infection and progression to clinical disease. Different patterns of disease resistance after infection have been observed in guinea pigs and inbred strains of mice (38). The rabbit model was extensively used by Lurie et al. to study resistance to disease progression and clearly represented two forms of genetically controlled resistance (39–41). The so-called "resistant" rabbits survived approximately twice as long as susceptible rabbits. Susceptible rabbits developed disseminated disease, while resistant rabbits developed cavitary TB (40, 42). An additional evaluation of Lurie's findings by Werneck-Barroso indicated that 20–40% of rabbits exposed to the bacterium did not develop disease and the majority of these did not become TST positive, even after prolonged exposure (43). This suggests that some animals had an intrinsic ability to resist natural *M. tuberculosis* infection and that the bacteria were eliminated without stimulating an acquired immune response (43).

No review of TB susceptibility would be complete without a discussion of the Lübeck disaster. During this tragedy, which took place from December 1929, 251 infants over a time period of 4 months were vaccinated with BCG accidentally contaminated with varying amounts of virulent *M. tuberculosis* [reviewed by Fox et al. (44)]. Clinical or radiological signs of TB were observed in 173 survivors, but 72 infants died from TB (44). Fox et al. pointed out three key lessons resulting from the accident. Firstly, 156 (68%) of those who had developed clinical disease, spontaneously resolved their symptoms, suggesting that newborn infants are remarkably resistant to TB. Secondly, based on available data, semiquantitative levels of *M. tuberculosis* contamination were inferred. At low levels of contamination, a wide range of clinical phenotypes was seen, revealing the extent of innate resistance to clinical TB. However, at high

doses of *M. tuberculosis* contamination, most babies were susceptible to disease, indicating that extreme exposure will eventually overcome host innate (genetic) resistance to disease (44). Therefore, the dose of *M. tuberculosis* is key to determine TB outcome. Lastly, two infants received vaccines with the lowest levels of contamination, but quickly progressed to disease and death, perhaps indicating that they were most susceptible to TB (44).

Isolated populations with little or no known past exposure to the TB bacterium, such as the indigenous populations of the Americas and sub-Saharan Africa, have a significantly higher TB mortality than non-indigenous populations whose ancestors had a longer mycobacterial exposure time (45–47). These observations support the "virgin soil" hypothesis, which postulates that the previous lack of exposure to a pathogen leads to hyper-susceptibility to infection, morbidity and mortality. Newer DNA evidence indicates that the *M. tuberculosis* complex was in fact already widespread around 4000 years ago in Ethiopia and TB infection was also found in western-central and western African individuals who arrived in Brazil as slaves during 1769–1830 (48, 49). The introduction of (European) TB strains possibly exacerbated by local host genetic factors and poor living conditions, contributed to the high mortality observed in these "naïve" populations (50). For example, analyses of the indigenous population of Canada, limited to the Qu'Appelle Agency located in Southeastern Saskatchewan, indicated an annual TB mortality rate of 10% in 1890 which fell to 0.2% after 40 years, but half of the population was eradicated (45). This is suggestive of a strong selection for TB resistance genes. More recent examples of the introduction of *M. tuberculosis* to underexposed populations is that of the Northern Aché of eastern Paraguay and the Yanomami Indians of the Brazilian Amazon (47, 51). Prevalence (18.2%) and infection (64.6%) rates in the Northern Aché quickly rose within 6 years of the first detected TB case (51). TST anergy, possibly reflecting reduced cell-mediated immune responses, and increased antibody responses were common in individuals with active TB from both these populations (47, 51). This could indicate that there has been no selection for TB resistance mechanisms in these groups. In contrast, the decline in TB incidence in North America and Europe during 1830–950, before the introduction of antimicrobial chemotherapy, has been ascribed to the increase of genetically determined resistance to TB due to natural selection after years of mycobacterial exposure (35, 48, 52–55). However, an estimation using Swedish fertility and mortality data, which included age-specific pulmonary TB mortality, from 1891 to 1900 indicated that changes in only the genetic make-up of the population would have been unlikely to account for the extreme decline in TB mortality (56). Although surviving individuals had a fitness advantage of 7–15% per generation compared to individuals who died, statistical calculations indicated that selection would only have reduced the frequency of rare susceptibility variants if these variants had large effects. In contrast, if rare resistance variants were in fact rare, 300 years would not have been sufficient for selection to increase the frequency of these variants to epidemiologically significant frequencies. Despite this, evidence for the role of natural selection in TB resistance is bolstered by findings from population genetic studies of the immune system which provide a context for the genetic interface between humans and mycobacteria (57–61).

## HERITABILITY, GENETIC EPIDEMIOLOGY, AND POPULATION GENETICS

Heritability, genetic epidemiology, and population genetic studies have made significant contributions to reveal the role of human genetic variation in susceptibility to TB infection. The investigations of TST and IGRA as quantitative traits have shown high heritability for both, conditional to *M. tuberculosis* exposure (TST above 50% and for IGRA between 30 and 50%). Heritability of quantitative TST reactivity (in mm) among young healthy children exposed to an active TB case was estimated at 92% in Chile (62). In the Gambia, the heritability of TST considered as a categorical trait and quantitative IGRA reactivity in healthy twins aged 12 to 83 years was estimated at 71 and 39%, respectively (63). In Colombia evidence was detected for a major co-dominant gene explaining ∼65% of TST variability (64), and in a South African familial sample, the heritability of quantitative IGRA responses was estimated to be between 43 and 58%, depending on the nature of the stimulating antigen (65). Recent data from Uganda, carefully adjusted for shared environment, also detected significant heritability of interferon gamma in response to *M. tuberculosis* culture filtrate (23–35%), ESAT6 (15–48%), and Antigen 85B (11–34%) (66).

Only a few molecular studies have investigated the genetic factors underlying *M. tuberculosis* infection resistance using TST reactivity. Candidate gene association studies have focused on TST response as a binary trait according to various thresholds (0, 5, or 10 mm) with a weak association reported for interleukin 10 (*IL10)* promoter variants (67, 68). Increased IL-10 production may contribute to the suppression of adaptive immune responses (68). A candidate gene association study of autophagy-related genes and LTBI, defined by a TST response greater than 5 mm, identified an association between a non-coding Unc-51 Like Autophagy Activating Kinase 1 gene (*ULK1)* variant and LTBI (69). A possibly associated role for ULK1 in the regulation of TNF secretion, both non-specific and *M. tuberculosis*–induced autophagy, and *M. tuberculosis* replication in monocytes was established (69). A genome-wide association study (GWAS) of TST reactivity in HIV positive individuals from Tanzania and Uganda pinpointed a polymorphism on chromosome 5q31.1 that protected against *M. tuberculosis* infection (**Table 1**) (80). This variant is located near the gene encoding IL-9, which is produced by mast and Th2 cells during inflammatory responses and has been associated with bronchial responsiveness, possibly linking resistance against *M. tuberculosis* infection and airway inflammation (80). A GWAS in Iceland detected associations between TST positivity (induration size not specified) and HLA class II variants (**Table 1**) (79). An imputed GWAS was done in 4,426 cases with a self-reported positive TST (defined as the presence of an induration) and 84 290 controls selected from more than 200 000 23andMe participants with European ancestry who completed a questionnaire on infection history

**TABLE 1 |** GWAS of TB infection and disease phenotypes. Adapted from Kinnear et al.(20).

| Population | Phenotype | Cases | Controls | Variant | Gene | Odds ratio [95% CI] | Reference |
|---|---|---|---|---|---|---|---|
| Ghana | TB | 921 | 1740 | rs4331426 | Gene desert (chromosome 18) | 1.19 [1.13–1.27] | (70) |
| Gambia | – | 1316 | 1382 | – | – | – | – |
| USA | Extrapulmonary TB | 48 | 57 | rs4893980 | *PDE11A* | 0.13 | (71) |
| – | – | – | – | rs10488286 | *KCND2* | 11.15 | – |
| – | – | – | – | rs2026414 | *PCDH15* | 3.11 | – |
| – | – | – | – | rs10487416 | Unknown gene | 5.56 | – |
| Thailand | Young TB | 433 | 295 | rs6071980 | *HSPEP1, MAFB* (intergenic, chromosome 20q12) | 1.73 [1.42–2.11] | (72) |
| Japan | – | 188 | 934 | – | – | – | – |
| Indonesia | Pulmonary TB | 108 | 115 | rs2273061 | *JAG1* | 1.8 [1.18–2.72] | (73) |
| – | – | – | – | rs4461087 | *DYNLRB2* | 1.62 [1.1–2.37] | – |
| – | – | – | – | rs1051787 | *EBF1* | 0.57 [0.38–0.88] | – |
| – | – | – | – | rs10497744 | *TMEFF2* | 0.55 [0.38–0.82] | – |
| – | – | – | – | rs1020941 | *TMEFF2* | 0.57 [0.38–0.83] | – |
| | | | | rs188872 | *CCL17* | 0.51 [0.33–0.78] | – |
| – | – | – | – | rs10245298 | *HAUS6* | 2.37 [1.09–5.16] | – |
| – | – | – | – | rs6985962 | *PENK* | 2.01 [1.12–3.61] | – |
| – | – | – | – | rs1418267 | *ERP44* | 3.19 [1.71–5.99] | – |
| Ghana | TB | 2127 | 5636 | rs2057178 | *WT1* (intergenic) | 0.77 [0.71–0.84] | (74) |
| Gambia | – | 1207 | 1349 | – | – | 0.80 [0.70–0.91] | – |
| Russia | – | 1025 | 983 | – | – | 0.91 [0.82–0.99] | – |
| Indonesia | – | 4441 | 5874 | – | – | 0.84 [0.68–1.03] | – |
| South Africa | Pulmonary TB | 642 | 91 | rs2057178 | *WT1* (intergenic) | 0.62 [0.50–0.75] | (75) |
| – | – | – | – | rs11031728 | *WT1* (intergenic) | 0.61(0.50–0.75) | – |
| Russia | Pulmonary TB | 5530 | 5607 | rs4733781 | *ASAP1* | 0.84 [0.79–0.89] | (76) |
| – | – | – | – | rs10956514 | *ASAP1* | 0.85 [0.80–0.90] | – |
| – | – | – | – | rs1017281 | *ASAP1* | 0.85 [0.81–0.90] | – |
| – | – | – | – | rs1469288 | *ASAP1* | 0.84 [0.79–0.89] | – |
| – | – | – | – | rs17285138 | *ASAP1* | 0.85 [0.80–0.90] | – |
| – | – | – | – | rs2033059 | *ASAP1* | 0.83 [0.79–0.88] | – |
| – | – | – | – | rs12680942 | *ASAP1* | 0.84 [0.79–0.89] | – |
| Morocco | Pulmonary TB | 556 | 650 | rs358793 | Intergenic | 0.68 [0.57–0.82] | (77) |
| – | – | – | – | rs17590261 | Intergenic | 6.24 [2.38–16.33] | – |
| – | – | – | – | rs6786408 | *FOXP1* | 1.47 [1.23–1.79] | – |
| – | – | – | – | rs916943 | *AGMO* | 1.86 [1.33–2.6] | – |
| Uganda | HIV positive TB resistance | 267 | 314 | rs4921437 | *IL12B* | 0.37 [0.27–0.53] | (78) |
| Tanzania | – | – | – | – | – | – | – |
| Iceland | TST positivity | 8162 | 277643 | rs557011 | Between *HLA-DQA1* and *HLA-DRB1* | 1.25 [1.17–1.33] | (79) |
| – | – | – | – | rs9271378 | Between *HLA-DQA1* and *HLA-DRB1* | 0.78 [0.73–0.84] | – |
| – | – | – | – | rs9272785 | *HLA-DQA1* | 1.14 [1.09–1.19] | – |
| Uganda | TST reactivity | 224 | 225 | rs877356 | *IL9* | 0.27 [0.17–0.42] | (80) |
| Tanzania | – | – | – | – | – | – | – |
| 23 and Me (European ancestry) | Positive TST | 4426 | 84290 | rs2894257 | *HLA* | 1.36 [1.33–1.39] | (81) |
| China (Han Chinese) | Pulmonary and extrapulmonary TB | 4310 | 6386 | rs4240897 | *MFN2* | 0.79 [0.75–0.83] | (82) |
| – | – | – | – | rs41553512 | HLA class II | 2.14 [1.78–2.57] | – |
| – | – | – | – | rs2269497 | *RGS12* | 1.51 [1.35–1.68] | – |
| Thailand | Non-Beijing lineage-infected old age onset | 182 | 489 | rs1418425 | *CD53* | 1.74 [1.43–2.12] | (83) |

(81). The *HLA* rs2894257 variant on chromosome 6p21.32 was significantly associated with the presence of a TST induration ($p = 8.16 \times 10^{-36}$, OR 1.36, 95% CI 1.33–1.39) and then after further fine mapping of the locus multiple independent associations between a history of a positive TST and HLA were detected (**Table 1**) (81). The HLA class II region could contribute to infection resistance by reduced presentation of *M. tuberculosis* antigens to T cells (79). In Uganda, a genome-wide linkage analysis (GWLA) reported suggestive, but not significant, linkage of persistent TST negativity (defined as a TST < 10 or 5 mm according to age and HIV status) with chromosomal regions 2q21-2q24 and 5p13-5q22 (84). The chromosome 2q region was subsequently investigated using an association scan in two independent cohorts from Uganda and associations were found with variants in the Zinc finger E-box-binding homeobox 2 (*ZEB2*) and Glycosyltransferase Like Domain Containing 1 (*GTDC1*) genes (85). These variants may regulate the histone deacetylase pathway, which has been implicated in infection resistance by transcriptomic investigations (discussed below) (86). Two loci were identified by GWLA in an HIV negative population from South Africa (87). *TST1* was identified on chromosome 11p14 by focusing on the phenotype of TST > 0 mm vs. TST = 0 mm, and captures innate resistance to infection with *M. tuberculosis*. *TST2* was mapped to region 5p15 and influences the intensity of TST reactivity - captured as TST induration in mm. Hence, *TST2* reflects intensity of T-cell mediated anti-mycobacterial immune responses. The mapping of *TST1* has been confirmed in an independent sample of different ethnic origins in France, and it was also shown that *TST1* cannot be distinguished by linkage from *TNF1*, a locus controlling TNF production in response to BCG/IFN-gamma (88, 89).

Compared to the study of *M. tuberculosis* infection resistance, a larger number of investigations have been published addressing the genetic factors that protect against or predispose to developing clinical TB. Indeed, 11 TB GWAS have been done using clinical TB as phenotype [**Table 1**, reviewed by (20, 21)]. Highlights included the identification of the 11p13 locus first identified in West Africa and replicated in Russia, Indonesia and South Africa (74, 75), a large Icelandic GWAS which identified HLA class II variants which was weakly replicated in Russia and Croatia (79) and a recent GWAS of TB resistance in HIV positive individuals from hyperendemic TB regions in Uganda and Tanzania (78). The latter study found an association with a locus at chromosome region 5q33.3. The associated variant is embedded in an H3K27A histone mark, but is also in a genomic region that includes *IL12B*, a gene known to underlie Mendelian susceptibility to mycobacterial disease (78).

Since the publication of previous reviews of clinical TB GWAS (20, 21), two additional studies have been completed using this study design. A three-stage replication approach was used in the Han Chinese and generated genotyping data (691 388 SNPs) for 972 TB cases and 1537 controls in the first stage (82). In the second stage, the top 45 loci were analyzed in 2278 TB cases and 2752 controls and the nine most significant variants were genotyped in 1060 TB cases and 2752 controls. Variants in three loci, namely *MFN2* (rs4240897, $p = 1.41 \times 10^{-11}$, OR 0.79, 95% CI 0.75–0.83), HLA class II (rs41553512, $p = 7.93 \times 10^{-11}$, OR 2.14, 95% CI 1.78–2.57), and *RGS12* (rs2269497, $p = 3.37 \times 10^{-8}$, OR 1.51, 95% CI 1.35–1.68) were significantly associated with TB in a meta-analysis of the three stages (4310 cases vs. 6386 controls). These are all excellent TB candidate genes and gene expression data supported the functional significance of two of the identified variants. The rs4240897 variant regulates *MFN2* expression suggesting that this variant could affect platelet count and macrophage differentiation. In addition, expression of this gene was increased in TB cases compared to controls (82). Another gene in close proximity to rs4240897 is *TNFRSF8* and expression of this immune gene was lower in TB cases than controls. Signaling of the *TNFSF8/TNFRSF8* pathway enhanced interferon gamma production in response to *M. bovis* BCG stimulation (82). A GWAS done in Thailand relied on *M. tuberculosis* pathogen lineage information and identified a chromosome 1p13 association between 489 healthy controls and 182 cases with non-Beijing lineage-infected old age onset (rs1418425, $p = 2.54 \times 10^{-8}$, OR 1.74, 95% CI 1.43–2.12) (83). The variant is located in the vicinity of the *CD53* gene and expression of this leukocyte surface glycoprotein was correlated with active TB (83). In addition, the rs1418425 variant is a known cis-expression quantitative trait locus in *M. tuberculosis* infected dendritic cells (83).

Clearly there is very little overlap with respect to the loci detected between the individual GWAS, but it seems that replication is more likely when populations with similar genetic backgrounds are compared. This was seen for the *WT1* locus in West and South Africa and it is possible that the same HLA class II factors are being tagged in Icelandic and other European populations, but this is not known at this point (74, 75, 79, 81). When GWAS data from Han Chinese and Gambians were combined in a meta-analysis, no significant associations were detected (82). Deciphering the complete genetic architecture of a complex trait requires more than a single ancestry, as was the case for skin pigmentation genes and other phenotypes (90, 91). For this reason, population genetics also has to be considered in investigations of TB resistance (92). Excess European, South Asian and East Asian ancestry protects admixed South African individuals against active TB, whereas excess African ancestry increased the risk for developing disease. These disparities in disease incidence were harnessed in a TB admixture mapping study (75, 93). The contribution of ethnicity to TB resistance may be due to selection after centuries of exposure to *M. tuberculosis* (as discussed in the section "Early epidemiological evidence of natural TB resistance"). This is supported by findings that individuals with diverse genetic backgrounds have different rates of TB infection and disease progression (not affected by socio economic circumstances) and the intensity of immune responses differ (35, 52–54, 59).

## FUNCTIONAL GENETICS

The mechanisms through which genetic variation contributes to TB resistance require functional follow-up to support statistical findings of epidemiological studies. Investigations of epigenetics,

microRNAs, and other products of transcription can provide functions to these genetic variants, but can also identify novel genes and pathways involved with TB resistance (94).

## Transcriptomics

Transcriptional profiles generated from blood cells have contributed to the elucidation of pathways involved in resistance to infection. Genome-wide transcriptional profiles from infected monocytes isolated from TST positive and persistently negative household contacts from Uganda who did not develop TB at least two years after follow-up were generated using microarrays (86). Pathways controlled by histone deacetylase were associated with resistance to *M. tuberculosis* infection and indicated that this function is vital in the early innate immune response to infection (86). Although this anti-inflammatory mechanism holds promise as a therapy, the *in vitro* findings may not extend to effects *in vivo*. The use of histone deacetylase inhibitors did not increase survival in a sepsis model (95) and histone deacetylase-related genes were also expressed in TST positive individuals (86). In a non-human primate model, a signature of 34 pre-infection transcripts could differentiate between animals that would progress to active disease or develop LTBI (96). Twelve of the upregulated transcripts were associated with interferon, cell cycle and inflammation processes. When the outcome was stratified based on $^{18}$F-fluorodeoxyglucose (FDG) positron emission tomography coupled with computed tomography (PET CT), 30 pre-infection transcripts were differentially expressed between animals with low and high FDG avidity. The differentially expressed genes did not correspond to the clinical status or lung avidity groups, but function in the same pathways related to inflammation and interferon (96). The inherent genetic ability of the host to upregulate these pathways may correspond to poor infection outcomes (96). Importantly, both studies underline that a balanced inflammatory response, regulated by the host genome, is critical to determine the outcome of infection (86, 96).

Although genome-wide transcriptomic studies of TB infection resistance are in their infancy, many studies have identified blood gene expression signatures for the classification of the TB pathogenesis stages (including the diagnosis of active TB) and monitoring of treatment efficacy (97–112). The value of these studies lies in their predictive accuracy, since TST and IGRAs cannot fulfill this function (21). However, transcriptomics cannot detect those genetically determined for disease progression before the onset of the process (21). Several predictive signatures of TB risk have been developed from whole blood RNA sequencing. Recently a four-transcript signature, labeled RISK4, could predict disease progression up to 2 years before TB symptoms presented in Africa cohorts from South Africa, The Gambia and Ethiopia (113). This signature consists of two upregulated (growth arrest–specific 6 and septin 4) and two downregulated (cluster of differentiation 1C and B lymphocyte kinase) genes. A 16 gene predictive signature of TB risk was developed from whole blood RNA sequencing of adolescents and could not only identify individuals at risk of developing active TB after LTBI, but could also distinguish active disease from LTBI and other disease forms in two African populations and three validation samples (114). The sixteen genes were Ankyrin repeat domain 22, Apolipoprotein L1, basic leucine zipper ATF-like transcription factor 2, ETS Variant 7, Fc Fragment Of IgG Receptor Ia, Fc Fragment Of IgG Receptor Ib, Guanylate Binding Protein 1,2,4, and 5, Scavenger Receptor Class F Member 1, septin 4, Serpin Family G Member 1, Signal Transducer And Activator Of Transcription 1, Transporter 1, ATP Binding Cassette Subfamily B Member and TRAF-Type Zinc Finger Domain Containing 1. In HIV positive drug users with and without TB, expression of the IL-13 and autoimmune regulator genes were predictive of developing disease even 8 months before the actual diagnosis (115), but this signature was not validated in the RNA sequencing study of HIV negative adolescents (114). To identify correlates of TB resistance using the LTBI phenotype as a proxy, network analysis was applied to a number of TB transcriptomic datasets. Here the focus was specifically on gene expression profiles of macrophages, as these cells can mount an antimicrobial response (116). IL-32 was identified as a functional marker of resistance to active TB and mediated interferon gamma vitamin D dependent antimicrobial immunity (16, 116). An *in vitro* investigation of monocytes (isolated from LTBI and active TB individuals) after *M. tuberculosis* infection indicated that IL-26 downregulation was beneficial to anti-mycobacterial activity, making it a plausible susceptibility candidate gene (94). A meta-analysis of 16 published studies identified a set of 380 genes that were differentially expressed in active TB in most investigations with interferon gamma as the most significant potential upstream regulated molecule (117).

## Epigenetics

The contribution of epigenetic mechanisms to the regulation of inflammatory immune responses in TB is an emerging field and evaluations of the genetic regulation of transcriptomic responses can assist in revealing the biology of TB host resistance (118). Epigenetic regulation incorporates all chromosomal modifications that alter gene expression without changing the underlying coding DNA nucleotide sequence, such as DNA methylation and histone acetylation. Methylation events in monocytes and granulocytes could discriminate between TB cases and healthy LTBI controls (119).

Histone modifications have been linked to mTOR dependent regulation of glucose and glutamine metabolism in BCG-trained monocytes and macrophages, with histone H3 trimethylation of lysine 4 (H3K4me3) found to be significantly increased at the promotors of mTOR, HK2 and PFKP, while trimethylation of lysine 9 (H3Kme3) was significantly decreased (120). Histone H3 hypoacetylation, specifically at lysine 14 (H3K14ac) was associated with active pulmonary TB (121) as well as being essential for the activation of several pro-inflammatory cytokines (122, 123). Interestingly, the macrophage response to different immune challenges can result in the generation of histone marks associated with *de novo* enhancer elements (124, 125). These marks have been hypothesized to cause the epigenetic reprogramming of the macrophages leading to a stronger transcriptional response to a second stimulus (124). In addition to histone modifications, recent data suggests that DNA methylation also plays a significant role

in in the reprogramming of innate immune cells and the regulation of transcriptional programs following *M. tuberculosis* infection (126).

Epigenetic modification of histone acetylation in monocyte-derived macrophages plays a significant regulatory role in *M. tuberculosis*-dependent gene expression and in the secretion of matrix metalloproteinase enzymes driving immunopathology. Modification of histone acetylases has implications for TB resistance too, based on the findings of Seshadri et al. discussed above (86). Variants in the regulatory regions of over 700 genes that were up- or downregulated after *M. tuberculosis* infection of monocyte-derived dendritic cells significantly influenced gene expression regardless of the stimulation status of cells. (127). These variants are expression quantitative trait loci (eQTL) and a subset of these (response-eQTL) were dependent on *M. tuberculosis* stimulation, indicating that epigenetic effects contribute to TB pathogenesis (126). Manipulating these regulatory mechanisms may have potential as host-directed therapy (128).

### MicroRNAs

MicroRNAs (miRNAs) play a crucial role in TB pathogenesis (129). MiRNAs are short, non-coding RNA molecules that regulate mRNA translation and degradation and affect the function of many immune cell types (129). Used as markers, miRNAs can distinguish between active disease, LTBI or other microbial infections (130–137), and also influence TB susceptibility, specifically miR-155 and miR-223 (138–141). MiR-223 directly targeted chemoattractants such as CXCL2, CCL3, and IL-6 to control neutrophil driven inflammation (138, 139). When miR-223 was deleted in a TB resistant mouse model these animals became extremely susceptible to TB, but the phenotype could be partly restored through the neutralization of the abovementioned chemoattractants (138). miR-155 was highly expressed after mycobacterial infection both *in vivo* and *in vitro* (139). The induced expression of miR-155 enhanced the autophagic response in macrophages thereby stimulating mycobacterial phagosome maturation and reducing the survival rate of intracellular mycobacteria. In contrast, when miR-155 was inhibited, there was increased mycobacterial survival. The mechanism of action of miR-155 is through the targeting of Ras homolog enrich in brain (Rheb), a known negative regulator of autophagy. When miR-155 was bound to the 3'-untranslated region of Rheb, both autophagy and intracellular killing of mycobacteria were increased (139). In addition to expression analysis, genetic variants in miR-499 and miR-146a were associated with pulmonary TB susceptibility in a case-control association study (142).

### Transgenic Animals

Although the animal models (discussed in the section "Early epidemiological evidence of natural TB resistance") were initially used to study the natural occurrence of TB resistance, advances in molecular genetic techniques have facilitated the creation of resistant species. Transgenic cattle with a knock-in SP110 nuclear body protein (*SP110*) gene were created using transcription activator-like effector nuclease (TALEN)-mediated genome modification (143). The mouse homolog of this gene, Ipr1, was previously shown to mediate innate immunity in sst1 congenic mice and *SP110* variants were associated in some settings with human pulmonary TB (144, 145), but not others (146–149). In the transgenic cattle apoptosis instead of necrosis was activated after infection (143). *In vivo* and *in vitro* experiments indicated that these animals could control growth and proliferation of *M. bovis*. Significantly, transmission experiments using tuberculous cattle indicated that the transgenic animals were resistant to low dose *M. bovis* infection (143).

## CONTROVERSIES, CURRENT RESEARCH GAPS, AND FUTURE DEVELOPMENTS

The four complementary theories of infectious disease propose that inter-individual variability in presentation depends on four factors, namely microbiological, ecological, immunological, and genetic (150). These elements not only intersect but are all required to dissect a complex infection phenotype such as TB resistance (150). To delineate the contribution of genetic factors will require innovative new approaches to combine available data sets to understand the resistance phenomenon, in particular in HIV-infected persons. Integrated clinical and laboratory defined phenotypes, whole genome sequencing, epigenetic and transcriptomic studies will be required to address this challenge.

The co-evolution of *M. tuberculosis* and humans has shaped host-pathogen interactions for thousands of years and has likely contributed to the diverse range of responses after infection including the phenotype of the TB resister (50, 61, 151). Host-pathogen interaction investigations are however complicated by the genetic heterogeneity of the bacterium (50). One approach is to use *M. tuberculosis* pathogen lineage information as was done for a TB GWAS in Thailand (**Table 1**) and several candidate gene association studies (83, 152–155). Despite the challenges, developments in this area could in future be used to design targeted vaccines and therapies directed to specific populations or individuals (50).

Several TB GWAS have interrogated resistance to disease, but most of these have been underpowered due to the extreme phenotypic heterogeneity. Meta-analyses can provide a solution to this problem and could provide insight into population-specific associations by harnessing linkage disequilibrium to fine map associations. The International TB Host Genetics Consortium has been established to collate TB GWAS data from individuals with pulmonary TB and healthy controls to do a large-scale meta-analysis (156). This large-scale approach will not be feasible to investigate persistently TST/IGRA negative individuals, since phenotyping is a costly process and requires several repeat assays to exclude those who revert and convert. Fortunately, since the TB resister phenotype is at the end of the TB susceptibility spectrum, it is possible that variants contributing to this extreme phenotype can be detected in limited sample sizes, as has been seen in investigations of HIV-infected participants who do not become infected or progress to active TB despite living in a TB endemic region (78, 86).

Finally, genome sequencing technologies, which are already used to diagnose individuals with Mendelian susceptibility to mycobacterial diseases, will deliver resistance variants not captured by microarray genotyping and imputing. Once genomes from TB resisters are available for data mining and analysis in system biology approaches—which will include transcriptomics, epigenomics, microbiomics, and other omics, we may be able to achieve prediction of individuals genetically determined as resistant.

## CONCLUSION

The involvement of a human genetic component in susceptibility to infection with *M. tuberculosis* and progression to active disease is incontestable. Findings from clinical genetics, genetic epidemiology, population and functional genetics have all contributed to identify TB susceptibility genes. More intriguing is the other side of the phenotypic coin—that of resistance to either initial infection or, after infection, resistance to progression to disease. Although the phenomenon is now recognized, the exact genetic variants and mechanisms that contribute still require elucidation. The most successful approaches in resistance/susceptibility investigation have focused on specific infection and disease phenotypes and the resister phenotype may hold the key to the discovery of actionable genetic variants in TB infection and disease.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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# Modulating Iron for Metabolic Support of TB Host Defense

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Tuberculosis (TB) is the world's biggest infectious disease killer. The increasing prevalence of multidrug-resistant and extensively drug-resistant TB demonstrates that current treatments are inadequate and there is an urgent need for novel therapies. Research is now focused on the development of host-directed therapies (HDTs) which can be used in combination with existing antimicrobials, with a special focus on promoting host defense. Immunometabolic reprogramming is integral to TB host defense, therefore, understanding and supporting the immunometabolic pathways that are altered after infection will be important for the development of new HDTs. Moreover, TB pathophysiology is interconnected with iron metabolism. Iron is essential for the survival of *Mycobacterium tuberculosis (Mtb),* the bacteria that causes TB disease. *Mtb* struggles to replicate and persist in low iron environments. Iron chelation has therefore been suggested as a HDT. In addition to its direct effects on iron availability, iron chelators modulate immunometabolism through the stabilization of HIF1α. This review examines immunometabolism in the context of *Mtb* and its links to iron metabolism. We suggest that iron chelation, and subsequent stabilization of HIF1α, will have multifaceted effects on immunometabolic function and holds potential to be utilized as a HDT to boost the host immune response to *Mtb* infection.

**Keywords: immunometabolism, host-directed therapy, host-directed prevention, iron chelation, tuberculosis, iron metabolism, *Mycobacterium tuberculosis*, HIF1α**

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## INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) and it is estimated that just under one quarter of the global population may be latently infected with *Mtb* (1, 2). TB is responsible for approximately 1.7 million deaths annually (2), making it the biggest infectious cause of death. *Mtb* is an airborne pathogen, spread through aerosols created by coughing. After inhalation and infection with *Mtb*, people attempt to mount an adequate innate immune response to eradicate the bacteria without the help of adaptive immunity (so called "early clearance") (2, 3). Only 5–10% of infected immunocompetent people progress to TB disease (4, 5). These figures suggest that the vast majority of immunocompetent people exposed to TB produce a robust and adequate immune response to clear the infection asymptomatically. This gives credence to the idea that a defective immune response causes TB disease, and can therefore be therapeutically corrected with host-directed therapies (HDT). We suggest that supporting macrophage metabolism, by manipulating iron availability, has potential as a HDT strategy.

The treatment regimen for TB is based on a combination of up to 4 drugs which have to be taken over protracted periods of time. Inadequate treatment and poor compliance however, have resulted in increasing incidences of multiple and extensively drug resistant TB (MDR and XDR TB respectively) (6). MDR and XDR TB patients have very limited treatment alternatives, thus there is an unmet clinical need for better therapeutic options (6). Basic cellular human research has shed light on many aspects of immunity to *Mtb* and has unveiled immune pathways that may be manipulated therapeutically (7–9). Early in *Mtb* infection, both pro and anti-inflammatory pathways are activated at the same time (7, 10). The aims of some HDT approaches are to manipulate this balance- in other words, to reduce the immune braking system and give the immune accelerator more gas. With this approach, a desired clinical outcome is to reduce the time required to clear the infection. A shorter, better treatment regimen would increase compliance and may reduce incidences of MDR and XDR TB. "Immunometabolism," (the metabolic changes that underpin the ability of immune cells to mount an immune response) has informed many aspects of immunity to *Mtb*. By understanding and manipulating metabolic pathways, we are seeking to redirect or accelerate the host to yield better clinical outcomes for patients. Iron plays a central role in modulating metabolic pathways (11–13). In this review, we present the evidence suggesting that iron chelation, and its effects on immunometabolism, may be a plausible adjunctive HDT option for TB.

## AN OVERVIEW OF IMMUNOMETABOLISM

Recent studies on macrophages and metabolic function have linked intermediate metabolism to immune phenotypes (14). It is now considered that immune cell function and cellular energy metabolism are closely coupled and that alterations in metabolic pathways are integral to the immune response, since they strongly influence cell fate and effector functions; these links have been thoroughly reviewed in T cells, macrophages, NK cells, dendritic cells (DCs), and neutrophils (15–20). The metabolic pathways of these cells must be tightly regulated to provide energy and biosynthetic precursors to meet the cells' functional requirements upon activation (21). It has been shown that, upon activation, various immune cells undergo metabolic reprogramming similar to oncogenic cells. For example, in 1927, Otto Warburg observed that neoplastic cells change their metabolism from oxidative phosphorylation (OXPHOS) to aerobic glycolysis (22). This switch to glycolysis also occurs in immune cells that are activated by pro-inflammatory signals, which differ depending on the cell type, and allows them to produce adenosine triphosphate (ATP) more rapidly (albeit less efficiently) and provides the necessary metabolic intermediates needed for cell growth, proliferation and effector mechanisms (23). Glycolytic shifts that take place in activated immune cells are not always classically Warburg (increased glycolysis and reduced OXPHOS) as both glycolysis and OXPHOS can be enhanced simultaneously (24). Increased activity of both glycolysis and OXPHOS is often observed in human immune cells upon activation (24). Furthermore, the metabolic profile of certain immune cells has also been shown to change during different stages of activation. The complex metabolic phenotype defines the function of the immune cell. For example, glycolytic metabolism is associated with classically activated pro-inflammatory macrophages (termed "M1"), effector T cells, cytokine activated NK cells and toll-like receptor (TLR) activated DCs (21, 23–26). This metabolic reprogramming toward increased glycolysis, resulting in increased inflammation, is mediated by two main signaling molecules; mTOR and HIF1α. On the other hand, OXPHOS is generally associated with the phenotype of tissue-resident and alternatively activated macrophages (termed "M2"), long-lived memory T cells, regulatory T cells and mature DCs in their antigen presenting phase (27). It is also worthwhile noting that oxidative metabolism supports immune cell longevity. For example, preserving OXPHOS in activated DCs results in an increased lifespan, and switching cellular metabolism from glycolysis to oxidative metabolism promotes a shift from short-lived M1 macrophages to longer-lived M2 macrophages (28, 29). Human alveolar macrophages (AMs), which are thought to be M2-like, demonstrate greater metabolic plasticity toward glycolytic metabolism upon inhibition of OXPHOS; this is despite a greater reliance of human AMs on OXPHOS at baseline compared to untreated human monocyte-derived macrophages (hMDMs) and IL4 treated hMDMs (30). However, this glycolytic reserve is attenuated in AMs from smokers and in AMs infected with both live attenuated *Mtb* H37Ra and irradiated *Mtb* H37Rv strains (30).

### Monocytes and Macrophages

Monocytes are phagocytic and are capable of antigen presentation but are best known as the precursor cells to macrophages and DC. Two key regulators of monocyte metabolism are mTOR and HIF1α, both of which enhance glycolysis (31). The activity and gene expression of these two molecular mediators is enhanced by β-glucan, one of the main components of the fungal cell wall, known to upregulate glycolysis in human monocytes (31). Interestingly, *M.bovis* BCG is also capable of inducing these changes to prime monocytes to respond more rapidly and with heightened activity when challenged by other pathogens, in a process known as innate training (32). The phenomenon of innate training relies fundamentally on changes in glycolytic and glutamine metabolism of monocytes which are crucial for the induction of histone modifications underlying BCG-induced trained immunity (32).

Monocytes extravasate from the blood into the tissue where they differentiate into macrophages or DCs (33, 34). Macrophages can have pro-inflammatory or pro-resolution phenotypes depending on the cytokine milieu they experience and pathogen- or damage- associated molecular patterns (PAMP/DAMP) signals they receive *in situ* (35). These are broadly classified as classically activated or M1-type macrophages and alternatively activated or M2-type macrophages (36). M1- and M2-type macrophages differ in terms of function and in the metabolic pathways they utilize; in fact, differences in metabolic function direct their differentiation and phenotype

(36, 37). M1 macrophages confer protection against bacterial infection via a pro-inflammatory response, involving several cytokines, nitrogen species and pro-inflammatory reactive oxygen species (ROS) (38). Murine macrophages, activated with LPS, have been demonstrated to rely on glycolysis to produce ATP, which is primarily mediated by HIF1α (39). Glycolysis in LPS-IFNγ stimulated murine bone marrow-derived macrophages (BMDMs) is also directed toward the pentose phosphate pathway (PPP) and the malate-aspartate shuttle to support NADPH synthesis, essential for ROS production (40). M2 macrophages, involved in tissue homeostasis and wound healing, mediate $Th_2$ cell immunity to parasitic infections, which are usually chronic and therefore energy demanding (41). In keeping with the longevity of their role, M2 macrophages independently stimulated with IL4, signal transducer and activator of transcription 6 (STAT6) and PPARγ-coactivator-1β (PGC-1β) engage an anti-inflammatory phenotype and rely on fatty acid oxidation (FAO) to generate ATP (42).

In murine BMDMs, LPS stimulation results in increased glycolysis (39) and a break in the tricarboxylic acid (TCA) cycle at two points; one at citrate and another at succinate. Succinate drives the production of IL1β, mediated by HIF1α (39) whereas citrate accumulation leads to the production of itaconate, a potent inhibitor of isocitrate lyase, which is necessary for *Mtb* persistence (43). Itaconate has anti-inflammatory and anti-oxidant properties, mediated by NRF2 signaling (44), as well as being directly able to effect *Mtb* growth (45). It has also long been known that lipid metabolism is significantly altered during infection and inflammation (46–48). Increased lipid uptake leads to foam cell formation, or foamy macrophages, which is characteristic of certain diseases such as atherosclerosis and TB (49, 50). Many experimental models also utilize LPS as a stimulus, however, the use of LPS can have its limitations (51).

Macrophages, especially alveolar macrophages, are crucial in *Mtb* infection as they are probably the first cell to encounter *Mtb* and become infected. Macrophages are both critical to the eradication of the infection but are also culpable of harboring *Mtb* thus, propagating the infection. We hypothesize that these divergent processes are hinged on the metabolic potential of the macrophage. Macrophage metabolism during *Mtb* infection will be further explored in the section below entitled "*Metabolic alterations within immune cells during Mtb infection.*"

## Neutrophils

Neutrophils play vital roles during infection, since they contribute directly to the elimination of pathogens via phagocytosis, the release of antimicrobial molecules such as hydrogen peroxide and netosis, a process whereby activated neutrophils form net-like structures to trap the pathogens (52). These key features of neutrophil activity rely on neutrophil phagocytosis and the switch toward aerobic glycolysis (52, 53). Interestingly, neither glucose nor glutamine are fully oxidized to produce ATP in these cells, indicating that glycolysis may be supporting alternative metabolic pathways through the production of intermediates to generate antimicrobial molecules (53). Indeed, glucose is metabolized to fuel the PPP to generate NADPH in healthy human neutrophils stimulated with PMA and amyloid fibrils (54). Moreover, degradation of glutamine to malate via the TCA cycle and the malate-aspartate shuttle, contributes to the generation of NADPH. NADPH production is essential for the microbicidal cytosolic NADPH oxidase (NOX) system, required for netosis and for production of antimicrobial molecules (55, 56). HIF1α also mediates glycolytic metabolism in murine neutrophils as conditional knockout of HIF1α drastically reduced ATP levels resulting in impaired bacterial killing (57). Neutrophils are implicated in early TB host defense but also contribute to tissue pathology, especially later on in disease. The metabolic profile of neutrophils during *Mtb* infection is not yet known, however; targeting metabolic pathways in these cells may help to fine-tune the immune response to promote clearance or inhibit tissue damage.

## Natural Killer Cells

Natural killer (NK) cells, activated by IL2, IL12, IL15 or combinations thereof, have increased glucose metabolism through aerobic glycolysis, which is necessary to meet the requirements for rapid growth and proliferation (58–60). Specifically, healthy human NK cells are classified into two distinct subsets based on their levels of CD56 receptor; $CD56^{DIM}$ cells are considered more cytotoxic whereas $CD56^{HI}$ cells are potent producers of IFNγ (24). Flow cytometric analysis showed that $CD56^{HI}$ cells express higher levels of the glucose transporter GLUT1 and exhibit higher glycolytic metabolism than $CD56^{DIM}$ cells. Although OXPHOS supports both CD56 cell subtypes, limiting glycolysis in $CD56^{HI}$ cells significantly impairs the production of IFNγ, a pro-inflammatory cytokine also central to host defense during *Mtb* infection (24). Both subpopulations of NK cells respond to *Mtb* and can directly kill *Mtb* infected phagocytes through the production of perforin, granzyme and the ligation of death receptors (24, 61). Indirectly, healthy human NK cells promote host defense in *Mtb* H37Ra-infected T cells by producing IFNγ and inducing $CD8^+$ T cell responses (62). Interestingly, memory-like antigen-specific $CD45RO^+$ NK cells, isolated from the pleural fluid from patients with tuberculosis, exhibit features of innate memory to *Mtb* antigens and may participate in the recall immune response to *Mtb* infection by producing IL22 (63, 64). This is similar to BCG-induced innate training observed in human and murine monocytes *in vitro*, which are dependent on glycolytic and glutamine metabolism (32). The metabolic changes in NK cells during *Mtb* infection are not yet characterized but are likely to be integral to its host defense mechanisms.

## Dendritic Cells

In DCs, similar to other immune cells, cell function is coupled to immunometabolism with the aim of meeting the bioenergetic and biosynthetic requirements for successful TLR induced activation and function (25, 65). TLR-activated DCs stimulated with LPS, heat killed *Propionibacterium acnes* or CpG, rely on aerobic glycolysis to generate ATP (65, 66). This switch to glycolysis is primarily regulated by HIF1α and the PI3K/Akt pathway (65, 67). Additionally, in real-time extracellular metabolic flux assays, the change to glycolytic metabolism has been shown to enhance nitric oxide (NO)

synthesis via the enzyme nitric oxide synthase 2 (NOS2), which inhibits OXPHOS in some populations of LPS stimulated human DCs (66). Therefore, glucose plays two roles in DC activation post TLR stimulation; in the early stages of activation, glucose provides the metabolic intermediates needed for DC maturation. However, in subsequent stages, NO production inhibits OXPHOS, making glycolysis necessary to synthesize ATP and support cell survival (66). Linking DC metabolism and function to adaptive immunity, glycolysis has also been shown to repress the pro-inflammatory output of BMDM-derived LPS-stimulated murine DCs and limit DC-induced T cell responses (27). Therefore, the lifecycle of the DC is marked by differences in metabolism intrinsic to the function of the DC at that stage. DCs play a crucial role in propagating T cell responses during *Mtb* infection, however, their metabolic phenotype is understudied. For example, one study characterized the cooperation between *Mtb*-infected human $CD1c^+$ DCs and plasmacytoid DCs which favors the stimulation of $CD4^+$ T cells, and another study has identified the rapid induction of glycolysis as an integral component of TLR signaling that is essential for the anabolic demands of the activation and function of murine DCs (25, 68). We hypothesize that metabolism may underpin DC function during *Mtb* infection, based on such observations.

## T Cells

Resting T cells rely primarily on OXPHOS, however, once activated by the T cell receptor and costimulatory molecule ligation, T cell subsets undergo a distinct metabolic reprogramming (69). In the early stages of inflammation, cytokines direct the differentiation of naïve $CD4^+$ T cells into effector ($T_{eff}$: $Th_1$, $Th_2$ or $Th_{17}$) or inducible regulatory T cell ($T_{reg}$) subsets (70–76). Effector T cell subsets show an increase in glycolytic metabolism following activation, namely $Th_{17}$ cells, $Th_1$ and $Th_2$ cells (21, 23, 77). Consistent with the different functions of these subsets, $T_{eff}$ and $T_{reg}$ cells utilize distinct metabolic programmes. Murine $T_{eff}$ cells depend on aerobic glycolysis to enable the rapid growth and proliferation essential for clonal expansion, migration and effector functions (21). Alternatively, $T_{reg}$ cells have less of the glucose transporter GLUT1 on the surface and rely on lipid oxidation and OXPHOS to generate ATP (21). Extracelular flux and flow cytometry analyses demonstrate that murine $CD8^+$ memory T cells primarily rely on lipid oxidation, the TCA cycle and OXPHOS, utilizing extracellular glucose to synthesize lipids rather than using extracellular fatty acids directly (78, 79) whereas activated effector $CD8^+$ T cells shift their metabolism toward glycolysis. Myc, HIF1α, estrogen related receptor-α and mTOR are some of the molecular mediators critical to driving these alterations in T-cells. Myc upregulates various genes involved in glucose and glutamine metabolism in the initial stages of T cell activation in primary murine cells (69). Similarly, the mTOR pathway promotes glucose metabolism in human $T_{eff}$ cells while inhibiting $T_{reg}$ generation (80). Moreover, in an mTOR dependent manner, HIF1α is a critical regulator of the $Th_{17}$ and $T_{reg}$ axis through the modulation of glycolytic metabolism in murine cells (77). In recent years, it has emerged that, in certain settings of inflammation, significant plasticity occurs between $Th_1$, $Th_{17}$ and $T_{reg}$ cell lineages (81, 82). Given their differential metabolic states, it is plausible that metabolic reprogramming underpins and directs the plasticity of these cells. The metabolic status of $CD3^+$ T cells was recently examined in an *in vivo* mouse model of *Mtb* infection where the authors showed that the T cell compartment in granulomatous regions of the lungs have increased transcripts encoding glucose transporters, glycolytic enzymes and enzymes of the pentose phosphate pathway (83). These alterations, and further increases in the expression of hexokinase-3 and lactate dehydrogenase A in co-localization analyses, may be indicative of increased glycolytic metabolism (83). Even though further studies are warranted to explore this link, T cell exhaustion in *Mtb* infection is postulated to be linked to metabolism, especially in the oxygen-deprived environment of the granuloma. Modulating T cell metabolism may therefore be beneficial in promoting a specific T cell response with the capacity to support *Mtb* clearance, particularly during the early stages of infection.

# METABOLIC ALTERATIONS WITHIN IMMUNE CELLS DURING *MTB* INFECTION

## Glycolysis and Oxidative Phosphorylation

Upon *Mtb* infection, the immune system aims to contain and eradicate the pathogen. However, infected cells such as macrophages, are sometimes unable to eliminate *Mtb*, thus favoring the formation of granulomas to contain the infection (84). As immune cells in these granulomatous structures need to be functionally committed to controlling the infection, it is crucial that their metabolic activity meets the bioenergetic and biosynthetic requirements needed to efficiently clear or contain the pathogenic burden. Infection of hMDMs, AMs and murine BMDMs with the irradiated *Mtb* H37Rv strain of *Mtb* is associated with increased extracellular lactate levels, indicative of an increase in glycolysis (7). Increased extracellular lactate levels were also enhanced in all macrophage cell types when infected with the live attenuated *Mtb* H37Ra strain and the live *Mtb* H37Rv strain (7). In addition, transcriptomic analysis of murine lungs infected with *Mtb* has revealed that during infection, genes involved in glucose metabolism are upregulated whilst genes that encode enzymes from the TCA cycle and OXPHOS are downregulated, indicating the occurrence of a Warburg effect (83). This switch is further evidenced in a NMR-based metabolomic profiling study showing increased concentrations of lactate in granulomas from *Mtb*-H37Rv-infected C57BL/6 mice (85). Moreover, the shift toward aerobic glycolysis during *Mtb* infection is linked to the ability of human macrophages to produce mature IL1β, subsequently demonstrated to be essential for bacteriocidal activity against *Mtb* when glycolysis was blocked with the glycolytic inhibitor 2-deoxyglucose (2-DG) (7).

Depending on their ontogeny, tissue resident macrophages and infiltrating macrophages have distinct roles, phenotypes and display differential metabolic profiles. In a mouse model of *Mtb* Erdman infection, *Mtb* has been shown to trigger

the accumulation of interstitial macrophages (IMs) ($Ly6C^{high}$, $CX_3CR1^+$, and $CD11b^{high}$) derived from blood monocytes that are phenotypically distinct from tissue resident AMs (Siglec $F^+$ and $CD11c^{high}$) (86). In this murine model, IMs were found to be more glycolytically active than AMs, with the latter cells relying more on FAO and fatty acid uptake (86). Interestingly, depletion of AMs reduced bacterial burden whereas deletion of IMs increased bacterial burden suggesting that AMs are permissive to *Mtb* (86). Furthermore, inhibition of glycolysis by 2-DG decreased the number of IMs and concomitantly increased bacterial burden thereby coupling metabolism with cellular function.

Human AMs exhibit significantly higher extracellular lactate levels, indicative of increased glycolysis, upon infection with the *Mtb* H37Ra and *Mtb* H37Rv strains (7). We have recently found that human AMs are also just as energetically responsive as hMDMs, which describes the ability of a cell to respond metabolically when stressed (30). For example, upon oligomycin-induced inhibition of OXPHOS in human AMs and hMDMs, AMs compensate by increasing glycolytic metabolism just as effectively as hMDMs (30). Others have also shown that human AMs contain *Mtb* better than monocytes (87). Furthermore, blocking glycolysis using the alternative carbon source, galactose, resulted in increased bacterial load in the human AMs, suggesting that this metabolic shift is required in AMs to allow them to exert bacillary killing (7). 2-DG reduced IL1β production in murine BMDMs, hMDM and human AMs, further supporting the idea that the switch to glycolysis is essential for optimal IL1β production, crucial to the control of bacillary replication (7). Production of IL1β is regulated by HIF1α, which is stabilized upon inhibition of the prolyl hydroxylase domain (PHD) proteins (88). As HIF1α lies at the crux of the glycolytic switch, HDTs that target PHD proteins and stabilize HIF1α may effectively boost glycolytic metabolism thereby supporting defense mechanisms within infected host immune cells.

## Amino Acid Metabolism

In addition to this glycolytic switch in energy metabolism, amino acid availability in the granuloma plays a key role in *Mtb* infection in human and murine studies (89–92). Amino acids are not only essential for cytokine and chemokine synthesis, but they play a role in the production of anti-microbial agents. More specifically, three amino acids, L-arginine, L-tryptophan and L-glutamine are key regulators of immunometabolism in TB (93–97). During TB infection, L-arginine is implicated in several immune cell effector functions, including the production of NO, and may therefore be important in the outcome of the infection (95). It has also been demonstrated that M2 macrophages express arginase-1 (Arg1), an enzyme that hydrolyses L-arginine to ornithine and urea (90). When macrophages express both Arg1 and inducible nitric oxide synthase (iNOS), NO synthesis is limited as both enzymes consume L-arginine. Abrogation of macrophage Arg1 exacerbates *Mtb* H37Rv growth and pathology in murine TB lung granulomas (89). Moreover, Arg1 plays an important role in L-arginine withdrawal from T cells within the same granuloma, leading to T cell inhibition (89). Hence these two functions of Arg1 may contribute in limiting the host cell's response to TB infection and protect the host from immune-mediated damage.

In response to *Mtb* infection, macrophages strongly upregulate the expression of indoleamine 2,3-dioxygenase enzymes (IDO1, IDO2, and TDO), that convert L-tryptophan into L-kynurenine. L-tryptophan catabolism has been demonstrated in transcriptomic and flow cytometry analyses to inhibit murine $T_{eff}$ cell function and induce $CD25^+Foxp3^+$ $T_{reg}$ subsets, reducing immune activity, limiting tissue damage and favoring pathogen survival (98). Furthermore, IDO-expressing DCs are essential for maintaining granulomas, which contain *Listeria monocytogenes* and enable mycobacterial survival (99). More recently, cerebral tryptophan metabolism has also been shown to be important for the outcome of tuberculous meningitis, where low cerebrospinal fluid tryptophan concentrations strongly predicted patient survival (100). Hence, modulating L-tryptophan metabolism could be used as a potential HDT strategy.

Glutamine is synthesized in a reaction catalyzed by the enzyme glutamine synthetase from L-glutamate, ammonia and ATP (96). Given the importance of this enzyme in nitrogen metabolism, it is believed to influence *Mtb* pathogenesis by altering ammonia levels within infected cells and thus may contribute to *Mtb*-mediated inhibition of phagosome-lysosome fusion and acidification (101). Conversely, L-glutamate exhibits potential to be utilized in the production of additional succinate, a TCA cycle intermediate now known to play an important role in the production of IL1β in LPS-stimulated murine BMDMs, mediated by the reverse electron transport process, in a ROS-HIF1α dependent manner (39, 102).

## Fatty Acid Metabolism

Fatty acid metabolism is another key aspect of TB that effects both *Mtb* and the infected host. Lung resident AMs are influenced by ongoing exposure to and uptake of surfactant, a lipid-protein complex that lowers surface tension and aids inhalation. When hMDMs are treated with surfactant *in vitro*, the growth of *Mtb* H37Rv is increased due to increased intracellular levels of the lipid, which the bacteria can use as a carbon source (103). Lipids serve as a key nutrient and energy source, but they also participate in regulating other immune responses. For instance, triacylglycerols (TAGs) can reduce *Mtb* H37Rv growth and antibiotic sensitivity, and the equilibrium between fatty acid synthesis and degradation may alter redox homeostasis in the cytosol (104, 105). Several studies have also demonstrated that *Mtb* utilizes cholesterol and fatty acids as essential nutrients during infection and *Mtb* preferentially metabolizes host lipids, although it can utilize a variety of nutrients to obtain energy (106, 107). Flow cytometry and co-localization analyses show that intra-phagosomal lipolysis is also markedly reduced in conjunction with the retention of host lipids further providing a potential source of nutrients for hMDMs and murine BMDMs infected with the *Mtb* CDC1551 strain (108).

The ability of *Mtb* to perturb fatty acid metabolism during infection results in the formation of foamy macrophages (106, 107). This is thought to be mediated by TLR2 signaling and increased PPARγ in human macrophages infected with *Mtb*

H37Rv, killed *Mtb* H37Rv, *M. smegmatis* and *M.bovis* resulting in lipid droplet accumulation (109). Low density lipoproteins containing cholesterol, TAGs and phospholipids, are sequestered within macrophages. Whilst TAGs and phospholipids are metabolized, the cholesterol is then either exported through ATP-binding cassette transporters, or esterified and accumulates as droplets, which leads to the formation of foamy macrophages (110). Traditionally, the function of foamy macrophages was thought to be restricted to lipid storage, however, it has now been shown that they may be essential for mycobacterial persistence and reactivation (111–113). For example, the bacterial glyoxylate shunt enzymes isocitrate lyase 1 and 2 are required for bacterial growth and virulence of *Mtb* Erdman-infected hMDMs and murine BMDMs *in-vitro*, and in an *in-vivo* murine model (111). *Mtb* H37Rv-infected murine BMDMs also require the utilization of cholesterol for survival during prolonged infection (112). Moreover, the accumulation of lipids has a significant impact on the metabolic pathways within *Mtb,* as the mycobacteria must produce more lipolytic enzymes to degrade these host lipids, especially cholesterol (107). Cholesterol degradation generates propionyl-CoA, which is a potential source of toxic metabolites that could compromise *Mtb* survival. Hence *Mtb* metabolizes this precursor toward different metabolites, by balancing acetyl-CoA and propionyl-CoA concentrations, some of which can be used to build the lipid elements of the cell wall, which not only support the structure, but are also important virulence factors (114). Thus the ability of *Mtb* to utilize host-derived lipids effectively is key to its success as a pathogen. Beyond providing a nutrient source and building blocks for bacterial growth, this accumulation of lipids in human cells can also block host autophagy and lysosome acidification, two other essential mechanisms for the control of *Mtb* (115). Others suggest that the accumulation of lipid droplets is the result of macrophage activation (not *Mtb*-induced perturbations) as it is dependent on IFNγ and HIF1α mediated glycolytic reprogramming in murine BMDMs (116). Interestingly, *Mtb* Erdman is able to acquire host lipids in the absence of lipid droplets, but not in the presence of IFNγ-induced host derived lipid droplets, thereby uncoupling macrophage lipid formation from bacterial acquisition of host lipids (116). These IFNγ-induced lipids, which require HIF1α for their synthesis, support the production of host protective eicosanoids including LXB4 and PGE2 (116). In addition, it has been demonstrated that lipid droplet formation is necessary for the production of host protective eicosanoids. Taken together, these changes in FA metabolism during *Mtb* infection suggest that targeting FA metabolism could result in the development of new and improved HDTs.

## HIF1α IS A KEY REGULATOR OF IMMUNOMETABOLISM DURING *MTB* INFECTION

HIF1α is central to reprogramming metabolism toward utilizing aerobic glycolysis; a process that functions as a key gate keeper in immune cell activation. As HIF1α is central to various preneoplastic and neoplastic diseases, it is not surprising therefore that HIF1α has been identified as a crucial molecular mediator during *Mtb* infection in humans and mice (8, 116–118). HIF1α is required for the production of NO, IL1β and prostaglandin E2 (PGE2) as demonstrated by murine HIF1α knockout macrophages which exhibit impaired production of these key cytokines in response to *Mtb* infection (8). HIF1α is also self-sustaining as stabilized HIF1α expression promotes glycolysis during *Mtb* infection, and this enhanced aerobic glycolysis promotes further stabilization of HIF1α (8). NO modulates macrophage responses to *Mtb* infection in murine BMDMs, through transcriptional and protein activation of HIF1α (119). HIF1α and iNOS are linked by a positive feedback loop that elicits further macrophage activation and regulate aerobic glycolysis (119). Specifically, $Nos2^{-/-}$ and $HIF1\alpha^{-/-}$ knockout results in significant transcriptional defects in various glycolytic genes including *GLUT1*, *LDHA,* and *PFKFB3* by RNAseq analysis (119). This results in significant reductions in extracellular glucose consumption in these BMDMs (119). Moreover, when murine BMDMs are activated with LPS, this results in an increase in the TCA metabolite succinate (39). Succinate is implicated in various different cellular mechanisms, such as inducing TLR synergy, participating in important post-translational modifications and in propagating further enhancement of glycolysis (39). Accumulation of succinate promotes the stabilization of HIF1α resulting in increased IL1β production from murine BMDMs (39).

HIF1α is also capable of binding to the promoter region of *pfkfb3* (120). The gene *pfkfb3* encodes an isoform of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB3). This enzyme regulates the production of fructose-2,6-biphosphate, a glycolysis intermediate which activates 6-phosphofructo-1-kinase, and this increases glucose uptake (121). Several studies have shown that *pfkfb3* levels increase after infection with *Mtb* in mouse, rabbit and human lungs (83, 122, 123). This upregulation is thought to be strongly dependent on HIF1α (83).

HIF1α is also crucial for the IFNγ-dependent control of *Mtb* in an *in vitro* and *in vivo* study of mice, as it mediates the metabolic switch to glycolysis in *Mtb* Erdman-infected BMDMs (8). IFNγ promotes M1-type macrophage polarization, cytokine production and synthesis of microbicidal mediators such as NO during infection with *Mtb* Erdman (124). Furthermore, HIF1α acts a positive feedback mediator during this process and acts to sustain the role of IFNγ in macrophage activation, helping to control and restrain the infection. This sustained metabolic transition to aerobic glycolysis is thought to be vital for IFNγ to successfully control the immune response to *Mtb* (8).

Mechanistically, the stabilization and subsequent activity of HIF1α is tightly regulated by a family of PHD proteins which continually target HIF1α for proteasomal degradation during homeostasis (125). If HIF1α is degraded, this turns off the regulation of all metabolic pathways where HIF1α is involved, such as the metabolic switch toward aerobic glycolysis. PHD enzymes require oxygen and α-ketoglutarate (αKG) as co-substrates, in addition to iron and ascorbate (126), hence the regulation of HIF1α by PHD enzymes is tightly associated with iron availability. Therefore, we suggest that the therapeutic

chelation of iron may serve to disable PHD activity and promote the stabilization of HIF1α which may in turn promote bacterial clearance during *Mtb* infection through enhanced immunometabolism and increased effector functions.

# IMMUNOMETABOLISM AND IRON ARE INTRINSICALLY LINKED.

## Controlling Physiological Levels of Iron

The activity of HIF1α can be significantly influenced by iron availability (127). Iron is crucially important for many physiological processes. The most common forms of iron in the human body are ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) iron; under physiological $O_2$ concentrations, the most stable form is $Fe^{3+}$ (128). Body stores of iron are usually assessed by transferrin saturation levels (normal range 20–30%), serum ferritin levels (normal range above 150 ng/mL), serum iron levels (normal range 60–110 ng/mL) and total iron binding capacity (normal range 240–300 ng/mL) but these levels can also vary between sexes (129, 130). Recent data examining iron distribution in freshly resected lungs of TB patients and healthy controls showed that the lungs of *Mtb*-infected patients contain more iron (54.7 ± 6.9 μg/g tissue) than healthy controls (19.4 ± 2.9 μg/g tissue) (131). Dietary iron is absorbed from the duodenum and upper jejunum (132). Here, the divalent metal transporter-1 (DMT1) transports $Fe^{2+}$ and $H^+$ into the cell. In the cell, $Fe^{2+}$ is stored within ferritin heavy and light chains, the primary iron storage protein, or it is transported into the blood when required. The iron exporter, ferroportin-1 enables the movement of iron out of cells (132). In the blood, oxidized $Fe^{3+}$ binds to transferrin and can be transported in this state until it reaches its target cell and binds to transferrin receptor-1 on the cell's surface (133). The transferrin-bound iron-transferrin receptor-1 (TBI-TfR1) complex is taken into the cell through the process of endocytosis, ultimately resulting in the release of $Fe^{3+}$ and recycling of transferrin and its receptor (133). Next, iron enters the mitochondria where it is a fundamental component in the synthesis of heme and iron-sulfur cluster-containing proteins which have a central function in the operation of the electron transport chain (134, 135). Thus iron metabolism plays a central role in regulating mitochondrial metabolism pathways. On a cellular level, iron levels are regulated by the iron regulatory protein (IRP)/ iron response element (IRE) system which controls the expression of several proteins essential for iron homeostasis, including DMT1 (136). The expression of hepcidin, a key regulator of the entry of intracellular iron stores into the circulation, is dependent on systemic iron levels (137). Hepcidin binds to ferroportin-1, inhibits it by promoting its internalization and degradation, thus negating iron export out of the cell subsequently lowering the amount of iron entering the circulation (136, 137). Iron is also responsible for oxygen transport and therefore regulates the bioavailability of oxygen in the cell (12). The presence of oxygen promotes the TCA cycle and OXPHOS (12). Conversely, when iron levels are low, there is less oxygen transport, and cells have a reduced oxygen supply. This can result in a decrease in mitochondrial metabolism, and an upregulation in anaerobic glycolysis to compensate for the reduction in ATP generated (12). Thus iron can be intrinsically linked to cellular metabolism and cell function in various ways, as **Figure 1** illustrates.

## Exploiting Iron Chelators for Therapeutic Gain

The therapeutic utilization of iron chelators has been widely reviewed (138–140). Iron-related pathologies occur when there is an excessive or insufficient level of iron (140). For example, iron overload typically arises from hereditary haemochromatosis. Iron chelation therapy is administered to prevent this, or reverse complications that may have already developed (138, 139). FDA-approved iron chelators, such as desferrioxamine (DFX), bind to free reactive iron in circulation (139). This complex is excreted from the body, thus reducing iron levels (139). Iron chelation therapy currently has many alternative applications. For example, deferiprone has been used in murine models of chronic obstructive pulmonary disease to transfer iron out of the mitochondria, and has been shown to ameliorate cigarette smoke-induced bronchitis and emphysema (141). Iron chelation also holds promise for the treatment of *Plasmodium falciparum*, which causes malaria, as use of deferiprone has been shown to reduce recovery time and increase clearance of the infection (142). DFX has also been shown to reduce the replication of HIV (143). Moreover, the iron chelators deferiprone, Apo6619, and VK28 have all been shown to possess antibacterial qualities against *Staphylococcus aureus* and *Escherichia coli* (144). Unsurprisingly, in addition to their chelating ability, specific iron chelators probably encompass additional properties that functionally set them apart from other iron chelators, elements of which are yet to be determined. For example, deferiprone is known to prevent the growth of coagulase-negative staphylococci but DFX promotes its growth (145). Therefore, the novel approach to treating bacterial infections with iron chelators could prove beneficial in TB, and may even hold promise against multi-drug resistant strains of the bacteria.

## Ironing out *Mycobacterium tuberculosis*

*Mtb* requires iron for survival and competes with the host for the same iron pool. To compete for iron, *Mtb* releases siderophores, namely exochelins, which have a high-affinity for iron and can remove it from the host's iron-binding molecules (146). Exochelins subsequently transfer iron to mycobactins in the cell wall of *Mtb* (146). Once iron is accessed, it is strictly controlled for the same reasons as seen in host cells; to maintain homeostatic levels, while preventing toxic accumulations. *Mtb* has readily evolved to utilize iron and controls iron uptake at a transcriptional level (147). The mycobacterial iron-dependent regulator (IdeR) is crucial to the maintenance of iron homeostasis in *Mtb* as experimentally-induced lack of IdeR results in an accumulation of iron, leading to oxidative damage and subsequent death of the mycobacterium, thus highlighting the importance of exochelins and IdeR as *Mtb*-survival mechanisms (147).

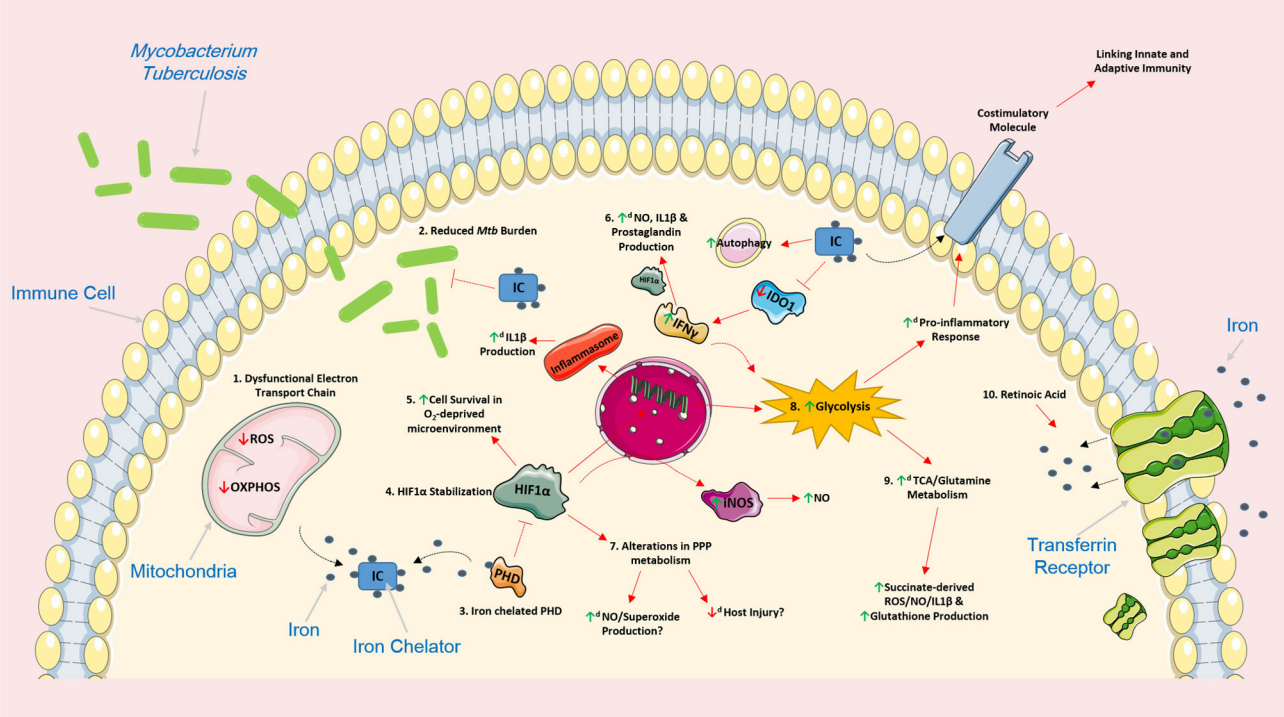


**FIGURE 1 |** Iron chelation encompasses the ability to support the host response by modulating cellular function and metabolism in various *Mtb*-infected immune cells. The use of iron chelators could potentially regulate a host of intracellular networks and support infected host cells by influencing several cellular processes. 1. Iron chelation results in a dysfunctional electron transport chain (ETC) as the ETC relies heavily on iron for optimal cellular function. A dysfunctional ETC could result in decreased production of ROS and a reduced capacity to undergo oxidative phosphorylation potentially promoting glycolysis. 2. Iron chelators have also been previously shown to have direct and indirect bacteriostatic and bactericidal effects on *Mtb*. 3. Iron chelation directly inhibits prolyl hydroxylase domain (PHD) proteins, proteins that normally function to silence HIF1α, by chelating one of its primary cofactors, iron. 4. Inhibition of the PHD proteins, through iron chelation, leads to the stabilization of HIF1α which can have various effects on cell function. 5. HIF1α plays an important role in promoting cellular survival in an oxygen-deprived microenvironment such as hypoxia. 6. HIF1α can also induce the production of IL1β, an important pro-inflammatory cytokine that helps control *Mtb* replication, by directly binding to the promotor of pro-IL1β. 6. IFNγ can boost production of nitric oxide (NO), IL1β and prostaglandin (e.g., PGE2) production, via HIF1α. Iron chelation can also inhibit IDO1, a key enzyme in tryptophan metabolism, and promote additional IFNγ production. Moreover, iron has been shown to increase the autophagic process. 7. The effect of HIF1α and iron chelators on pentose phosphate pathway (PPP) metabolism remains unclear, however, such alterations in this pathway could be beneficial. Increased NO and superoxide production can help kill unwanted infectious agents, and as the PPP is linked to NADPH and ROS production, decreased activity of this pathway could potentially reduce host injury and increase flux through glycolysis. 8. Iron chelators also encompass the ability to significantly boost glycolysis; such boosts in glycolysis are linked to the production of a host of pro-inflammatory mediators and the expression of various costimulatory molecules which could also link innate and adaptive immunity during *Mtb* infection. 9. By supporting glycolysis, iron chelators could also simultaneously enhance the activity of both the TCA cycle and glutamine metabolism which are intrinsically linked to the production of succinate, ROS, NO, IL1β, and glutathione. 10. The effect of iron chelation on these cellular processes could also be further augmented when administered in combination with other host directed therapies during *Mtb* infection. For example, retinoic acid can promote internalization of the transferrin receptor and further limit intracellular iron stores thereby reinforcing the effect of iron chelation. Image produced with the aid of Servier Medical Art software (see copyright license at https://smart.servier.com).

Macrophages play a key role in recycling iron. Excessive levels of iron have been documented in macrophages and hepatocytes from populations in Sub-Saharan Africa (148, 149). These populations are linked with a 3.5-fold increase in the probability of developing pulmonary TB (149). Excessive levels of iron are also seen in the macrophages of HIV patients, due to chronic blood transfusions or inflammation (150). Additionally, smoking increases the risk of developing active TB; this may be due in part to increased iron loading AMs. In fact, iron levels in AMs are over 3.2-fold higher in asymptomatic smokers and up to 5.6-fold higher in symptomatic smokers compared with nonsmokers; this could rise to 5.4-fold and 9.2-fold respectively when experimental variation is taken into account (151). It is also well known that iron starvation greatly affects *Mtb*'s ability to proliferate. *Mtb* also adapts to low iron levels by upregulating the expression of various factors such as the ESX-3 secretion system, which facilitates its survival (152). Although there is no direct proof of cause or effect, clinicopathological analysis of iron distribution within human lung tissue shows that *Mtb* severely disrupts iron homeostasis in distinct microanatomic locations of the human lung thus potentially contributing to lung immunopathology (131).

Targeting the hepcidin-ferroportin axis may have clinical utility and could be exploited as a means to alter intracellular iron levels. TLR agonists, except TLR2, were shown to polarize murine BMDMs into pro-inflammatory macrophages and upregulate hepcidin transcript levels (153). By measuring a

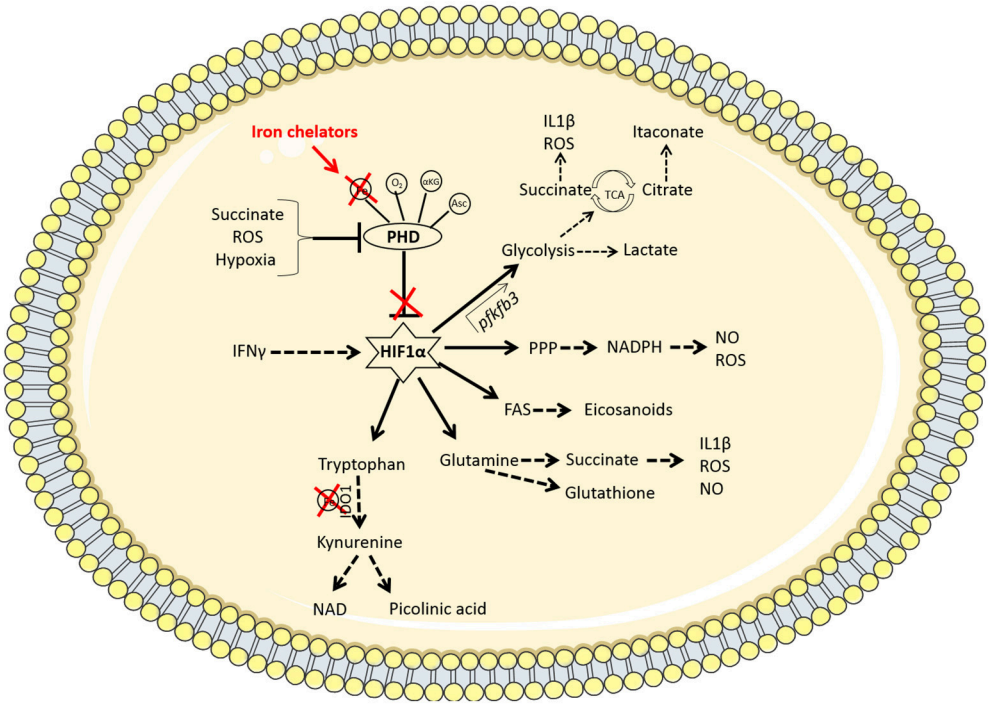


**FIGURE 2 |** Iron chelators modulate multiple immunometabolic pathways via the stabilization of HIF1α. Under homeostatic conditions, the PHD enzymes hydroxylate HIF1α marking it for degradation. These enzymes require oxygen ($O_2$), iron (Fe), α-ketoglutarate (αKG), and ascorbate (Asc) as cofactors to function. When oxygen is low (hypoxia), the PHD enzymes are disabled, resulting in the stabilization of hypoxia-inducible factor 1α (HIF1α). Other factors such as the accumulation of the metabolite succinate or reactive oxygen species (ROS) can also inhibit PHD enzymes. The therapeutic use of iron chelators will reduce the availability of iron inside the cell and therefore deny the PHD enzymes of the iron they require to function. Iron chelators thereby stabilize HIF1α which promotes enhanced flux through glycolysis by binding to the promoter region of the *pfkfb3* gene. This increased rate of glycolysis produces lactate and synthesizes the required building blocks for cellular proliferation and effector function. PAMP or DAMP signals in the macrophage (such as LPS stimulation or infection with *Mtb*, for example) leads to increased aerobic glycolysis and a break in the TCA cycle at 2 points; succinate (which promotes IL1β and ROS production as well as further inhibiting PHD enzymes) and citrate (which leads to the accumulation of the anti-bacterial metabolite, itaconate, via the enzyme IRG-1). HIF1α also mediates increased flux through the pentose phosphate pathway (PPP) which provides NADPH required from NO and ROS production. HIF1α promotes fatty acid synthesis (FAS), leading to the accumulation of lipid droplets and the production of eicosanoids. There is also a role for HIF1α in promoting amino acid metabolism. Glutamine can be used to produce succinate or the antioxidant glutathione. Tryptophan is processed by the iron-dependent enzyme IDO1, which results in a net anti-inflammatory response but can also produce NAD or picolinic acid, which has anti-microbial properties. Thus iron chelation may be a useful tool for manipulating macrophage metabolism during *Mtb* infection through the stabilization of HIF1α.

combination of transcript and protein levels of hepcidin and ferroportin, another study showed that differential TLR signaling can induce intracellular iron sequestration in THP-1 human macrophages (154). Specifically, agonists to TLR1/2, TLR2, and TLR6 significantly reduced transcript levels of ferroportin in THP-1 cells without affecting transcript levels of hepcidin (154). Conversely, TLR4, TLR7, and TLR8 agonists significantly induced both transcript and protein levels of hepcidin without affecting transcript levels of ferroportin (154). More significantly however, both alterations in hepcidin and ferroportin resulted in iron sequestration, suggesting that targeting these may be therapeutically beneficial. For example, by targeting hepcidin, this may reduce intracellular iron sequestration potentially affecting the growth of siderophilic bacteria, such as *Mtb*, while enhancing metabolism though HIF1α stabilization. Indeed, the same study shows that hMDMs infected with the *Mtb* Erdman strain induce high protein levels of hepcidin (154). Interestingly, heparin treatment has recently been shown to reduce hepcidin transcript and protein levels in THP-1 human macrophages infected with BCG and *Mtb* Erdman (155). Moreover, heparin treated macrophages exhibited higher ferroportin transcript and protein levels, promoting iron export and decreasing iron availability to intracellular bacilli. These infected heparin-treated cells also induce increased protein levels of IL1β further rendering hepcidin and ferroportin as attractive therapeutic targets (155).

Macrophage membrane-bound compartments, such as phagosomes and lysosomes, contain the natural resistance-associated macrophage protein-1 (NRAMP1). Murine studies using *M. avium*-infected BMDMs have shown that NRAMP1 acts to protect the host (156, 157). Moreover, several 3′UTR polymorphisms in this protein in humans have been shown to increase susceptibility to TB in specific populations (158–160). Mechanistically, NRAMP1 creates $Fe^{2+}$ efflux from the cell, and TB patients with these NRAMP1 polymorphisms are deprived of this protective method which would normally restrict *Mtb*

growth (156, 158, 161). The use of iron chelators on individuals with NRAMP1 polymorphisms could potentially provide the protection that they require. It is also likely that current anti-TB drugs, and other HDTs, administered in combination with iron chelators may result in better clinical outcomes. For example, retinoic acid has re-emerged as a potential HDT as it has been shown to promote cell-mediated clearance of *Mtb* H37Ra-infected BMDMs, hMDMs and human AMs (162). Moreover, retinoic acid has been reported to significantly reduce transferrin receptors on the membrane of macrophages, thus reducing the amount of iron available to the cell (163). Furthermore, as iron levels have also been shown to significantly reduce the efficacy of the anti-TB antibiotics isoniazid and ethambutol, the use of iron chelators may restore the effectiveness of such antibiotics (164). Targeting iron metabolism has the potential to directly inhibit the growth of *Mtb*, by interfering with *Mtb*-specific iron pathways and its survival mechanisms. Additionally, restricting iron availability in host immune cells may also serve to fight *Mtb* infection by stabilizing HIF1α to enhance important inflammatory and metabolic processes central to eradicating the infection. Therefore, we hypothesize that therapeutic iron chelation will function as a double-edged sword by boosting host immunometabolism via the stabilization of HIF1α and by directly starving *Mtb* of iron.

## FINE-TUNING HIF1α AND IRON; A MECHANISM TO SUPPORT INNATE HOST CELL FUNCTION DURING *MTB* INFECTION?

Iron chelation may be utilized to artificially trigger HIF1α-mediated pro-inflammatory and glycolytic pathways in host immune cells during *Mtb* infection. In normoxia, HIF1α is usually undetectable due to the inhibitory action of the PHD proteins (165). PHD proteins act by hydroxylating the oxygen-dependent degradation (ODD) domain on HIF1α (166). To function optimally, PHD proteins require oxygen, 2-oxoglutarate, ascorbate and $Fe^{2+}$ to successfully modify the ODD domain on HIF1α (126). When $Fe^{2+}$ levels are low, the activity of the PHD proteins is reduced (167). This is in contrast to hypoxic conditions, where the lack of oxygen inhibits the PHD proteins, thus stabilizing HIF1α (126). This allows heterodimerization of HIF1α with its β-subunit, and translocation into the nucleus where HIF1α binds to hypoxia-response elements linked with a variety of genes involved in various cellular processes, including inducing *pfkfb3* and *IL1β* transcription (168, 169). Moreover, GLUT1, as well as a number of other glycolytic enzymes such as phosphofructokinase, are upregulated to promote anaerobic glycolysis, to compensate for the lack of OXPHOS (170–172). Iron chelator-induced inhibition of PHD proteins and the resulting HIF1α stabilization encompasses the potential to trigger this molecular cascade during *Mtb* infection under aerobic conditions, thus, boosting the pro-inflammatory response of the infected host macrophage and promoting clearance of the infection. Indeed, several studies have shown that HIF is stabilized upon iron chelation in various cell types, including human renal Hep3B cells, human breast cancer MDA468 cells, and hMDMs (173–177). If iron chelation could induce such pro-inflammatory and pro-glycolytic effects during *Mtb* infection, then one may expect the opposite to occur with the addition of iron itself. Indeed, iron has been shown to promote intracellular and extracellular growth of *Mtb* H37Rv in J774A.1 macrophages (178). Moreover, addition of iron significantly reduces TNFα, IL1α, IL1β, and IL6 transcripts, along with TNFα protein levels, during *Mtb* infection (178). Hence, this work demonstrates that the modulation of iron metabolism can potentially regulate the functional relationship between the infected host cell and *Mtb*. Additionally, DFX has been shown to boost the autophagic process, to promote eradication of *Mtb* (179, 180). Western blot and immunofluorescence analyses of murine BMDMs incubated with the iron chelators deferiprone or desferasirox have also been shown to reduce the intracellular growth of *Chlamydia psittaci* and *Legionella pneumophilia* further suggesting that iron chelation may be therapeutically beneficial in the context of *Mtb* infection (181). Even though host-directed iron chelation may bring about reductions in intracellular iron levels, stabilize HIF1α, and trigger pro-inflammatory and glycolytic responses, intrinsic homeostatic mechanisms are still in place to correct for low iron levels thereby limiting host cell stress and toxicity. For example, ferritin, a key intracellular iron storage protein, helps to maintain optimal cellular function upon iron deprivation (154). Indeed, extensive flow cytometry analysis, extracellular metabolic flux analysis and mass spectrometry analysis show that complete ferritin deficiency in myeloid cells dysregulates host energy metabolism and increases susceptibility to *Mtb* H37Rv infection (131). Furthermore, the use of iron chelators have been shown to have no effect, and even reduce, the production of superoxide in *Mtb* H37Rv–infected U937 macrophage cells and THP-1 monocytes while simultaneously reducing the number and viability of *Mtb* mycobacteria (182, 183). Harnessing the potential of PHD proteins and their interconnectivity with HIF1α, through the use of iron chelators, may hold future promise for the development of HDTs for the treatment of TB infection and other infectious diseases. It must also be acknowledged that prolonged induction of HIF1α may cause detrimental damage to lung tissue by promoting excessive inflammation and oxidative stress. For example, in hMDMs and human respiratory A549 cells, HIF1α enhances the expression and secretion of matrix metalloprotease-1 (MMP-1), the main protease implicated in the uncontrolled destruction of lung tissue in TB (184). In fact, HIF1α, which is expressed highly in lung biopsies from patients with pulmonary TB, is necessary for MMP-1 gene expression and secretion (184). Moreover, HIF1α, and DFX, has been shown to positively regulate transcript levels of heme oxygenase−1 (HO-1), an oxidative stress response protein that catalyzes the degradation of heme to $Fe^{2+}$ and other intermediaries (185, 186). HO-1 expression is also markedly increased in rabbits, mice, and non-human primates during experimental *Mtb* Erdman and *Mtb* H37Rv infection and its expression gradually decreases during subsequent successful therapy (187). Moreover, systemic levels of HO-1 are dramatically increased in individuals with active pulmonary

and extra-pulmonary tuberculosis (188). Therefore, a thorough understanding of the underlying molecular mechanisms governed by HIF1α during *Mtb* infection would undoubtedly help fine-tune the development of combinatorial host-directed therapeutic approaches, while helping to reduce damage caused to the lung, thus preventing further TB dissemination. Another advantage of stabilizing HIF1α through the use of iron chelators is the potential ability to also boost host immune cell function through the modulation of alternative metabolic pathways such as the PPP, fatty acid metabolism and amino acid metabolism.

## THE POTENTIAL EFFECT OF IRON, IRON CHELATORS AND HIF1α STABILIZATION ON ALTERNATIVE METABOLIC PATHWAYS

Alternative metabolic pathways are also crucial for cellular growth and function particularly during *Mtb* infection. These alternative metabolic pathways may play a crucial role in the host response to infection and could potentially be targeted by iron chelation therapy. As HIF1α is a well-documented regulator of glycolysis, it is plausible that it can potentially regulate specific alternative metabolic pathways and support immunity during *Mtb* infection. These metabolic pathways include the PPP, fatty acid metabolism and the metabolism of important amino acids, namely glutamine and tryptophan.

The PPP is tightly coupled to glycolysis through the glycolytic intermediate glucose-6-phosphate (G6P), which can be shunted to the PPP to generate NADPH, ribose-5-phospate and other biosynthetic intermediates also utilized in the glycolytic process (189). Coupled with the fact that glycolysis has been shown to be induced upon *Mtb* infection in human AMs, this may also be indicative of an upregulation of the PPP during *Mtb* infection (7). Indeed, the lungs of *Mtb*-infected mice exhibit upregulated gene expression of enzymes involved in both glycolysis and the PPP (83). In this study, transcript levels of the PPP genes *Gpi1*, *G6pdx,* and *Pgd* were analyzed (83). The first enzyme of the oxidative phase of the PPP, glucose-6-phosphate dehydrogenase (G6PD), is induced by HIF1α in several different cancer cell lines (171, 190–192). Moreover, metabolomic analyses show that HIF1α overexpression results in increased levels of PPP metabolites in murine BMDMs (26). Importantly, the PPP is a major source of NADPH, which is necessary for the production of free radicals like NO and superoxide, and for protecting the cells against oxidative stress (193). The importance of the PPP for ROS production during *Mtb* infection has also been suggested by one study linking G6PD deficiency in humans with increased susceptibility to BCG infections due to impaired ROS production by neutrophils and monocytes (194). Another study investigating metabolomic profiles in murine macrophages treated with iron show increased levels of NADPH and 6-phosphogluconic acid, indicating the potential involvement of other factors, in addition to HIF1α, in the iron-mediated regulation of the PPP (195). Since the upregulation of glycolysis for rapid ATP production is an important host response against *Mtb*, downregulation of the PPP could further increase flux through the glycolytic pathway thus supporting host immune cells further. Whether these observations reflect the findings in iron chelated-*Mtb*-infected host cells has yet to be examined and needs to be investigated.

HIF1α has also been shown to be involved in fatty acid metabolism. Research shows that upon infection with *Mtb*, host cells differentiate into lipid forming foamy macrophages due to pathogen-induced dysregulations in lipid metabolism (113, 115). In an ESAT-6 mediated feedback mechanism, another study shows that *Mtb* actively manipulates host cells into metabolizing fatty acids, by diverting glycolytic metabolism toward ketone body synthesis, by enabling feedback activation of the anti-lipolytic G protein-coupled receptor GPR109A resulting in lipid body accumulation (115). Studies in cancer cells also show that hypoxia boosts the expression of fatty acid synthase and lipin-1 through HIF1α and the sterol regulatory element binding protein resulting in elevated fatty acid synthesis and lipid storage (196, 197). In accordance with that, murine peritoneal macrophages exposed to hypoxia show increased accumulation of lipid droplets, fatty acid synthesis and TAG synthesis (198). Importantly, hypoxic conditions also result in the downregulation of acyl-CoA synthase and acyl-CoA dehydrogenase, two key enzymes of the fatty acid β-oxidation pathway (199). These metabolic conditions could favor growth of mycobacteria, which use host derived fatty acids as a major carbon source (107). However, it remains to be investigated if iron chelation, and subsequent stabilization of HIF1α affects fatty acid metabolism in *Mtb*-infected host cells.

Glutamine metabolism represents another important metabolic pathway during *Mtb* infection. Glutamine metabolism is also a metabolic target of HIF1α signaling. During *Mtb* H37Rv infection, nuclear magnetic resonance analysis of infected C57BL/6 murine lungs shows an upregulation of succinate, which can be generated from glutamine through glutaminolysis (85). Silencing of PHD2 in skeletal cells, which stabilizes HIF1α, results in an increase in glutamine uptake and an increase in the expression of glutaminase-1, the enzyme that catalyzes the conversion of glutamine to glutamate. Glutamate can then be fed into the TCA cycle to produce αKG and succinate. Importantly, glutamate-derived αKG may also be used by the TCA cycle to produce succinate. Increased succinate oxidation by the succinate dehydrogenase (SDH) enhances the production of mitochondrial ROS, which in turn boosts HIF1α and IL1β levels in LPS-stimulated murine BMDMs (102). Furthermore, metabolizing glutamate through the arginosuccinate shunt, which links the TCA cycle with the urea cycle, results in the production of NO (40). Thus, increases in glutamine metabolism may potentially support anti-microbial immune responses in *Mtb*-infected host cells. However, glutamine can be preferentially used for glutathione production rather than being shunted into the TCA cycle (200). Glutathione is known to be an important antioxidant and reducing agent protecting cells from being damaged by oxidizing conditions thus may be critical during *Mtb* infection to protect the delicate lung tissue (201). Whether HIF1α stabilization through iron chelation has similar effects on glutamine metabolism in *Mtb*-infected host cells remains to be seen.

Tryptophan metabolism is another important metabolic pathway regulated through iron and HIF1α. Tryptophan is a crucial amino acid for intracellular bacterial growth and depletion of tryptophan through activation of the kynurenine pathway has been shown to inhibit growth of *Toxoplasma gondii* and *Legionella pneumophila* in monocytes and fibroblasts (202). Mycobacterial growth, however, is unaffected by tryptophan starvation in murine peritoneal macrophages, due to the bacteria's capacity to synthesize tryptophan *de novo* (97). However, picolinic acid, a natural degradation product of tryptophan, inhibits intra-macrophagic growth of *M.avium* and *Mtb in vitro* (93, 202). Nevertheless, IDO1, the first rate-limiting enzyme in kynurenine metabolism, is upregulated in murine BMDMs upon infection with *M.avium*, however, its deficiency does not impact on the outcome of the infection (93). Increased IDO1 activity is known to suppress the protective immune response in rhesus macaques, particularly the production of IFNγ by $CD4^+$ T cells, and correlates with a higher bacterial *Mtb* CDC1551burden (203). Therefore, inhibition of IDO1 may be beneficial for TB, in the context of persistent live bacterial infection. Interestingly, IDO1 is a heme-containing enzyme; iron chelation reduces its activity and iron supplementation increases its activity thus the effect of iron chelation on tryptophan metabolism in *Mtb*-infected cells may be promising and warrants further investigation (204).

## CONCLUSION

Despite various treatment options available to treat active TB, the prevalence of drug-resistant TB is increasing, further highlighting the need for novel therapies to fight the bacteria. The majority of individuals infected with *Mtb* mount an adequate innate immune response which results in early clearance of the bacteria. This suggests that supporting myeloid cell function could serve as a host directed preventative or therapeutic strategy. We hypothesize that restricting iron availability, through the use of iron chelators, may be an effective host-directed approach to supporting protective *Mtb*-infected macrophage responses which may enhance early clearance of the infection. As **Figure 2** depicts, by depriving macrophages of iron and stabilizing HIF1α, this could potentially function as a double-edged sword by boosting host immunometabolism and by directly starving *Mtb* of iron. In addition to boosting multiple metabolic pathways, HIF1α can directly and indirectly support many key cellular mediators, such as the multi-functional effects of IFNγ. Thus future studies need to investigate the use of iron chelators and their potential to be utilized as a HDT to boost the host immune response to *Mtb* infection.

## AUTHOR CONTRIBUTIONS

JP conceptualized, planned, wrote, proof read, and edited all drafts of the manuscript. SB and JK wrote, planned, proof read and edited all drafts of the manuscript. ST wrote and proof read the manuscript. SM and JS wrote the manuscript.

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# Future Path Toward TB Vaccine Development: Boosting BCG or Re-educating by a New Subunit Vaccine

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Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*), kills 5,000 people per day globally. Rapid development and spread of various multi drug-resistant strains of *Mtb* emphasize that an effective vaccine is still the most cost-effectives and efficient way of controlling and eradicating TB. Bacillus Calmette-Guerin (BCG), the only licensed TB vaccine, still remains the most widely administered human vaccine, but is inefficient in protecting from pulmonary TB in adults. The protective immunity afforded by BCG is thought to wane with time and considered to last only through adolescent years. Heterologous boosting of BCG-primed immune responses using a subunit vaccine represents a promising vaccination approach to promote strong cellular responses against *Mtb*. In our earlier studies, we discovered lipopeptides of ESAT-6 antigen with strong potential as a subunit vaccine candidate. Here, we have investigated that potential as a booster to BCG vaccine in both a pre-exposure preventive vaccine and a post-exposure therapeutic vaccine setting. Surprisingly, our results demonstrated that boosting BCG with subunit vaccine shortly before *Mtb* challenge did not improve the BCG-primed immunity, whereas the subunit vaccine boost after *Mtb* challenge markedly improved the quantity and quality of effector T cell responses and significantly reduced *Mtb* load in lungs, liver and spleen in mice. These studies suggest that ESAT-6 lipopeptide-based subunit vaccine was ineffective in overcoming the apparent immunomodulation induced by BCG vaccine in *Mtb* uninfected mice, but upon infection, the subunit vaccine is effective in re-educating the protective immunity against *Mtb* infection. These important results have significant implications in the design and investigation of effective vaccine strategies and immunotherapeutic approaches for individuals who have been pre-immunized with BCG vaccine but still get infected with *Mtb*.

**Keywords: vaccine, tuberculosis, subunit, BCG, booster, cellular immunity**

## INTRODUCTION

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (*Mtb*), is the leading cause of death due to an infectious disease, claiming ∼2 million lives globally each year (1). The rise in strains of *Mtb* resistant to almost all of the available TB drugs is making this deadly infectious disease ominous. Moreover, one third of the world's population harbors *Mtb* in a latent state serving as a large reservoir. These individuals are potentially at risk of developing active disease again and further spreading it easily to the remaining population (2, 3). Currently, Bacille Calmette-Guerin (BCG), which was developed nearly 100 years ago, is the only available vaccine to prevent TB. BCG remains the most widely administered vaccine around the world and is effective in providing 60–80% protection against childhood and extra-pulmonary forms of TB (4–7). But the beneficial effect of the vaccine given to children wanes over time. Further, BCG affords highly inconsistent and inadequate protection against pulmonary TB and is ineffective when given in adulthood. Because BCG has minimal effect on pulmonary TB, it has not had major effect on the global burden of TB (4–7). The reasons for the varying protective efficacy of BCG are still unclear. It is thought that pre-exposure with environment mycobacteria may prevent or mask the protective immunity induced by BCG. Also, BCG strain(s) (have) lost some of the genes encoding immunodominant antigens during attenuation (8). However, considering the fact that BCG has been administered to >3 billion infants in countries/settings with a high incidence of TB, and this will continue in the foreseeable future, developing a new and more efficient vaccine to replace BCG is a formidable task (9). Therefore, improving, boosting or supplementing BCG in different clinical settings appears to be a more logical path for new vaccine/immunotherapy approaches for TB.

In attempts to improve the efficacy of BCG vaccine, a phase 1 clinical trial for safety and tolerability was conducted using a recombinant BCG that expressed a) immunodominant antigens seen in active infection and during reactivation from latency (Ag85A, Ag85B and Rv3407) and b) a mutant perfringolysin (PFOG137Q) derived from *Clostridium perfringens*, able to perforate the endosomal membrane (10, 11). However, for some unknown reasons, 2 of 8 patients developed reactivation of varicella zoster virus, resulting in the discontinuation of further development of this vaccine (11).

Heterologous prime-boost strategies using viral-vectored vaccines or adjuvanted protein/peptide subunit vaccines have been a hopeful approach to TB vaccination. Several viral vectors, such as adenovirus (human Ad5, human Ad35, Chimp ChAdOx1), vesicular stomatitis virus (VSV), modified vaccinia Ankara (MVA) expressing antigen 85A, a highly conserved molecule across mycobacterial species, have been shown to provide superior protection when administered in BCG-primed animals compared to BCG alone (12–17). However, despite being highly immunogenic, none of these experimental vaccines provided satisfactory results in clinical testing yet (18).

Recombinant fusion protein-based vaccines may have potential as a booster to BCG vaccine and may also possess a number of advantages such as high level of safety, purity and cost-effectiveness. However, these vaccines would require effective and safe adjuvant to strengthen the immune responses generated. Some of the fusion protein-based vaccines being tested in clinical trials include H56:IC31 [a fusion protein of three *M. tuberculosis* antigens (85B, ESAT-6 and Rv2660c) formulated in the proprietary adjuvant IC31® from Valneva], H4:IC31 [a recombinant fusion protein of *Mtb* antigens 85B and TB10.4 combined with IC31® adjuvant] and M72 + ASO1E [immunogenic fusion protein (M72) derived from two *M. tuberculosis* antigens (MTB32A and MTB39A), and the GlaxoSmithKline's proprietary adjuvant AS01E] (19–24). The efficacy of these experimental vaccines remains to be determined.

Early secreted antigenic target 6 kDa protein (ESAT-6) is a potent T-cell antigen expressed in pathogenic *Mtb* and contains an unusually high number of permissive T cell epitopes spanning the entire sequence of the molecule (25, 26). ESAT-6 is an interesting antigen associated with active *Mtb* infection; however, the gene encoding ESAT-6 belongs to the RD-1 region, and all BCG vaccine strains distributed worldwide have deleted RD1 regions (27). ESAT-6 based subunit vaccines have shown tremendous potential in animal models (28, 29).

We have earlier reported the lipopeptides of ESAT-6 antigen corresponding to immunodominant epitopes as a promising candidate for TB vaccine. We demonstrated that mucosal immunization with lipopeptides of ESAT-6 antigen with or without adjuvant monophosphoryl lipid A (MPL) promoted strong mucosal and systemic immune responses, which were effective in reducing the *Mtb* burden in a mouse model of *Mtb* infection, supporting its efficacy as a prophylactic vaccine (30).

In the present study, we investigated the potential of lipopeptides of ESAT-6 based subunit vaccine to boost protective immunity induced by BCG both before (pre-exposure) and after (post-exposure) infection. We hypothesized that lipopeptides of ESAT-6 based subunit (LP-ESAT-6) vaccine could be potentially used to boost BCG as a prophylactic vaccine and used as immunotherapeutic vaccine for BCG vaccinated but still infected individuals. Intriguingly, while the pre-exposure boost with our LP-ESAT-6 vaccine did not lead to significant improvement in efficacy of BCG upon infection of mice with *Mtb*, post-exposure LP-ESAT-6 boost in BCG primed mice led to a significant decrease in *Mtb* bacterial loads compared to the BCG vaccine group, which was associated with increased immune responses both locally in lungs and systemically in spleen. While these results further expose the difficulties encountered in designing effective strategies to boost BCG vaccine, they provide a new paradigm to the concept of boosting BCG vaccine in efforts to investigate novel approaches for TB vaccine.

## MATERIALS AND METHODS

### Mice

All animal experiments used in this study were approved by the University of Alberta's Animal Care and Use Committee (ACUC) for Health Sciences and conducted in accordance with the guidelines of the Canadian Council of Animal Care (CCAC). Five-to six-week-old female BALB/c mice were purchased from

Charles River Laboratories and housed in biocontainment BSL 2/3 animal facility (HSLAS) at the University of Alberta.

## Synthetic Peptides, Antigen and Adjuvants

Synthetic lipopeptides derived from ESAT-6 [P1 (ESAT-$6_{1-15}$): MTEQQWNFAGIEAAAK(palmitate)G; P4 (ESAT-$6_{31-55}$):EG KQSLTKLAAAWGGSGSEAYQGVQK(palmitate)G; P5 (ESAT-$6_{46-70}$): SGSEAYQGVQQKWDATATELNNALQK(palmitate) G; P6 (ESAT-$6_{61-85}$):TATELNNALQNLARTISEAGQAMASK( palmitate)G; P7 (ESAT-$6_{76-95}$):ISEAGQAMASTEGNVTGMFA K(palmitate)G], were custom synthesized by Genscript Inc. (NJ, United States) (30). All lipopeptides were dissolved in DMSO at 10 mg/ml, stored at −20°C, and diluted with PBS or medium prior to use. MPL (Sigma Aldrich) was used as adjuvant. PPD antigen was obtained from Statens Serum Institut (Denmark).

## *Mycobacterium bovis* BCG and *Mtb* H37Ra Strains

BCG vaccine (Copenhagen) and *M. tuberculosis* (H37Ra) (ATCC, Rockville, MD) were grown in Middlebrook 7H9 broth supplemented with 10% oleic acid albumin dextrose complex (BD), 0.05% Tween 80, and 0.5% glycerol to mid-log phase before freezing at −80°C. Bacterial viability was determined by plating samples on Middlebrook 7H11 medium supplemented with ODAC and counting the number of colony-forming units (CFUs).

## BCG Vaccination and Immunization Schedule

For vaccination, $1 \times 10^6$ live BCG (100 μl/mouse) in PBS was administered subcutaneously (s.c.) at the base of the tail of each mouse. For subunit boost immunization, mice were administered intranasally (30 μl, 15 μl in each nostril) with a pool of lipopeptides (LP-ESAT-6, containing lipopeptide mix of P1, P4, P5, P6, and P7, each at 12 μg/mouse, total 60 μg/mouse) in the absence or presence of 10 μg of the adjuvant MPL. Control mice were immunized with an equal amount of PBS. The schedule of administration of BCG and LP-ESAT-6 are shown in **Figure 1I** in both pre-exposure and post-exposure settings.

## Mycobacterial Challenge of Mice and CFU Assay

For mycobacterial infection, mice were injected with $5 \times 10^5$ CFU/mouse of *Mtb* H37Ra intravenously. Four weeks after *Mtb* infection, mice were euthanized, and lungs, liver and spleen were removed aseptically and individually homogenized in 5 ml of saline. A 100 μl aliquot was taken from each organ homogenate of individual mice, serially diluted and plated on 7H11 Middlebrook agar plates (BD Biosciences). The plates were incubated at 37°C for 3–4 weeks prior to counting the colonies. CFUs of *Mtb* obtained in the unvaccinated group from individual mice in different experiments were in the ranges 5–6.5 $\times 10^4$ (lungs); 1.8–3.5 $\times 10^4$ (liver) and 0.7–1.4 $\times 10^4$ (spleen).

## Bronchoalveolar Lavage (BAL)

To harvest BAL fluid and cells, lungs were lavaged with 500 μl of ice-cold sterile PBS [with 0.3% wt/vol bovine serum albumin (BSA)] and two 500 μl PBS washes. Fluids were centrifuged at 1,500 rpm for 10 min, and RBC lysis was performed on cell pellets. For RBC lysis, the cell pellet was resuspended in 500 μl of sterile distilled water and vortexed briefly. Immediately after, 500 μl of 2X PBS was added, the tube was vortexed briefly and the volume was made to 2 ml with 1X PBS. The obtained lymphocytes were used for staining. The supernatants of the initial 500 μl BAL fluid were used for cytokine analyses.

## Cytokine ELISA

Cytokines secreted in BAL were measured using sandwich ELISA kits (IFN-γ, IL-17A, IL-22 and IL-10) following the manufacturer's protocol (eBioscience, CA, United States). A dilution of 1:2–1:10 was used for the samples with the standards ranging from 2 to 2,000 pg/ml. The ELISA plates were read with an automated ELISA plate reader (Fluostar Optima, BMG Labtech GmbH, Ortenberg, Germany).

## T Cell Proliferation Assay

Antigen-specific T cell proliferation assays were performed using splenocytes purified by nylon wool as reported previously by us Krishnadas et al. (31) Respective lipopeptides were used as recall antigen at a concentration of 10 μg/ml, and purified protein derivative of *Mtb* (PPD) was used at 1 μg/ml. Plates were incubated for 4 days, and cells were pulsed with 0.5 μCi/well [$^3$H]-thymidine (Amersham) for 12–18 h and harvested on filter papers. The levels of [$^3$H]-thymidine incorporated into the DNA of proliferating cells were counted in a Microbeta Trilux liquid scintillation counter (Perkin Elmer). Stimulation indices were calculated as CPM of antigen-stimulated culture/CPM of medium stimulated culture. Data are represented as the mean stimulation indices ± SD (standard deviation) of triplicate cultures.

## Flow Cytometry Analysis of Immune Cells

A total of $1 \times 10^6$ cells in BALs from immunized and challenged mice were stained with extracellular (anti-mouse CD3e-FITC, CD4-APC, CD8-APC-Cy-7, CD69-PECy-5) (eBioscience, CA, United States) markers using established procedures (32). For intracellular cytokine staining, $2 \times 10^6$ splenocytes were cultured for 4 days with medium only, peptide pools (1 μg/ml of each lipopeptide) or PPD (1 μg/ml). On day 5, brefeldin A (1.5 μg/ml, 1 X; eBioscience) was added, and cultured for 5 h at 37°C and subsequently stained for extracellular markers CD3-PE Cy7, CD4-PE Cy5, CD8-APC-Cy7, and intracellular cytokines IFN-γ-PE and IL-10-FITC using our previously reported procedures. For intracellular GrB staining, 2 x $10^6$ splenocytes obtained from mice were stained for extracellular markers CD3-PE Cy7, CD49b-Alexaflour 700, CD8-APC-Cy7, and intracellular GrB-Alexaflour 647, without *ex vivo* stimulation, using our previously reported procedures (31). Samples were run on LSR Fortessa SORP flow cytometer and analyzed using FACS-DIVA software (Becton Dickinson, Mountain View, CA, United States). Respective isotype-matched control antibodies were used to gate non-specific staining in each

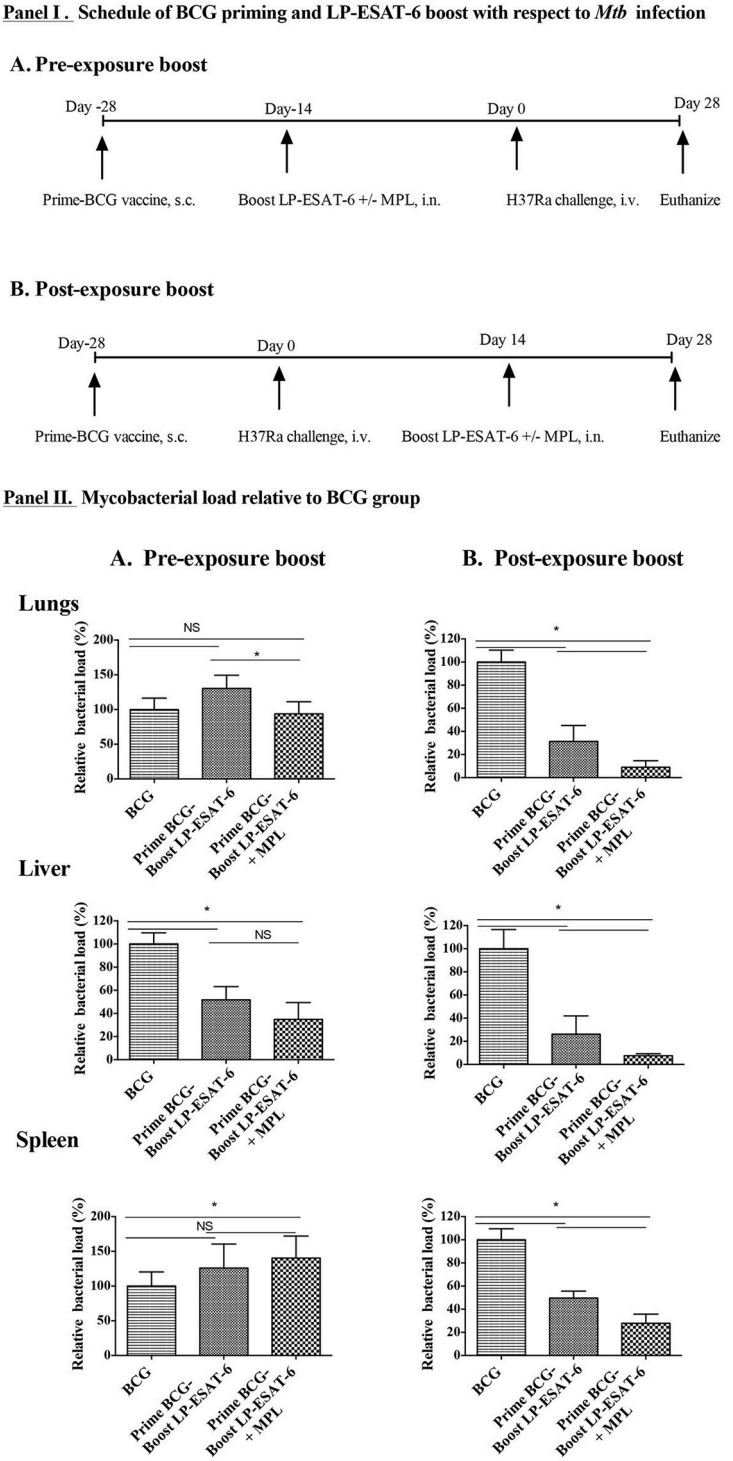


**FIGURE 1 |** Decrease in *Mtb* loads is dependent on schedule of the boost in heterologous prime-boost with BCG/LP-ESAT-6. **(I)**. Experimental design of BCG priming (s.c.) and LP-ESAT-6 boost (i.n.) using pre- and post-exposure schedules. **(II)**. Female BALB/c mice ($n = 5$/group) were immunized subcutaneously with BCG ($1 \times 10^6$ live BCG, 100 μl/mouse) or PBS (unvaccinated group). A mixture of P1 and P4-P7 lipopeptides (12 μg each) alone and combined with MPL was administered intranasally as LP-ESAT-6 boost according to the schedule shown in **(I)**, either before or after infection with H37Ra ($0.5 \times 10^6$ cfu) intravenously. Four weeks after *Mtb*-challenge, mice were euthanized and lungs, liver and spleens were collected from all groups. Bacterial loads were determined in lungs, liver and spleen by CFU assay. Mycobacterial reduction in **(A)** pre-exposure boost and **(B)** post-exposure boost of immunization is presented as percent relative bacterial load with respect to the corresponding BCG-alone group. All results are shown as mean ± standard deviation of percent relative bacterial load from individual mice. Data are representative of two different repeated experiments. *Indicates significant difference ($P < 0.05$) between the compared groups and "NS" represents not significant ($P > 0.05$).

experiment. Gates were set to exclude 95% of isotype control antibody stained cells in all extracellular and intracellular staining experiments.

## Statistical Analysis

Data were analyzed using Graphpad Prism software (Graphpad Software Inc., CA, United States). Data were presented as mean ± SD of 3-5 replicate values and significant differences between two groups or more than two groups were analyzed using two-way ANOVA using Tukey's multiple comparisons test. A $P \leq 0.05$ was considered to be statistically significant.

# RESULTS

## Scheduling of the Intranasal Boost With LP-ESAT-6 After BCG Priming Influences Protection Against *Mtb* Infection

In conventional prime-boost strategies for immunization both priming and boosting vaccines are given in a prophylactic manner to prevent an infection from occurring. However, besides examining the role of LP-ESAT-6 boost to BCG vaccine in a conventional manner, we also wanted to examine if LP-ESAT-6 boost will be useful for *Mtb* infection in the settings where one had been primed with BCG vaccine and yet became infected with *Mtb*. We designed two prime-boost schedules (**Figures 1IA,B**) of immunizations. In schedule A, pre-exposure boost, mice were primed with BCG vaccine (s.c.), followed by LP-ESAT-6±MPL boost (intranasally) at 14 days, and infection with *Mtb* subsequently on day 14 after the boost. In schedule B, post-exposure boost, mice were primed with BCG vaccine (s.c.), followed by infection with *Mtb* on day 14, and boost with LP-ESAT-6±MPL (intranasally) subsequently 14 days after the infection. The memory responses would take 4–6 weeks to emerge, however, we wanted to examine the effect of primary immunization. A significant number of papers in immunization field use 8–14 days post-immunization to evaluate the induction of adaptive immunity. Vast literature suggests that at 14 days after immunization, both humoral and cellular adaptive immune responses can be measured. Further, the effect of adjuvant (MPL) used is on innate immunity and not expected to last for 14 days, as we had 14 days between immunization and challenge (schedule A) or euthanization (schedule B). This schedule allowed us to maintain a consistent time-frame after initial BCG priming in both pre- and post-exposure boost schedules. Mice vaccinated with BCG alone or unvaccinated mice were used as controls. Mice in both prime-boost schedules A and B were euthanized 28 days post infection with *Mtb*. The outcomes of infection were determined by examining viable counts of *Mtb* in lungs, liver and spleen, using colony forming unit (CFU) assays. Immunization with BCG-alone led to ∼30–60% reduction in CFUs in lungs (∼30%), liver (∼60%), and spleen (∼50%), compared to unvaccinated controls in both sets of experiments. CFUs of *Mtb* obtained in the unvaccinated group from individual mice in different experiments were in the ranges 5.0–6.5 $\times$ $10^4$ (lungs); 1.8–3.5 $\times$ $10^4$ (liver) and 0.7–1.4 $\times$ $10^4$ (spleen). To determine the effect of LP-ESAT-6 subunit vaccine boost over BCG priming in both schedules, we calculated % *Mtb* viable counts relative to the BCG-alone group as shown in **Figures 1IIA,B**. Boosting with LP-ESAT-6 subunit vaccine in pre-exposure settings did not enhance the protection over BCG-alone based on lungs and spleen but not liver (**Figure 1IIA**). In fact, the *Mtb* loads were slightly higher in the LP-ESAT-6 ± MPL boosted groups in both lungs and spleen compared to BCG-alone group.

Intriguingly, when BCG-primed animals were boosted with LP-ESAT-6 subunit vaccine after *Mtb* infection as in post-exposure schedule, there was a remarkable reduction in pulmonary (∼30%) and extra pulmonary bacterial loads (30–50% in liver and spleen) when compared to BCG-alone vaccinated mice (**Figure 1IIB**). Moreover, addition of adjuvant MPL to LP-ESAT-6 subunit vaccine further enhanced the protective efficacy of booster vaccine (**Figure 1IIB**). Overall, these results demonstrate that the LP-ESAT-6 subunit boost with or without MPL imparts superior protection over BCG when administered after infection.

## Pre- and Post-exposure Boosting With LP-ESAT-6 Subunit Vaccine Distinctly Influences Cytokines in Bronchoalveolar Lavage (BAL) Fluid

The presence of cytokines in BAL fluid reflects the immune responses ongoing in lungs, the primary site of *Mtb* infection. To determine the pulmonary immune responses underlying the enhanced protection observed with post-exposure LP-ESAT-6 boost, we next analyzed the concentrations of IFN-γ, IL-17A, IL-22, and IL-10 in BAL fluids of vaccinated and *Mtb*-infected mice. BALs of unvaccinated animals exhibited very low levels of IFN-γ, IL-17A, and IL-22 but high levels of IL-10 (**Figures 2A,B**). Vaccination with BCG alone led to increased levels of IFN-γ, IL-17A, and IL-22 and a decrease in IL-10 compared to the unvaccinated group. However, interesting changes in BAL cytokines were observed upon the LP-ESAT-6 boost. Correlating to *Mtb* loads, when BCG primed mice were boosted with LP-ESAT-6 subunit vaccine after infection with *Mtb*, there were higher levels of IFN-γ, IL-17A, and IL-22 and lower levels of IL-10 compared to BCG-vaccinated mice, demonstrating the increase in protective immunity (**Figure 2B**). In contrast, our results showed significant decreases in the levels of IFN-γ and IL-10, and increase in IL-17A levels in the BAL fluid of BCG-primed mice boosted with LP-ESAT-6 vaccine before *Mtb* challenge, compared to BCG alone vaccinated animals (**Figure 2A**). No significant difference in IL-22 levels was found between BCG alone vaccinated and pre-exposure boosted groups. Addition of adjuvant MPL either enhanced or had no effect on cytokines induced in BALs by boosting with LP-ESAT-6 subunit vaccine (**Figure 2**).

## Boosting With LP-ESAT-6 Subunit Vaccine Enhances the Infiltration of T Cells in BALs

T cells infiltrating airways have been shown to be critical for improved protection after mucosal vaccination. Thus, we next

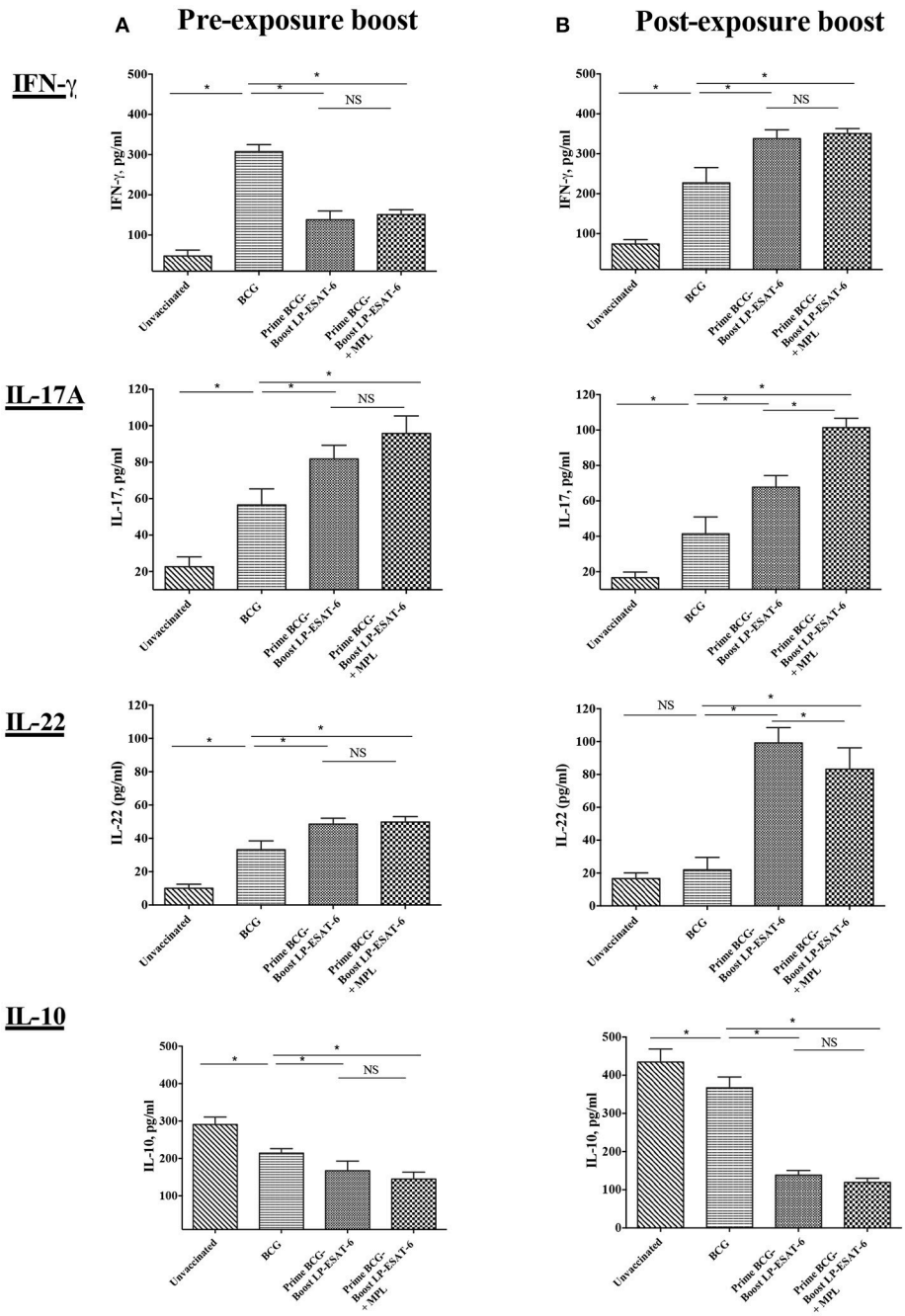


**FIGURE 2 |** Schedule of boosting with LP-ESAT-6 subunit vaccine in BCG-primed mice leads to differential induction of cytokines in bronchoalveolar lavage fluids. Female BALB/c mice ($n = 5$/group) were immunized according to the pre-exposure or post-exposure schedule shown in **Figure 1**. Four weeks after infection, mice were euthanized and lung lavages were collected from **(A)** pre-exposure boost and **(B)** post-exposure boost groups to determine IFN-γ, IL-17A, IL-22 and IL-10 by ELISA. Mean ± standard deviation of cytokine concentrations from five individual mice are shown. *Indicates significant difference ($P < 0.05$) between the compared groups and "NS" represents not significant ($P > 0.05$). Data are representative of two different repeated experiments.

sought to determine T cell infiltration in BALs to see how pre- and post-exposure LP-ESAT-6 subunit boost might modify the BCG-primed T cell responses in mouse lungs. Due to the presence of multiple T cell epitopes in both BCG as well as LP-ESAT-6 subunit vaccine, we chose to examine gross changes in total T cell population, instead of peptide epitope-specific T cells. The results obtained demonstrated that the frequency of infiltrating $CD4^+$ T cells was higher in LP-ESAT-6 subunit boosted mice compared to BCG-alone groups, regardless of the time of boost (**Figures 3IA,B**). In contrast, frequencies of

$CD8^+$ and $CD4^-CD8^-$ T cells (double negative T cells, DN T cells) were significantly enhanced in the post-exposure boost group compared to BCG-alone and pre-exposure boost groups (**Figures 3II,IIIA,B**). Strikingly, a significantly higher frequency of $CD8^+$ T cell subsets that expressed activation marker CD69 ($CD8^+CD69^+$) was found in the post-exposure boost group compared to BCG-alone (**Figures 3IIA,B**). The frequencies of $CD4^+$ and $CD4^-CD8^-$ T cells expressing CD69 were found to be comparable in both LP-ESAT-6 pre-and post-exposure subunit boosts (**Figures 3I,IIIA,B**). Inclusion of MPL in LP-ESAT-6 subunit vaccine led to an overall increase in the infiltration of T cells in BAL irrespective of the schedule of the boost (**Figure 3**). Overall, these data suggest that boosting BCG-primed responses with LP-ESAT-6 subunit vaccine results in an enhanced infiltration of activated $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T cells in BALs, with selectively increased infiltration of activated $CD8^+$ T cells in the post-exposure boost schedule. This could at least partially account for the enhanced protection over BCG-alone.

## Schedule of Boost With LP-ESAT-6 Subunit Vaccine Distinguishes the Proliferative Response of Splenocytes Upon *ex vivo* Stimulation With the Respective Antigen

Next, we sought to determine whether intranasal boosting with LP-ESAT-6 subunit vaccine at different schedules i.e., pre- and post-exposure, distinguishes the systemic T cell responses induced against the boosting antigen. We performed a bulk T cell proliferation assay using splenocytes obtained from different experimental groups after *ex vivo* stimulation with purified protein derivative (PPD) of *Mtb* or individual lipopeptides of the LP-ESAT-6 subunit vaccine and results are expressed as stimulation indices, obtained by dividing the counts per minute of antigen-stimulated culture with that of the corresponding medium-solvent control culture (**Figure 4**). Our results demonstrated that the unvaccinated group showed some proliferation in response to PPD antigen due to intrinsic T cell priming upon infection with *Mtb* in the mice. BCG vaccination led to a significant increase in PPD-specific proliferation. Interestingly, however, boosting with LP-ESAT-6 subunit vaccine in the presence or absence of adjuvant MPL did not lead to a significant difference in PPD-specific proliferation, which was also not affected by the schedule of boosting (**Figures 4IA,B**). In contrast, responses to *ex vivo* stimulation with individual lipopeptide components of the LP-ESAT-6 subunit vaccine were remarkable (**Figures 4IIA,B**). While there was a significant T cell proliferative response against ESAT-6 lipopeptides in splenocytes from mice which were given a boost before infection compared to the BCG-vaccinated or unvaccinated group, the proliferative response was dramatically increased in mice given the post-exposure boost with LP-ESAT-6 subunit vaccine (**Figure 4II**). Further, the addition of adjuvant MPL did not result in an enhancement of antigen-specific proliferation in the pre-exposure boost group, but resulted in a stronger enhancement in T cell proliferation in the post-exposure boost group, compared to the respective LP-ESAT-6 subunit group (**Figure 4II**). These results suggest that immunization with BCG possibly leads to modulation of T cells that compromises response to the subunit vaccine, whereas if the boost is given after *Mtb* infection, it is able to re-educate the immune system and lead to prolific subunit vaccine-induced T cell responses culminating in improved efficacy by reducing the *Mtb* burden.

## Schedule of the Boost With LP-ESAT-6 Subunit Vaccine Dictates the Quality of Systemic $CD4^+$ and $CD8^+$ T Cell Responses

The functional quality of T cell responses induced with a subunit vaccine boost would determine their protective efficacy and ability to boost BCG-primed responses. We observed that a post-exposure boost with LP-ESAT-6 subunit vaccine provided higher reduction in *Mtb* viable counts, higher IFN-γ levels in the BALs and higher proliferation of splenocytes in antigen-dependent manner compared to BCG vaccination only and pre-exposure boost (**Figures 1**, **2**, **4**). We then sought to examine the functional attributes of $CD4^+$ and $CD8^+$ T cells in splenocytes stimulated *ex vivo* with PPD or LP mix as recall antigens (**Figure 5**). Bulk splenocyte cultures incubated with medium, PPD or LP mix for 4 days were used to determine intracellular cytokine production in $CD4^+$ and $CD8^+$ T cells by flow cytometry. The data are expressed as % of medium control culture in the corresponding experimental groups, to determine the antigen specificity/dependency of the induced cytokine responses. Among the $CD4^+$ T cells, IFN-γ induced in response to *ex vivo* stimulation with PPD was increased in the BCG-immunized group compared to the unvaccinated group, but was unaffected in the groups with an LP-ESAT-6 boost both in pre- and post-exposure groups (**Figures 5IA,B**). In contrast, there was a significant increase in IFN-γ-producing $CD4^+$ T cells in the LP-ESAT-6 post-exposure groups, both compared to pre-exposure, unvaccinated and BCG only vaccinated groups (**Figures 5IA,B**). Interestingly, in PPD recall groups an increase in IL-10-producing $CD4^+$ T cells was observed upon BCG vaccination, which was maintained upon boosting with LP-ESAT-6 in both schedule. In contrast, however, *ex vivo* stimulation of splenocytes with LP mix did not lead to a significant increase in IL-10-producing $CD4^+$ T cells in unvaccinated, BCG-alone and LP-ESAT-6±MPL groups, suggesting that boosting with the LP-ESAT-6 subunit did not lead to $CD4^+$ T cells producing high levels of IL-10 (**Figures 5IA,B**). Examination of cytokines produced in $CD8^+$ T cells demonstrated an entirely different but interesting pattern (**Figures 5IIA,B**). While in response to PPD there was induction of IFN-γ and IL-10-producing $CD8^+$T cells after BCG vaccination, they were similar in LP-ESAT-6-boosted groups irrespective of the schedule of boosting. In contrast, in response to *ex vivo* stimulation with ESAT-6 LP mix, only in the post-exposure group there was a significantly higher % of IFN-γ as well as IL-10 producing $CD8^+$ T cells, which were further enhanced in the groups that received MPL in addition to LP-ESAT-6 vaccine (**Figures 5IIA,B**). There seemed to be almost a

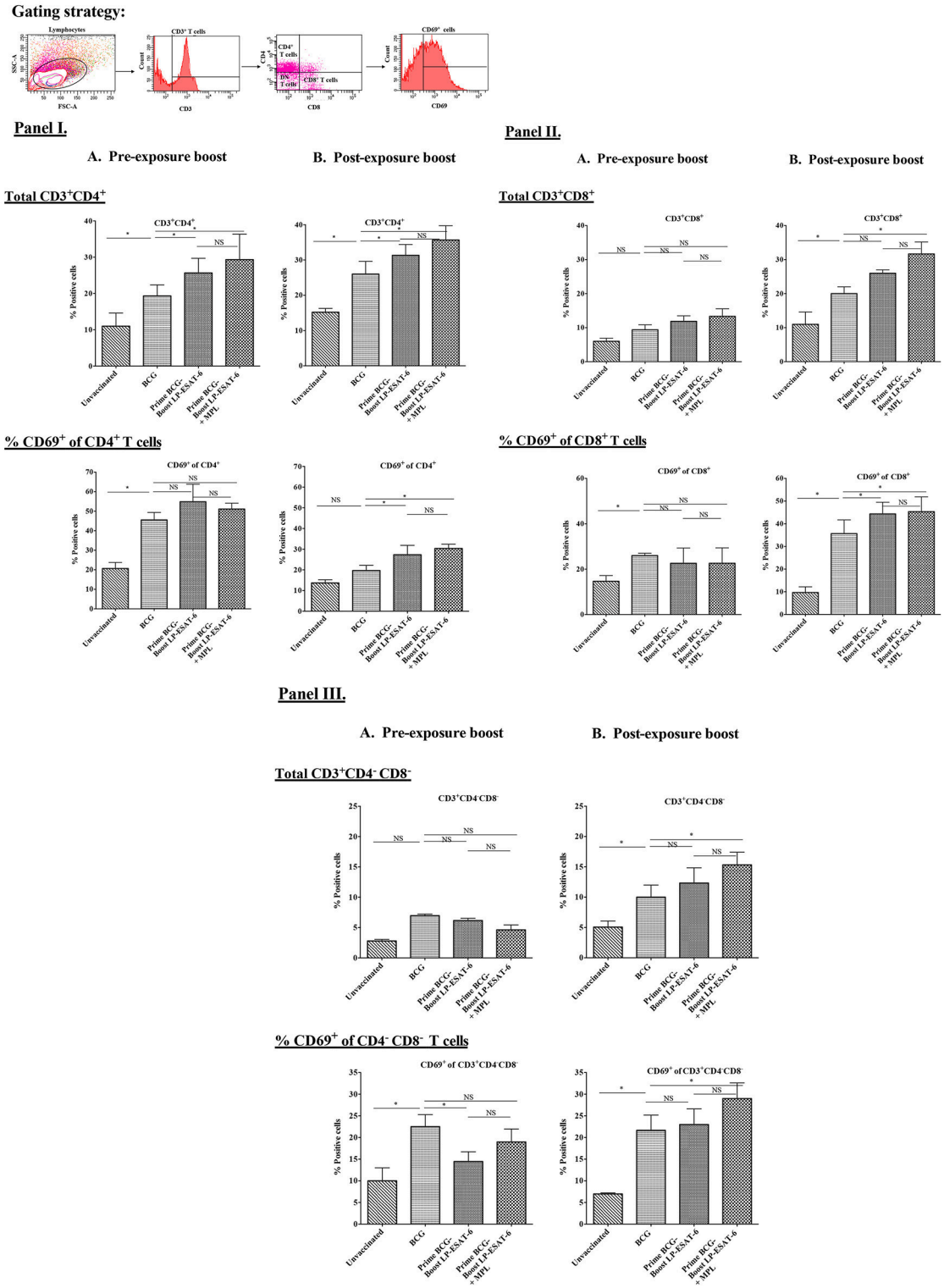


**FIGURE 3 |** Boosting with LP-ESAT-6 subunit vaccine in BCG-primed mice leads to enhanced recruitment of immune cells in bronchoalveolar lavage fluids. Female BALB/c mice ($n = 5$/group) were immunized according to the pre-exposure or post-exposure schedule shown in **Figure 1**. Four weeks after infection, mice were euthanized and lung lavages were collected from **(A)** pre-exposure boost and **(B)** post-exposure boost groups to examine the presence and activation ($CD69^+$) of $CD3^+CD4^+$ **(I)**, $CD3^+CD8^+$ **(II)** and $CD3^+CD4^-CD8^-$ (DN) T cells **(III)** by flow cytometry. The gating strategy shown above the bar graphs in **(I)** was used to detect the different subsets of T cell based on the expression of CD3, CD4, CD8 and CD69 markers by flow cytometry analysis. Mean ± standard deviations of percent positive cells from five individual mice are shown. *Indicates significant difference ($^*P < 0.05$) between the compared groups and "NS" represents not significant ($P > 0.05$). Data are representative of two different repeated experiments.

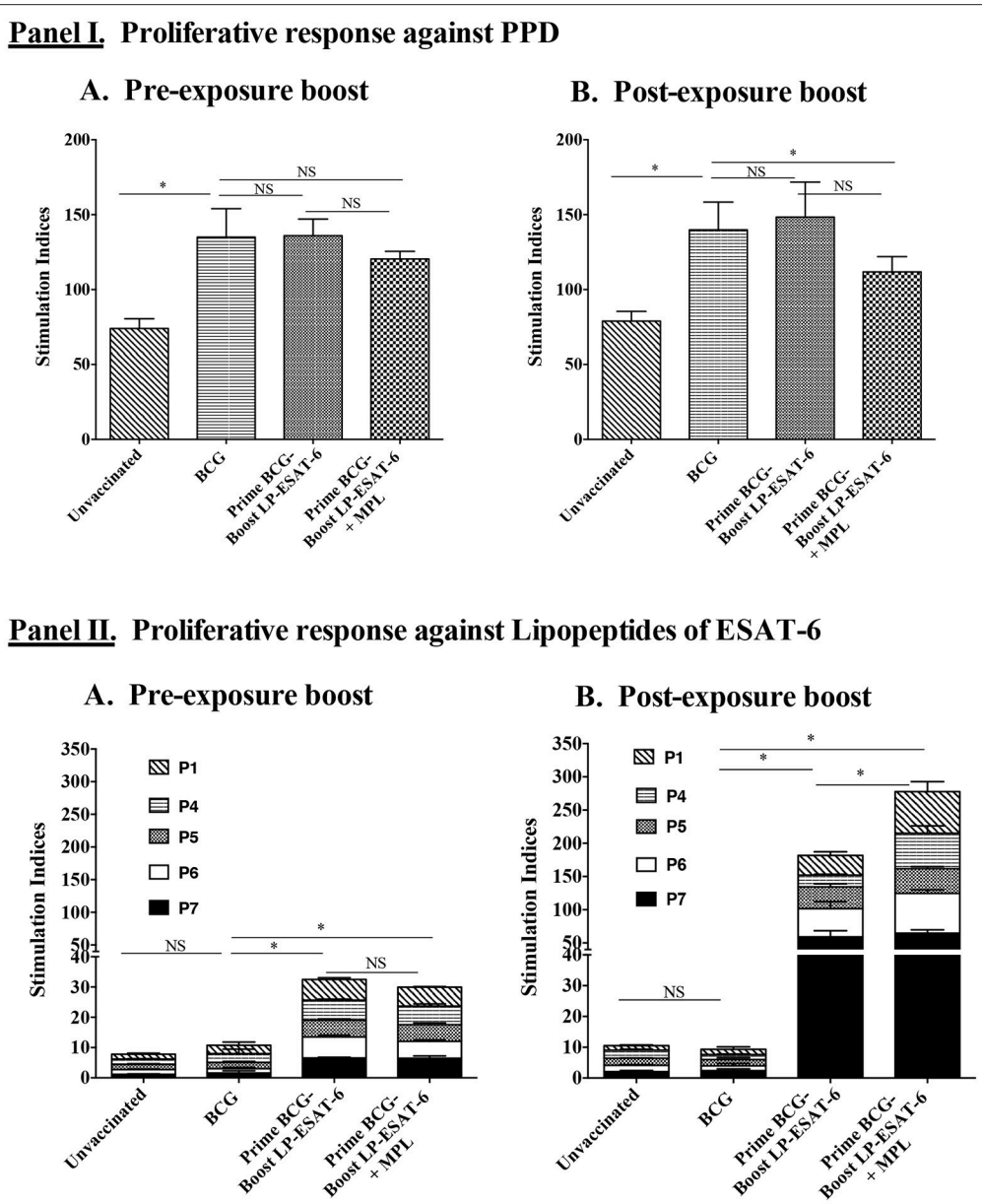


**FIGURE 4 |** Proliferative responses of splenocytes upon *ex vivo* stimulation with PPD or individual lipopeptide components of LP-ESAT-6 subunit vaccine. Female BALB/c mice ($n = 5$/group) were immunized according to the pre-exposure or post-exposure schedule shown in **Figure 1**. Four weeks after infection, mice were euthanized and spleens were collected from **(A)** pre-exposure boost and **(B)** post-exposure boost groups to examine the antigen-specific proliferation of T cells. Briefly, T cells obtained from splenocytes (pooled from 5 mice of a group) were cultured with irradiated APCs (splenocytes from unimmunized mice) and with medium, PPD (1 μg/ml) **(Panel I)** or respective lipopeptides P1 and P4-P7 at 10 μg/ml concentration **(Panel II)** for 4 days. T cell proliferation was measured by [$^3$H] thymidine incorporation. Stimulation indices were calculated as CPM of the antigen-containing cultures/CPM of medium control group. Mean ± standard deviation of stimulation indices from triplicate wells are shown. *denotes significant difference ($P < 0.05$) between the compared groups and "NS" represents not significant ($P > 0.05$).

complete lack of induction of antigen-specific/dependent IFN-γ and IL-10-producing CD8$^+$ T cells in pre-exposure boost groups, which received LP-ESAT-6 subunit vaccine with or without MPL. These data indicate that there is a significant difference in the induction of effector CD8$^+$ T cells depending on the timing of the subunit vaccine given pre- or post-exposure. It is not clear whether this difference is due to immunomodulation induced by BCG vaccine, which cannot be overcome by a subunit vaccine as such. But upon infection, the subunit vaccine is either able to amplify the primary response induced by infection or the immunomodulation induced by BCG vaccine is overcome by the productive infection.

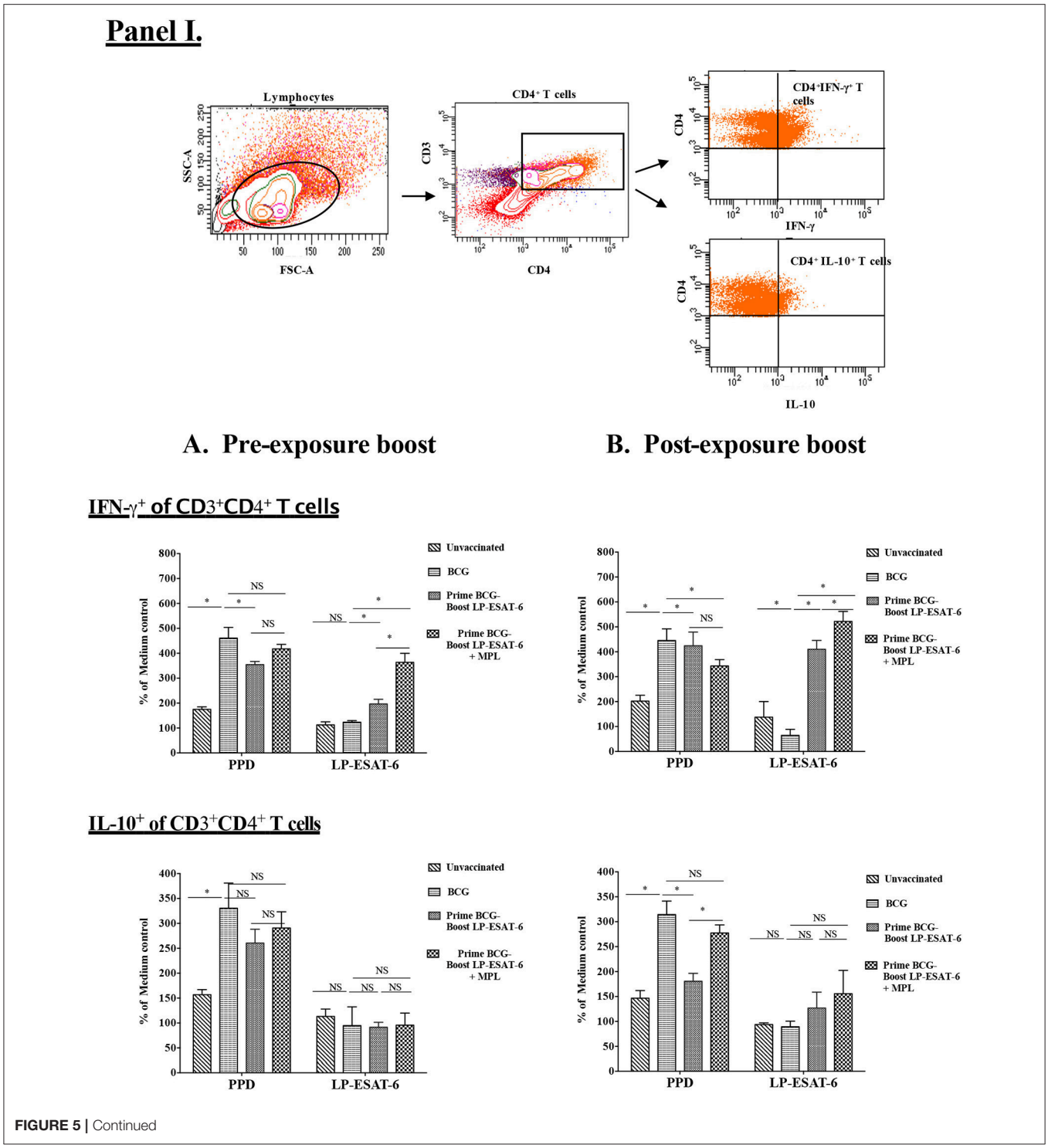


**FIGURE 5 |** Continued

## Boosting With LP-ESAT-6 Subunit Vaccine Enhances Granzyme B (GrB)-Producing Effector Lymphocytes Including NK, NKT and CD8$^+$ T Cells

For an intracellular pathogen like *Mtb*, besides effector cytokines, cytotoxic mechanisms are important to rid the infected host cells of bacteria, and Granzyme B (GrB) is a marker of functional effector cytotoxic cells. NK, NKT and CD8$^+$ T cells are prominent cytotoxic lymphocytes and mediate killing of infected cells through the secretion of GrB. NK and NKT cells have been classically considered as innate immune cells but have recently been also shown to be stimulated upon antigen-based vaccinations and have memory responses like

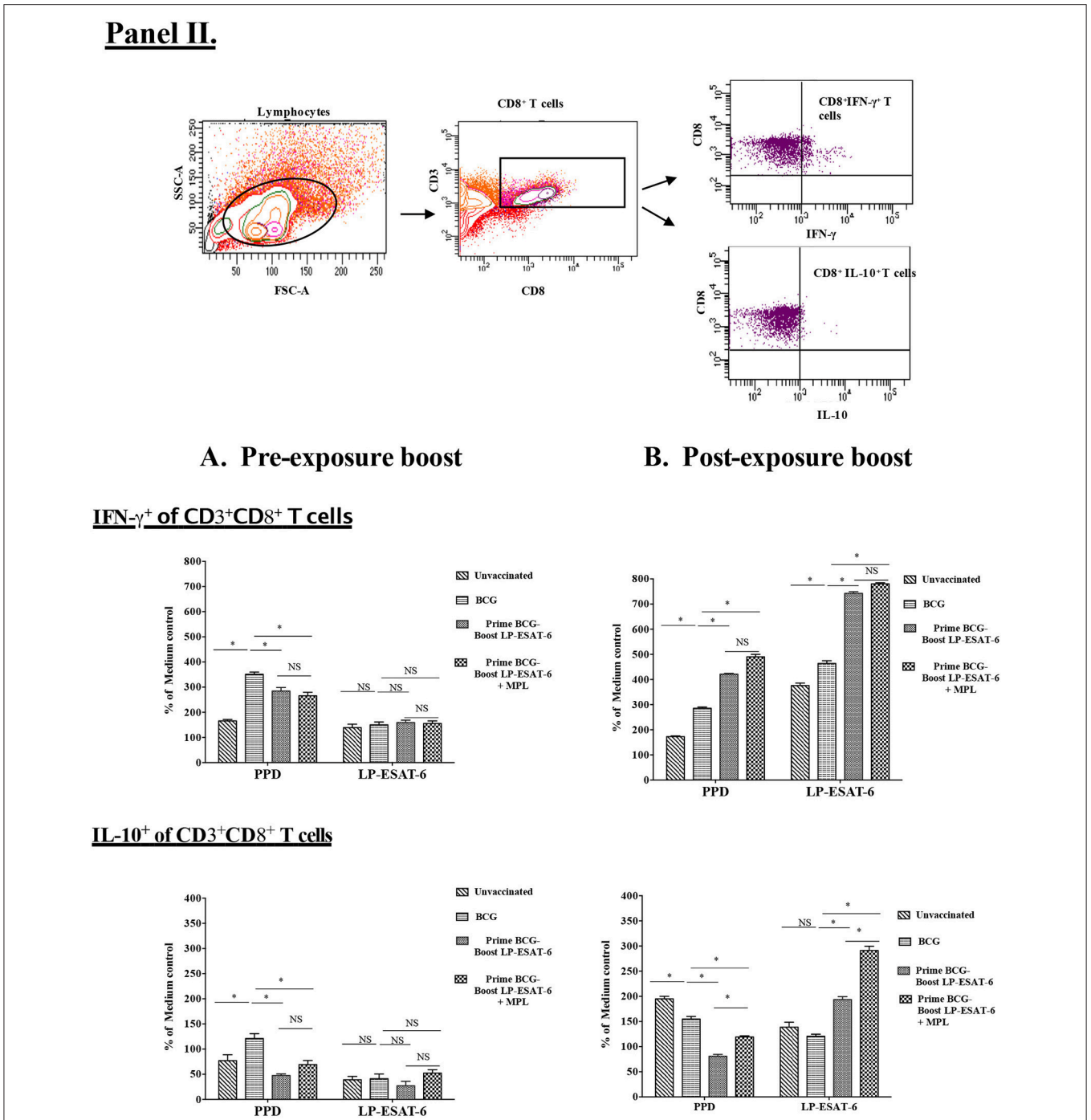


**FIGURE 5 |** Intracellular IFN-γ and IL-10 are differentially expressed in antigen-specific $CD4^+$ **(I)** and $CD8^+$ **(II)** T cells in BCG-primed mice boosted with LP-ESAT-6 subunit vaccine in pre-exposure or post-exposure schedule. Female BALB/c mice ($n = 5$/group) were immunized according to the pre-exposure or post-exposure schedule shown in **Figure 1**. Four weeks after infection, mice were euthanized and spleens were collected from **(A)** pre-exposure boost and **(B)** post-exposure boost groups. Spleen cells obtained from 5 mice immunized with lipopeptide P1 and P4-P7 were cultured for 4 days with or without PPD (1 μg/ml) or lipopeptide mix (each of P1 and P4-P7 at 1 μg/ml), cultured with Brefeldin A (1.5 μg/ml) 1X; eBioscience) for 5 h, and labeled with antibodies against CD3, CD4 and CD8 for extracellular staining along with intracellular IFN-γ and IL-10. The cells were gated for $CD3^+CD4^+$ and $CD3^+CD8^+$, which were subsequently analyzed for IFN-γ or IL-10 expression as shown in the gating strategy shown above the bar graphs in each panel. Data are shown as the percentage of IFN-$\gamma^+$ or IL-$10^+$ of $CD4^+$ **(I)** and $CD8^+$ T cells **(II)** upon stimulation with PPD or lipopeptide mix normalized to medium control in each of the experimental groups: unvaccinated, BCG alone, BCG prime/LP-ESAT-6 boost and BCG prime/LP-ESAT-6+MPL boost in **(A)** pre-exposure and **(B)** post-exposure schedules. Mean ± standard deviation from triplicate cultures from spleen cells pooled from five individual mice are shown at the bottom. *denotes significant difference ($P < 0.05$) between the compared groups and "NS" represents not significant ($P > 0.05$). Data are representative of two repeated experiments.

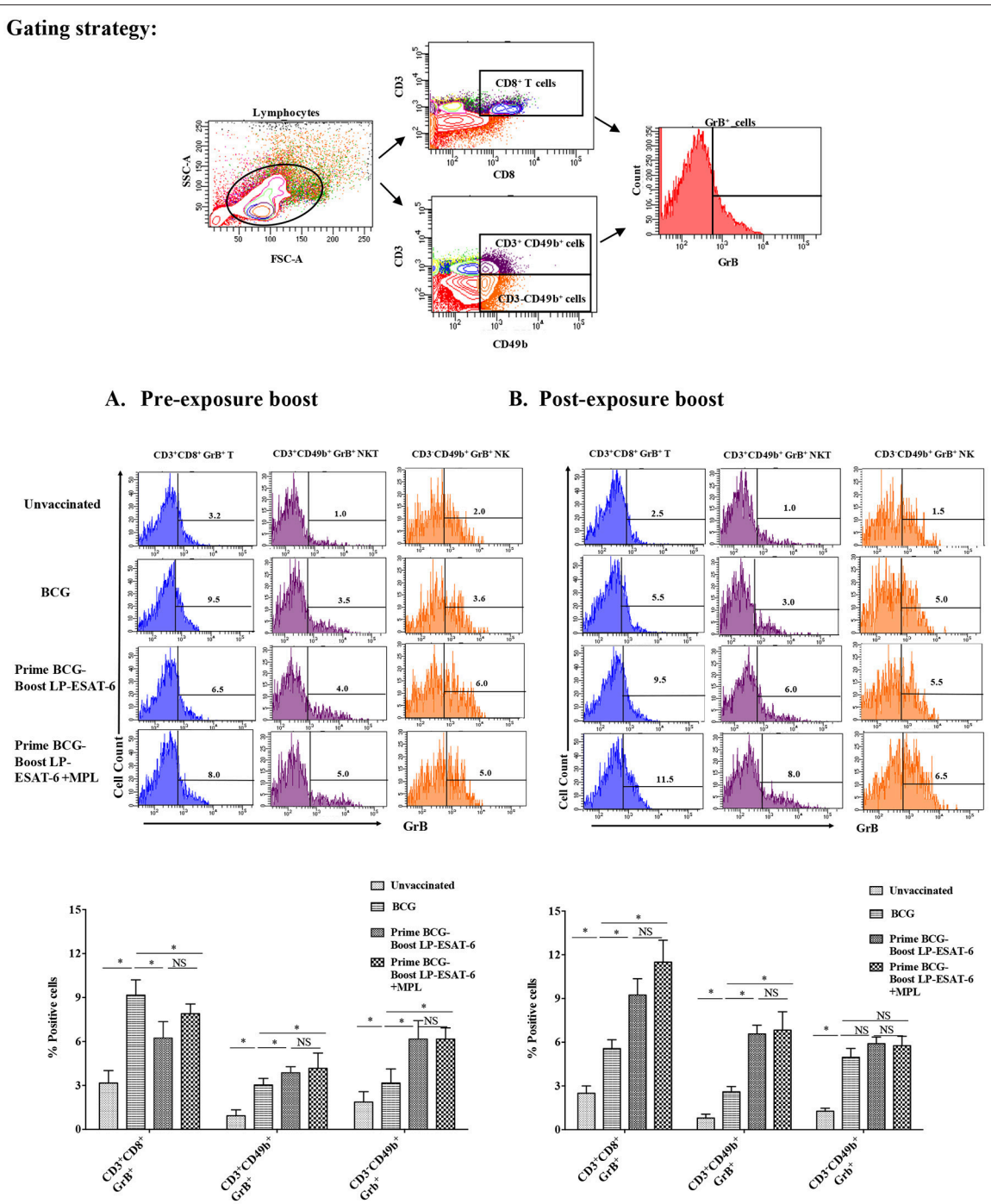


**FIGURE 6 |** Induction of granzyme B (GrB)-producing effector lymphocytes including NK and NKT and CD8+ T cells in BCG-primed mice boosted with LP-ESAT-6 subunit vaccine in the pre-exposure or post-exposure schedule. Female BALB/c mice ($n = 5$/group) were immunized according to the pre-exposure or post-exposure schedule shown in **Figure 1**. Four weeks after infection, mice were euthanized and spleens were collected from **(A)** pre-exposure boost and **(B)** post-exposure boost groups. Spleen cells obtained from individual mice were cultured for 5 h with Brefeldin A (1.5 μg/ml) 1X; eBioscience), and labeled with antibodies against CD3, CD8 and CD49b for extracellular staining along with intracellular GrB. The cells were gated for $CD3^+CD8^+$, $CD3^-CDCD49b^+$ (NK) and $CD3^+CDCD49b^+$ (NKT) cells, which were subsequently analyzed for GrB expression as shown in the gating strategy above the bar graphs in each panel. Data are shown as the percentage of $GrB^+$ of $CD3^+CD8^+$, $CD3^-CDCD49b^+$ and $CD3^+CDCD49b^+$ cells from each of the experimental groups: unvaccinated, BCG alone, BCG prime/LP-ESAT-6 boost and BCG prime/LP-ESAT-6+MPL boost in **(A)** pre-exposure and **(B)** post-exposure schedules. Histograms of $GrB^+$ cells are shown in the top panel and bar graphs representing mean ± standard deviations from five individual mice are shown in the bottom panel in each of the schedules **(A,B)**. *denotes significant difference ($P < 0.05$) between the compared groups and "NS" represents not significant ($P > 0.05$). Data are representative of two repeated experiments.

adaptive T cells (33). To examine the effect of LP-ESAT-6 subunit vaccine boost on effector cytotoxic lymphocytes, splenocytes obtained from mice were examined for GrB expression in NK, NKT and $CD8^+$ T cells without *ex vivo* re-stimulation (**Figures 6A,B**). Intriguingly, the percent of $CD8^+$T cells expressing GrB increased in the order: unvaccinated<BCG alone<LP-ESAT-6 boost<LP-ESAT-6+MPL boost in the post-exposure schedule, whereas in the pre-exposure schedule, boosting with LP-ESAT-6±MPL led to a decrease compared to the BCG group (**Figures 6A,B**). GrB expression in both NK ($CD3^-CD49b^+$) and NKT cells ($CD3^+CD49b^+$) showed a trend as unvaccinated<BCG alone<LP-ESAT-6 boost<LP-ESAT-6+MPL in both pre- and post-exposure groups (**Figures 6A,B**). These experiments indicate that boost with LP-ESAT-6 subunit vaccine leads to overall increase in GrB-expressing effector cytotoxic cells compared to BCG alone.

## DISCUSSION

There is clearly an urgent unmet need to investigate new vaccine/immunotherapy approaches for tuberculosis. Heterologous prime-boost vaccination appears to be a promising strategy in the investigation of new vaccine approaches. However, despite an increased focus in the last two decades, clinical progress in heterologous prime-boost strategies has been limited due to insufficient understanding of the protective immune response required, target antigen and its delivery, and route and schedule of boosting. While viral vector derived boosting vaccines have been investigated, there has been limited clinical success and concerns of serious side effects (12–18). A number of fusion protein-based subunit vaccines are being tested as boosters to BCG. However, weak immunogenicity of protein/peptide-based vaccines requires a safe and effective adjuvant, which in itself poses issues (19–24, 34).

Our earlier studies demonstrated that lipid-modified permissive T cell epitopes from ESAT-6 (LP-ESAT-6) induce strong protective immunity against *Mtb* upon mucosal (intranasal) administration, which is further enhanced by addition of an adjuvant MPL (30). With the premise that ESAT-6-based subunit vaccine by itself may not be sufficient to induce broad multifunctional protective immunity required to protect from a complex pathogen such as *Mtb*, we sought to investigate its potential as a boosting or supplementing vaccine after priming with BCG. This approach would not stringently conform to the classical definition of boosting since BCG vaccine does not encompass ESAT-6 antigen, but would rather provide an expansion to the concept of prime-boost vaccine strategies. However, numerous earlier examples of not-so-successful preclinical and clinical testing with BCG-prime and heterologous-boost prompted us to re-examine this approach in an unconventional manner. Most of the post-exposure or therapeutic vaccines previously studied were evaluated in BCG-naïve or BCG-vaccinated healthy individuals (14, 18, 19). Traditional prime-boost vaccine approaches require administration of the booster vaccine at various times after the priming vaccine to prevent an infection. But the timing, dose and route of which still continues to be debated. Although BCG-induced immunity is known to wane after time, it is known to be good for 10–15 years after childhood immunization in humans (6, 35, 36). This suggests that only minor changes in the timing of booster vaccination may not show quantifiable differences. While there are scarce studies, which thoroughly examined the timing of boosting, homologous boosting with BCG after BCG priming has not demonstrated beneficial effect compared to heterologous boosting (37). Effect of timing with different boosting vaccines has been variable: the boosting potential of MVA85A was not found to be influenced by the timing of the boost after BCG-priming in children whereas prolonging the time between BCG priming and boosting was found to be favorable for another subunit vaccine that used a recombinant mycobacterial adhesin heparin-binding haemagglutinin (rHBHA) (38). It has been speculated that repeated activation of already activated T cells may lead to increased apoptosis following a subunit-boost resulting in a suboptimal booster effect observed at the peak of a BCG response, whereas boosting at the contraction/effector memory phase of a T cell response may provide a better effect (39–41). However, for a heterologous boosting antigen (ESAT-6), which is absent in BCG, these mechanisms may neither be relevant nor limiting, and therefore it may offer an advantage due to at least not having a detrimental effect caused by apoptosis of already activated T cells. Further, BCG is known to not protect against pulmonary TB and our LP-ESAT-6 subunit vaccine demonstrated promising efficacy against pulmonary *Mtb* infection when given intranasally. Above all, since many of the BCG-primed individuals still develop active *Mtb* infection and TB disease, and one third of the world's population is latently infected with *Mtb*, there is a desperate need to develop post-exposure vaccine for TB.

Our experimental protocol (**Figures 1IA,B**), was designed to test LP-ESAT-6 as both a prophylactic (pre-exposure) and a therapeutic (post-exposure) vaccine in mice primed with BCG. Surprisingly, LP-ESAT-6 subunit vaccine was not able to confer enhanced protection in the pre-exposure schedule (except in liver) but exhibited promising enhancement of protection in the post-exposure schedule when compared to the BCG-alone group (**Figures 1IIA,B**). It is possible that LP-ESAT-6 vaccination post-infection also leads to reduction in *Mtb* loads in BCG unprimed mice, however, this group was not included due to our experimental design focusing on BCG primed animals. The lack of improvement in protection in the pre-exposure schedule was unexpected as in our earlier studies LP-ESAT-6 subunit vaccine by itself conferred significant protection against *Mtb* infection (30) and we were anticipating at least an additive effect. The exact mechanism for this discrepancy is not clear. However, we speculate that priming with BCG modulates and regulates T cells in a way that could not be overcome by LP-ESAT-6 subunit boost prior to infection, whereas upon infection, either T cells get primed due to *de novo* infection with *Mtb* which then get boosted upon administration of the LP-ESAT-6 subunit vaccine or the productive infection allows re-education of recent thymic emigrant (RTE) T cells. These possibilities will be investigated in future studies. It has been shown in a mouse model of *Mtb* infection that recent thymic emigrants contribute

to peripheral T cell responses during acute *Mtb* infection (42). On the other hand, chronic infection with *Mtb* leads to exhaustion of antigen-specific T cells. Further, central tolerance is not a major factor limiting T cell responses during *Mtb* infection, and infection with *Mtb* is capable of supporting the activation of recent thymic emigrants (42), opening the possibility of re-educating them with a subunit booster vaccine. Although BCG vaccine is a live vaccine and expected to replicate in the host, the short duration (14 days) between BCG priming and LP-ESAT-6 subunit vaccine in the pre-exposure schedule in our study is not expected to lead to exhaustion or apoptosis of T cells. Furthermore, since ESAT-6 antigen is not present in the BCG vaccine strain, these mechanisms should not blunt the response induced against ESAT-6 antigen. It is possible that priming with BCG induced $T_{regs}$, which then dampened the induction of responses against LP-ESAT-6. It has been shown earlier that BCG vaccine triggers the specific activation of $CD4^+$ and $CD8^+$ $T_{regs}$ (43–45). However, to what extent the $T_{regs}$ affect boosting with another vaccine is not clear, and we did not evaluate them in our experiments as we performed all of our studies in mice with productive infection with *Mtb*.

The examination of BAL fluid for cytokines demonstrated an interesting pattern in IFN-γ vs. IL-17A production (**Figures 2A,B**). While IFN-γ has been suggested to be the single most important cytokine in protective immunity against *Mtb*, the role of IL-17A could be protective, inflammatory and/or regulatory (46). Compared to healthy people, $CD4^+$ T cells producing IL-22 and IL-17A have been reported to be upregulated in BAL fluids of pulmonary TB patients (47), indicating their probable contribution to anti-mycobacterial responses. Interestingly, the post-exposure boost in our study led to enhanced IFN-γ, IL-17A and IL-22 levels, in contrast to pre-exposure boost, where IL-17A and IL-22 was enhanced in the absence of upregulation in IFN-γ; IL-10 level was reduced proportionally. The exact cellular source of these cytokines in BAL fluids is also not clear and could be from innate and/or adaptive lymphocytes. The examination of BAL infiltrating lymphocytes demonstrated an interesting pattern with a clear distinction between pre-exposure and post-exposure boost (**Figures 3I,II,IIIA,B**). While BCG priming led to increased recruitment of $CD4^+$ T cells compared to unvaccinated mice, boosting with ESAT-$6^+$MPL led to the significant enhancement in $CD8^+$ and $CD4^-CD8^-$ (double negative, DN) T cells infiltrating BAL only in the post-exposure boost regimen. The reason why there was no enhancement in $CD8^+$ and $CD4^-CD8^-$ T cells infiltrating BAL in the pre-exposure boost schedule, is not clear. However, it appears that the increased $CD8^+$ and $CD4^-CD8^-$ T cells in BAL in the post-exposure boost may at least be partially contributing to increased reduction in *Mtb* viable counts (**Figures 1**, **3**). For an intracellular pathogen like *Mtb*, besides cytokine-mediated mechanisms, cytotoxic lymphocytes are expected to play a significant role in removing the infected host cells, thereby clearing the infection (48). Interestingly, BCG vaccine has been known to predominantly induce $CD4^+$ T cells and attempts to broaden the response toward both $CD4^+$ and $CD8^+$ T cell responses have included boosting with recombinant adenovirus vector containing Ag85A and B, and making recombinant BCG more amenable to inducing $CD8^+$ T cell response by including multiple antigens and perfringolysin (49–52). BCG is also known to induce $CD8^+$ $T_{regs}$ (43, 53), which may have compromised the ability of the LP-ESAT-6 boost to enhance $CD8^+$ T cell infiltration in the pre-exposure boost, but which could be overcome by productive infection with *Mtb* resulting in a significant increase in the post-exposure boost. Further, in the post-exposure boost with LP-ESAT-6, there was a significant increase in double negative T cells ($CD3^+CD4^-CD8^-$) in BALs. The expression of early activation marker CD69 on both $CD8^+$ and DN T cells, although increased over unvaccinated mice, was not significantly different compared to the BCG-alone group. Since CD69 is expressed transiently early after activation of T cells, the timing of experiments could have contributed to seemingly no significant increase. The significant enhancement in percentage of DN T cells in BAL upon post exposure boost is interesting, although the current studies did not clearly identify the function of these cells in contributing to the reduction of *Mtb* loads. Double negative T cells have been suggested to be of thymic and extra-thymic origin, prevalent in lungs, liver and genital tract, and may have inflammatory and/or regulatory potential (54). Interestingly, in response to a live vaccine against respiratory infection with intracellular bacteria *Francisella tularensis* in mice, DN T cells were shown to be the major responding T cells (55). DN T cells have been shown to produce IL-17A earlier than CD4 T cells and demonstrate features of antigen-experienced T cells including lower threshold of stimulation, proliferation, cytokine production, and also contribute to clearance of infection (56). Further, IL-10 has been shown to limit the expansion of DN T cells (57). Our results also demonstrated a reciprocal regulation of IL-10 and DN T cells in BALs in post-exposure boosted mice (**Figures 2**, **3**). Double negative memory T cells in lungs with functional properties similar to $CD8^+$ T cells have also been shown to react to influenza virus infection. Further, they were shown to express CD69, representing the activated memory phenotype (58). Overall, our results demonstrated an enhancement of $CD8^+$ and DN T cells in BAL upon post-exposure boosting with LP-ESAT-6±MPL, which may have contributed to increased reduction in *Mtb* loads.

Proliferation and expansion of antigen-specific T cells is a signature of the activation of adaptive immunity. In gross splenocyte proliferation assays, the recall response to purified protein derivative (PPD) did not show an enhancement in the LP-ESAT-6-boosted group when compared to BCG alone. However, recall response to individual LPs of the LP-ESAT-6 subunit vaccine was substantially increased in the post-exposure group, compared to pre-exposure group (**Figure 4**). Addition of MPL further enhanced this effect (**Figure 4**). Our results demonstrated that the T cell proliferation response to ESAT-6 was very poor in the pre-exposure group where LP-ESAT-6 was given 14 days after BCG priming (**Figure 4II**). This result was in contrast to our earlier studies where LP-ESAT-6±MPL immunized mice, which were not primed with BCG, showed excellent T cell responses against LP-ESAT-6

(30); this was likely due to immune-modulation induced by priming with BCG vaccine. We observed that proliferation in response to PPD recall was not affected in both pre- and post-exposure boost with the subunit compared to the BCG-alone group. But the proliferative response to LPs became very highly exaggerated in the post-exposure boost, further indicating that LP-ESAT-6 subunit vaccine was not efficient in boosting the BCG priming *per se* but either could re-educate the *Mtb*-specific response after infection or that the productive infection could overcome the BCG-induced regulatory effects.

Analyses of antigen-dependent IFN-γ and IL-10 production in $CD4^+$ and $CD8^+$ T cells from *ex vivo*-stimulated splenocytes of BCG-alone and LP-ESAT-6 subunit vaccine boost groups (**Figures 5I,IIA,B**) demonstrated clear superiority of post-exposure LP-ESAT-6 subunit vaccine in inducing IFN-γ producing $CD8^+$ T cells, compared to pre-exposure boost. IFN-γ-producing $CD8^+$ T cells have been demonstrated to be efficient effectors for intracellular pathogens (59). Intriguingly though, there also was a significant increase in IL-10-producing $CD8^+$ T cells (**Figure 5II**). In a mouse model of coronavirus infection-induced encephalitis, IL-10 produced by $CD8^+$ T cells has been demonstrated to reflect cytotoxic T lymphocytes with a superior effector (cytolytic) function (60). Unlike IL-10 production by $CD4^+$ T cells, which have been shown to be at least partially associated with persistent infections, IL-10 producing $CD8^+$ T cells have been shown in acute respiratory infections with influenza virus, respiratory syncytial virus and simian virus (61–63).

Granzyme B, produced by activated innate and adaptive cytotoxic lymphocytes, is a determinant of cytotoxic granules and perforin-dependent cytolytic activity. Clearance of an intracellular pathogen such as *Mtb* would be highly facilitated by activation of cytotoxic lymphocytes. We conducted GrB expression analyses in splenocytes directly obtained from the animals without *ex vivo* culturing, so as to avoid non-specific *ex vivo* activation of effector lymphocytes. It has been reported that cytokines such as IL-2 and IL-15 can stimulate GrB expression in T cells even without antigen stimulation (64). Our examination of intracellular GrB expression in splenocytes demonstrated that boosting with LP-ESAT-6 subunit vaccine increased GrB expression in $CD8^+$ T cells compared to BCG vaccination alone in the post-exposure boost group but not in the pre-exposure group (**Figure 6**). In contrast, GrB expression on NK ($CD3^-CD49b^+$) and NKT ($CD3^+CD49b^+$) cells was upregulated following BCG vaccination compared to the unvaccinated group, which showed a trend toward further enhancement with LP-ESAT-6 boosting in both pre and post-exposure schedules (**Figure 6**). These results indicate that boosting with LP-ESAT-6 may be inducing memory-like effects in NK and NKT cells. It has been reported that homologous BCG boosting in adult humans, besides stimulating $CD4^+$ and $CD8^+$ T cells, also stimulates memory NK and NKT cells expressing GrB as an antimycobacterial effector molecule (33).

A key question in attempts to generate a more effective TB vaccine is the quality and quantity of immune responses required for optimal protection against TB. Our studies clearly suggest that mucosal boosting with LP-ESAT-6 subunit vaccine allows the expansion of immune responses with respect to the target mycobacterial antigen, and overall quality and quantity, compared to BCG vaccine alone. A less-explored but crucial question is the timing of the boost after BCG priming. Our studies have shed important light on this key question and perhaps partially help to explain the many unsuccessful attempts directed toward a prime-boost strategy for TB vaccine. Further work is needed to determine the detailed reasons/mechanisms for the inability of a pre-exposure boost with LP-ESAT-6 subunit vaccine to enhance protection compared to BCG alone. Nevertheless, they represent a significant advancement demonstrating that scheduling the subunit vaccine boost post-exposure, and therefore re-educating the T cells, may be a promising future path toward a successful TB vaccine. Additionally, these studies provide immense promise for development of a subunit vaccine for BCG-vaccinated individuals who still get active TB infection.

## AUTHOR CONTRIBUTIONS

RK, NG, DK, and BA: Conception; NG, SG, SV, and RK: Experimental planning and execution; NG, RK, and BA: Data analyses and manuscript writing.

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# The Host Microbiota Contributes to Early Protection Against Lung Colonization by *Mycobacterium tuberculosis*

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Tuberculosis (TB), caused by the airborne bacterial pathogen *Mycobacterium tuberculosis*, remains a major source of morbidity and mortality worldwide. So far, the study of host-pathogen interactions in TB has mostly focused on the physiology and virulence of the pathogen, as well as, on the various innate and adaptive immune compartments of the host. Microbial organisms endogenous to our body, the so-called microbiota, interact not only with invading pathogens, but also with our immune system. Yet, the impact of the microbiota on host defense against *M. tuberculosis* remains poorly understood. In order to address this question, we adapted a robust and reproducible mouse model of microbial dysbiosis based on a combination of wide-spectrum antibiotics. We found that microbiota dysbiosis resulted in an increased early colonization of the lungs by *M. tuberculosis* during the first week of infection, correlating with an altered diversity of the gut microbiota during this time period. At the cellular level, no significant difference in the recruitment of conventional myeloid cells, including macrophages, dendritic cells and neutrophils, to the lungs could be detected during the first week of infection between microbiota-competent and -deficient mice. At the molecular level, microbiota depletion did not impact the global production of pro-inflammatory cytokines, such as interferon (IFN)γ, tumor necrosis factor (TNF)α and interleukin (IL)-1β in the lungs. Strikingly, a reduced number of mucosal-associated invariant T (MAIT) cells, a population of innate-like lymphocytes whose development is known to depend on the host microbiota, was observed in the lungs of the antibiotics-treated animals after 1week of infection. These cells produced less IL-17A in antibiotics-treated mice. Notably, dysbiosis correction through the inoculation of a complex microbiota in antibiotics-treated animals reversed these phenotypes and improved the ability of MAIT cells to proliferate. Altogether, our results demonstrate that the host microbiota contributes to early protection of lung colonization by *M. tuberculosis*, possibly through sustaining the function(s) of MAIT cells. Our study calls for a better understanding of the impact of the microbiota on host-pathogen interactions in TB. Ultimately, this study may help to develop novel therapeutic approaches based on the use of beneficial microbes, or components thereof, to boost anti-mycobacterial immunity.

**Keywords: microbiota, MAIT cells, macrophage, tuberculosis, IL-17**

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## INTRODUCTION

Tuberculosis (TB) remains a major public health issue, with over 10 million cases each year and 1.7 million deaths in 2016, according to the World Health Organization (WHO Annual report 2017). Understanding the host factors that modulate susceptibility or resistance to *Mycobacterium tuberculosis*, the etiological agent of TB, might help develop novel intervention strategies, including a more efficacious alternative to the Bacillus Calmette-Guérin (BCG) vaccine, as well as, host-directed therapies (1).

A key parameter controlling susceptibility to TB is the balance between the virulence of the infecting *M. tuberculosis* strain, and the immune status of the infected host. To date, the parameters involved in this balance mostly included the host genetic background, gender, age, nutrition status, and the occurrence of human immunodeficiency virus (HIV) co-infection or diabetes co-morbidity (2). The host microbiota, which represents the entirety of the microbial communities present on the skin and the mucosal surfaces of the body, might constitute an important but yet poorly explored additional factor influencing *M. tuberculosis* interaction with its host, and susceptibility to TB (3, 4).

The microbiota includes mostly bacteria, and profoundly influences human health and disease (5, 6). In mice, some evidence demonstrate its beneficial role, including resistance to infection by pathogenic microorganisms (7, 8). For example, segmented filamentous bacteria (SFB) present in the gut are sufficient to induce the accumulation of T helper (Th)17 cells in the lamina propria and to protect against infection by intestinal pathogens, such as *Citrobacter rodentium* (9). SFB were also reported to promote host resistance to pathogens at distal tissue sites, including the lung (10), which is now commonly referred to as the "gut-lung axis" (11, 12). In addition, the host microbiota is known to promote tolerance and immune homeostasis, such as through sustaining the development of FoxP3$^+$ regulatory T cells (Tregs) in the colon (13, 14). Although the microbiota predominates in the gut, in which it has been mostly studied, it is present in all other mucosal surfaces of the body. The lung is no exception and it has become increasingly apparent over the past years that, although considered sterile for decades, the mammalian respiratory tract harbors a genuine microbiota as well (15–17). Yet, the exact role that these microbial communities play in susceptibility or resistance to respiratory diseases still remains largely enigmatic.

In TB, variations in the composition of the host intestinal and lung microbiota have been reported in various settings, including in patients and in animal models (18–20). However, from a functional viewpoint, whether the host microbiota contributes to resistance or susceptibility to TB, and how it does so, is still poorly understood. In particular, it is not known whether the microbiota modulates lung immunity in response to *M. tuberculosis* infection; a question that we have addressed in the present study.

To explore the role of the microbiota in physiological processes, including immunity, two mouse models are classically used, namely germ-Free (GF) mice, which are born and raised in sterile conditions, and mice treated with various cocktails of broad-spectrum antibiotics. Because the microbiota is involved in the education of the immune system, particularly during infancy, it is well-known that GF mice display abnormal immune functions, such as an immature and underdeveloped lymphoid system (21). This is not the case in antibiotics-treated mice, which are only transiently depleted of their microbiota and possess a mature immune system (22).

Here, we adapted an antibiotic treatment-based model of microbiota dysbiosis (23) in order to study the role of the microbiota on host susceptibility to *M. tuberculosis* infection and anti-mycobacterial immunity. We found that dysbiosis following antibiotic treatment strongly altered the diversity of the gut microbiota, but not that of the lung community, and increased early lung colonization by the TB bacillus. These phenotypes were reversed after microbial reconstitution of antibiotics-treated mice through fecal transplantation (FT). Strikingly, impaired protection against *M. tuberculosis* infection in antibiotics-treated mice correlated with a decreased number of lung mucosal associated invariant T (MAIT) cells, a population of innate-like lymphocytes associated with protection against pulmonary pathogens (24, 25) and whose development is known to be microbiota-dependent (26). Moreover, MAIT cells in antibiotics-treated mice produced less interleukin (IL)-17A, and MAIT cells from FT mice proliferated more than those from antibiotics-treated mice following infection.

Altogether, our results reveal that the presence of a healthy microbiota promotes host resistance to early colonization by *M. tuberculosis*, possibly through sustaining the function(s) of MAIT lymphocytes.

## MATERIALS AND METHODS

### Animal Models of Dysbiosis, Microbiota Restoration and *M. tuberculosis* Infection

Six-to-eight week-old female C57BL/6 mice were purchased from Charles River Laboratories. Mice were given a combination of broad-spectrum antibiotics *ad libitum* in drinking water. Mice were given ampicillin (1 g/L, Sigma Aldrich), vancomycin (500 mg/L, Alfa Aesar) and neomycin sulfate (1 g/L, Fisher) during 2 weeks, and this treatment was complemented with metronidazole during the third (0.5 g/L, Alfa Aesar) and fourth (1 g/L) weeks. Antibiotics-containing water was changed twice a week, and treatment was stopped 2 days before *M. tuberculosis* infection or fecal transplantation. Microbial depletion was confirmed by checking for the absence of live microorganisms in the feces using the colorimetric Releasat biological indicator (Mesa Labs) at 37°C, in either aerobic or anaerobic conditions. Following microbial depletion, mice were maintained in a sterile isolator during all the experiment. Recolonization with a complex microbiota was performed through a single gavage with the intestinal content from two non-treated mice. Repopulation occurred after 1 week, as assessed using the colorimetric Releasat biological indicator. Control mice received normal water during all experiment.

*M. tuberculosis* (H37Rv) was grown in 7H9 liquid medium (Difco) supplemented with ADC (Middlebrook) and tyloxapol

0.05%. For infection, mice were anesthetized using isoflurane and infected intranasally with $10^3$ colony-forming units (CFUs) of *M. tuberculosis*.

## Preparation of Lungs Homogenates for Microbiological, Molecular, and Immunological Analyses

Mice were sacrificed, lungs were harvested aseptically, homogenized using a gentleMACS dissociator (C Tubes, Miltenyi), and incubated with DNAse I (0.1 mg/mL, Roche) and collagenase D (2 mg/mL, Roche) during 30 min at 37°C under 5% $CO_2$. *M. tuberculosis* bacterial load was determined by plating serial dilutions of the lung homogenates onto 7H11 solid medium (Difco) supplemented with OADC (Middlebrook). The plates were incubated at 37°C for 3 weeks before bacterial CFUs scoring. Lungs homogenates were filtered on 40 μm cell strainers and centrifuged at 329 × *g* during 5 min. Supernatants were conserved for cytokine content analysis. A part of the cellular pellet was conserved in TRIzol reagent for cellular RNA analysis. In the remaining fraction, red blood cells were lysed in 150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.2) for immunological staining. For microbial composition analysis, lungs were harvested aseptically, homogenized using a gentleMACS dissociator (M Tubes, Miltenyi), and filtered on 40 μm cell strainers.

## *In vitro* Stimulation, Antibody Staining, and Flow Cytometry Analysis

Single-cell suspensions from lungs homogenates were prepared as described above. In case of intracellular staining of lymphocytes, cytokine production was stimulated in RPMI (Difco) supplemented with phorbol myristate acetate (PMA) and ionomycin (50 ng/mL and 1 μg/mL, respectively, Sigma Aldrich), and blocked with brefeldin A and monensin (5 μg/mL, Becton Dickinson) for 4 h at 37°C under 5% $CO_2$. In case of intracellular staining of myeloid cells, cytokine production was stimulated in RPMI (Difco) supplemented with lipopolysaccharide (LPS) (1 μg/mL, Sigma Aldrich), and blocked with brefeldin A (5 μg/mL, Becton Dickinson) for 4 h at 37°C under 5% $CO_2$. Cells were stained with Zombie Aqua Viability for dead cells exclusion and non-specific binding to Fc receptors was blocked by incubating the cells with anti-CD16/32 antibodies (BioLegend). For MAIT cell staining, mouse MR1-5-OP-RU tetramer labeled with PE and MR1-Ac-6-FP tetramer labeled with APC were incubated for 45 min at room temperature in the dark. The MR1 tetramer was obtained from the NIH Tetramer Core Facility. For extracellular staining, cells were incubated with a panel of surface antibodies for 20 min at 4°C in the dark. For intracellular staining, cells were fixed and permeabilized with Fixation and Permeabilization Reagents (eBioscience) and subsequently stained with the antibodies of interest for 40 min at room temperature in the dark. All antibodies used are referenced in **Supplementary Table 1**. Cell staining was analyzed using LSR II or LSR Fortessa flow cytometers (BD) and FlowJo software version V10. Cells were first gated on singlets (FSC-H vs. FSC-W and SSC-H vs. SSC-W) and live cells before further analyses. An average of 10–25,000 alveolar macrophages were sorted using a FACSAria Fusion cytometer. Sorted cells were incubated in TRIzol reagent to isolate RNA.

## Bacterial DNA and Cellular RNA Isolation, and Real Time-qPCR

DNA from fecal samples and lungs homogenates were extracted according to manufacturer's instructions (QIAamp Fast DNA Stool Mini Kit, Qiagen). DNA was extracted from the fecal samples, using lab tissue-specific technique. The 16S rDNA present in the samples was measured by Real Time-qPCR (RT-qPCR) in triplicate and normalized using a plasmid-based standard scale. The amount of bacterial DNA was assessed using the "Universal 16S Real Time qPCR" workflow established by Vaiomer (Vaiomer SAS, Labège, France). In the case of detection of bacterial phylums from lungs and feces DNA, RT-qPCR was performed using phylum-specific primers [**Supplementary Table 2**, (8, 27)] using TB green Premix Ex Taq (Takara), according to the manufacturer's protocol. All RT-qPCR reactions were carried out using a 7,500 RT-PCR System, and data were analyzed using the 7,500 Software version v2.3 (Applied Biosystems). Values were normalized using the universal 16S rRNA gene, and expressed as a fold change in experimental samples (antibiotics-treated mice or reconstituted mice) relative to control samples (non-treated mice). RNA from lungs homogenates and sorted cells were extracted using TRIzol reagent (Ambion) and RNeasy spin columns according to manufacturer's instructions (RNeasy kit, Qiagen). RNA were reverse transcribed into cDNA using M-MLV Reverse transcriptase (Invitrogen). RT-qPCR was performed using gene-targeted primers (**Supplementary Table 2**) as described above. Values were normalized using the *Hprt* housekeeping gene, and expressed as a fold change in experimental samples (antibiotics-treated mice) relative to control samples (non-treated mice).

## Cytokine Quantification

Cytokines present in the supernatants from lung homogenates were quantified using a customized LEGENDplex™ Mouse Inflammation Panel and a LSRII flow cytometer, using manufacturer's instructions (BioLegend). CCL20 was detected by ELISA (Invitrogen), according to the manufacturer's instructions.

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 software. An Agostino and Pearson normality test was performed to determine whether samples followed a normal distribution. Data following a normal distribution are represented as mean ± SD; data following a non-normal distribution were represented as median with interquartile range. Unpaired *t*-test (for normal data) or Mann-Whitney (for non-normal data) were performed when two samples were compared; ANOVA (for normal data) or Kruskal-Wallis (for non-normal data) tests were performed when more than two samples were compared. For all analyses, * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, and **** indicates $P < 0.0001$. Data obtained from FT mice were included in the figures only when statistical difference between antibiotics-treated and control mice were detected.

# RESULTS

## Microbiota Dysbiosis Increases Early Lung Colonization by *M. tuberculosis*

In order to assess whether the microbiota is involved in the control of *M. tuberculosis* infection, we adapted a model of microbiota dysbiosis consisting in the administration of a cocktail of broad-spectrum antibiotics composed of ampicillin, neomycin sulfate, metronidazole, and vancomycin over the course of 4 weeks. This procedure has previously been shown to deplete all detectable commensals and bacterial products (23). We observed that adding metronidazole at the start of antibiotic treatment resulted in animal morbidity and mortality, presumably due to dehydration caused by a reluctance of the mice to drink metronidazole-containing water (28). Thus, the protocol was modified and metronidazole was added only during the last 2 weeks of antibiotic treatment (**Figure 1A**). In this way, mice did not suffer from dehydration and did not lose weight during the course of treatment (**Figure 1B**). Microbial depletion before *M. tuberculosis* infection was confirmed by quantification of total 16S rDNA in feces (**Figure 1C**) and by inoculation of fecal samples in reporter liquid medium incubated under aerobic (**Figure 1D**) or anaerobic (data not shown) conditions. Bacterial diversity was analyzed at the phylum level in control and antibiotics-treated (ABX) mice. *Bacteroidetes* and *Firmicute*s, the major phylums present in the gut microbiota (29) were strongly reduced, while *Beta-proteobacteria* were increased in the gut of ABX mice, compared to control animals (**Figure 1E**). An increase in *Proteobacteria* is known to be associated with gut inflammation and a bad prognostic during intestinal diseases (30, 31). We also verified if the lung community could be altered by antibiotics treatment. Colorimetric analyses did not reveal the presence of viable bacteria in the broncho-alveolar lavages (BALs) of normal, specific pathogen-free (SPF) mice (data not shown). A similar trend was observed in the lungs for the major phyla (17, 32), although statistical significance was reached only for Proteobacteria (**Figure 1F**).

In order to confirm the direct contribution of the host microbiota to the observed phenotypes, we generated groups of ABX mice reconstituted with the intestinal content from untreated control mice through fecal transplantation (FT). Some features of normal mice were restored in ABX mice after FT (**Figures 1D,E**). In particular, the diversity of the major phylums (e.g., *Bacteroidetes* and *Firmicutes*) was similar between control and FT mice (**Figure 1E**).

We next verified that the antibiotic treatment did not alter the level and functionality of several lung immune cell populations at the steady state (**Supplementary Figure 1**). The total numbers of macrophages, dendritic cells, neutrophils, natural killer (NK), CD4 T lymphocytes, and B cells were quantified by flow cytometry. We found no difference in the number of these cells (**Supplementary Figure 1A**), and in the ability of macrophages and CD4 T cells to produce cytokines (**Supplementary Figure 1B**) between control and ABX mice.

Control (non-ABX) and ABX mice were infected intranasally with *M. tuberculosis* H37Rv 2 days after cessation of antibiotics treatment, and the lungs of the infected animals were collected 7, 14, and 21 days after infection, homogenized and plated onto agar medium for colony-forming unit (CFUs) scoring. Compared to control mice, ABX mice contained about twice as many bacteria in their lungs at day 7 post-infection (p.i., **Figure 2A**). This phenotype was only transient since there was no difference in the lung bacterial load between control and ABX mice after 14 or 21 days p.i. (**Figure 2B**); similarly no differences in bacterial loads were observed in the spleen of control vs. ABX animals 21 days p.i. (data not shown). This phenotype correlated with a reduced amount of total gut microbial rDNA in ABX mice 7 days p.i, while this difference was abrogated 21 days p.i. (**Figure 2C**), indicating that ABX mice were recolonized following cessation of the antibiotic treatment. Yet, microbial recolonization over time in ABX mice was only partial, which correlated with the presence of an enlarged caecum in ABX mice, as previously observed in GF mice (21) (**Figure 2D**). The relative abundance of *Firmicutes* was no more different in infected ABX and control mice 7 days p.i., however the relative abundance of *Bacteroidetes* and *Proteobacteria* was still altered in ABX animals at this time point (**Figure 2E**), which likely reflected ongoing recolonization.

Reintroduction of a complex microbiota (FT) restored all the examined phenotypes, i.e., prevented the increased lung colonization by *M. tuberculosis* 7 days p.i. (**Figure 2A**), caecum enlargement (**Figure 2C**), and restored, at least partially, microbial diversity (**Figure 2E**).

Altogether, these data demonstrate that the host microbiota contributes to early resistance against lung colonization by *M. tuberculosis*.

## Microbiota Dysbiosis Correlates With Impaired Functions of MAIT Cells During Early Lung Colonization by *M. tuberculosis*

In order to understand the increased susceptibility of ABX mice to *M. tuberculosis*, we next quantified the early expression and production of several key inflammatory factors known to be involved in immunity to TB (33). Seven days after *M. tuberculosis* infection, we found no difference in the mRNA expression and/or protein production of TNFα, IL-1β, IL-6, transforming growth factor (TGF)β, interferon (IFN)γ, IL-17A and IL-12 (p70) in the lungs of ABX vs. control mice (**Supplementary Figure 2**). We also quantified the expression of cathelicidin-related antimicrobial peptide (CRAMP), whose production is known to be stimulated by the microbiota (34), and found no difference between ABX and control mice (**Supplementary Figure 2A**).

We next analyzed the early accumulation of innate myeloid and lymphoid cell populations in the lungs of infected ABX and control mice by flow cytometry. We found no difference in the number of macrophages, dendritic cells, neutrophils and natural killer (NK) cells between the two groups of animals 7 days p.i. (**Supplementary Figure 3**). Macrophages are known to be the main host cell target for *M. tuberculosis*, and are able to control mycobacterial proliferation upon activation by inflammatory cytokines, including TNFα (33). In particular, alveolar macrophages (AMs) were recently reported to sustain

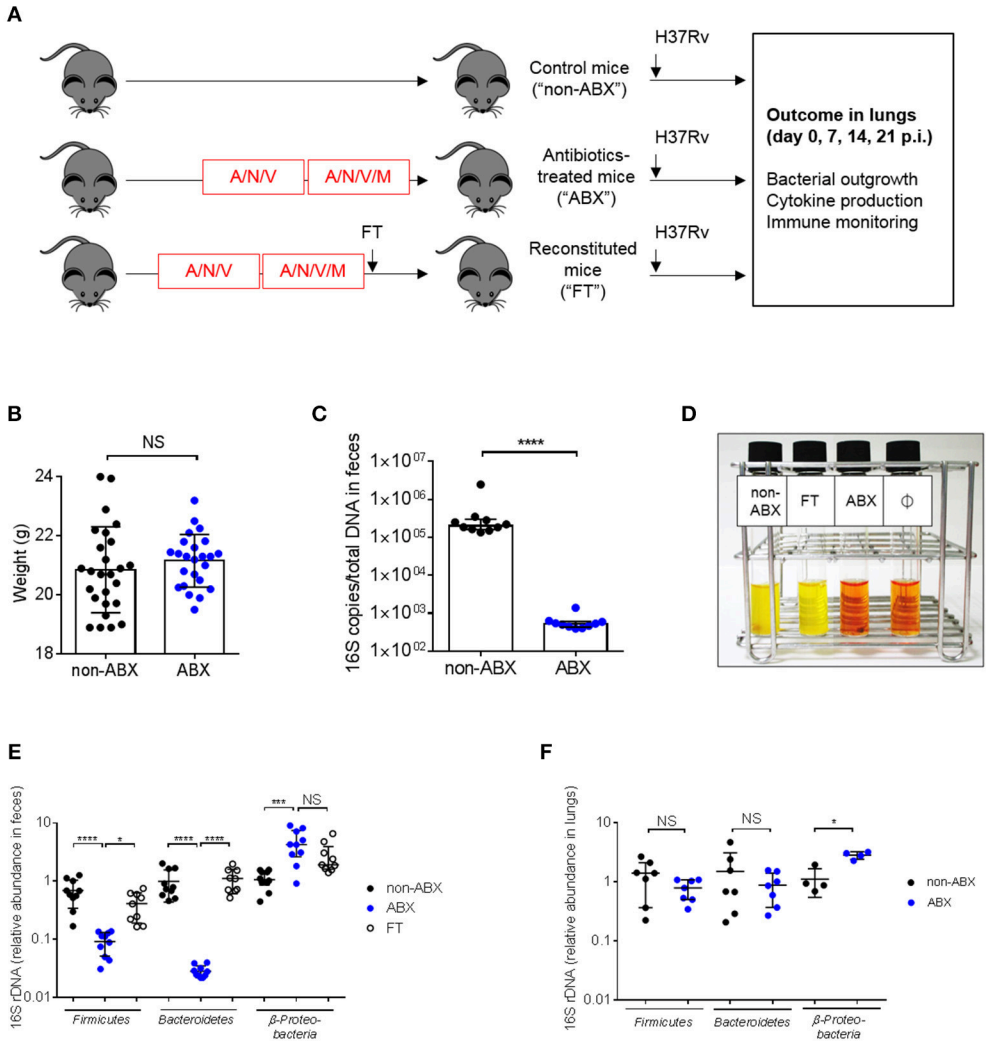


**FIGURE 1 |** Mouse model to study the impact of microbiota dysbiosis during *M. tuberculosis* infection. **(A)** Experimental design. Six-to-eight weeks old C57BL/6 female mice were treated for 4 weeks with a cocktail of broad-spectrum antibiotics (ampicillin, A, neomycin sulfate, N, vancomycin, V, and metronidazole, M, which was added during the last 2 weeks) in drinking water (ABX) or were given water without antibiotics (non-ABX). Microbiota reconstitution was realized through fecal transplantation (FT) after a single oral gavage with the intestinal content from 2 control mice and occurred during 1 week. The three groups of mice received an intranasal challenge of *M. tuberculosis* H37Rv ($10^3$ CFUs/mouse). Bacterial outgrowth, cytokine production and immune monitoring were determined 0, 7, 14, or 21 days post-infection (p.i.) in the lungs. **(B)** Mouse weight in the ABX vs. non-ABX groups at the time of *M. tuberculosis* infection. Data from 4 independent experiments ($n$ = 4–14 mice/group/experiment) were pooled and the graph represent mean ± SD of the pooled data. Data were analyzed using the unpaired Student's $t$-test. **(C)** The amount of bacterial rDNA was assessed using the "Universal 16S RT-qPCR" kit, calculated as 16S rDNA gene copies per total DNA extracted from feces of non-ABX and ABX groups before infection. **(D)** Colorimetric evaluation of the presence of microbial flora in the feces of the different groups of animals before infection; Ø indicates negative control. Yellow color indicates the presence of live microorganisms. Image depicted in **(D)** is representative of 3 independent experiments. Fecal **(E)** and lung **(F)** microbiota were detected by RT-qPCR using phylum-specific primers. Relative Ct value compared to universal 16S rRNA gene Ct value (ΔCt) and the mean of ΔCt values in the control group (ΔΔCt). **(E, F)** data from 2–3 independent experiments ($n$ = 2–4 mice/group/experiment) were pooled. The graph **(E)** show mean ± SD (*Firmicutes* and *Bacteroidetes*) and median with interquartile range (*Proteobacteria*) of the pooled data. Data were analyzed using ANOVA (*Firmicutes* and *Bacteroidetes*) and the Kruskal-Wallis test (*Proteobacteria*). The graph **(F)** show median with interquartile range of the pooled data. Data were analyzed using the Mann-Whitney test. NS, not significant; *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

mycobacterial proliferation while interstitial macrophages (IMs) are more bactericidal (35). Here we found no difference in the number of AMs and IMs between ABX and control mice 7 days p.i. (**Figures 3A–C**); however after sorting the AM population, we found that these cells expressed less TNFα mRNA in ABX mice, as compared to in control animals (**Figure 3D**, left panel). Yet, the production of TNFα by AMs in ABX and control mice was similar at the protein level, as assessed by flow cytometry (**Figure 3D**, right panel); similar results were obtained in IMs (data not shown).

Since the microbiota is known to play a part in the effector functions of innate-like lymphocytes (36), we next explored the

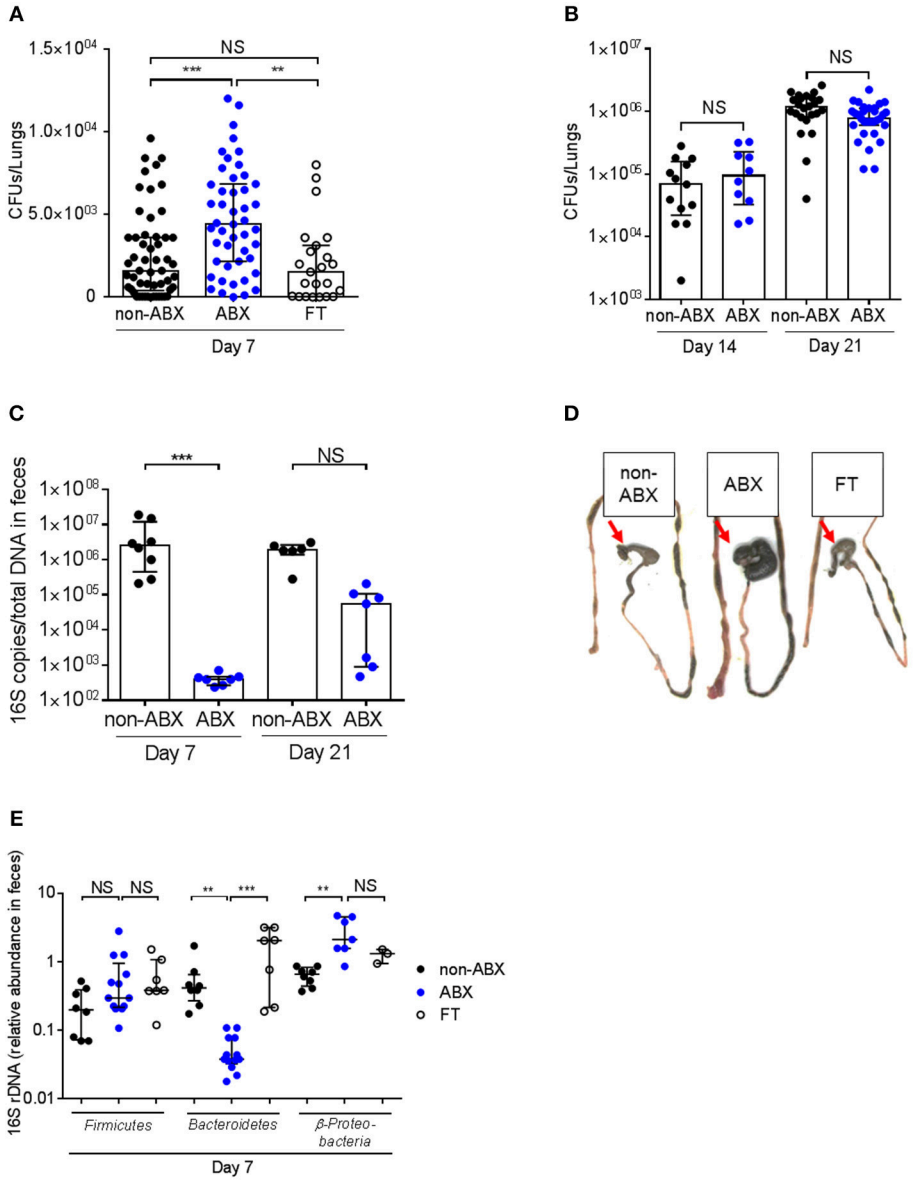


**FIGURE 2 |** The host microbiota protects against early lung colonization by *M. tuberculosis*. **(A)** *M. tuberculosis* bacterial load (CFUs) was measured in the lungs of control (non-ABX), ABX and FT mice 7 days p.i. **(B)** *M. tuberculosis* bacterial load was measured in the lungs of control (non-ABX) and ABX mice 14 or 21 days p.i., Data from 3–6 **(A)** or 2 **(B)** independent experiments ($n$ = 4–8 mice/group/experiment) were pooled and the graphs show median with interquartile range of the pooled data. Data were analyzed using the Kruskal-Wallis test. **(C)** The amount of bacterial rDNA was assessed using the "Universal 16S RT-qPCR" kit, calculated as 16S rDNA gene copies per total DNA extracted from feces of non-ABX, ABX, and FT groups 7 days p.i. **(D)** Morphology of the caecum in the non-ABX, ABX, and FT groups 7 days p.i., Red arrow indicates the caecum. Image depicted in **(D)** is representative of 3 independent experiments. **(E)** Fecal microbiota from non-ABX, ABX and FT groups was analyzed by RT-qPCR using phylum-specific primers 7 days p.i., Data were analyzed as in **Figure 1**. **(C,E)** Data from 1–4 independent experiments ($n$ = 2–5 mice/group/experiment) were pooled and the graphs show median with interquartile range of the pooled data. Data were analyzed using the Kruskal-Wallis test. NS, not significant; $^{**}p < 0.01$; $^{***}p < 0.001$.

accumulation of NKT cells, γ/δ T cells and MAIT cells in the lungs of ABX and control mice during *M. tuberculosis* infection. The total number and percentage of NKT (NK1.1+CD3+) and γ/δ T (TCRγ/δ+CD3+) lymphocytes were similar in the lungs of infected ABX and control mice (**Supplementary Figure 4**). Strikingly, the only difference we observed between the two groups of mice was related to the accumulation of mucosal-associated invariant T (MAIT) cells, characterized by their MR1-5-OP-RU tetramer+TCRβ+ phenotype (**Figure 4A**), with a marked decrease in the total number and percentage of these cells in ABX mice, compared to in control mice 7 days p.i. (**Figures 4B,C**). This phenotype was reversed in FT mice

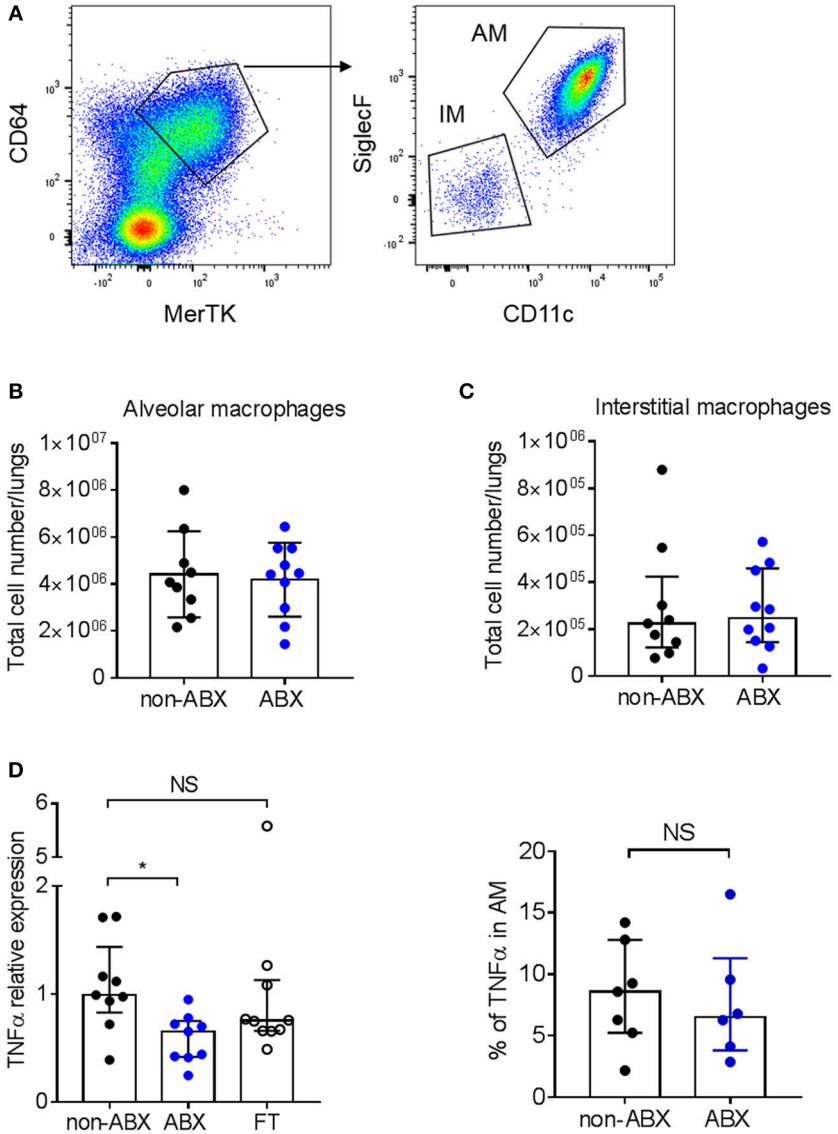


**FIGURE 3 |** Microbiota dysbiosis modify TNFα mRNA expression but not protein production by alveolar macrophages. **(A)** Gating strategy to analyze alveolar ($MerTK^+CD64^+SiglecF^+CD11c^+$) and interstitial ($MerTK^+CD64^+SiglecF^-CD11c^-$) macrophages by flow cytometry. The total number of **(B)** alveolar and **(C)** interstitial macrophages was quantified by flow-cytometry in lung homogenates from *M. tuberculosis*-infected ABX vs. non-ABX mice 7 days p.i., Data from 2 independent experiments ($n = 4$–5 mice/group/experiment) were pooled and the graphs show mean ± SD **(B)** or median with interquartile range **(C)**. Data were analyzed using the Student's *t*-test **(B)** or the Mann-Whitney test **(C)**. **(D)** Left panel, $MerTK^+CD64^+SiglecF^+CD11c^+$ alveolar macrophages were sorted and the expression of TNFα was measured by RT-qPCR in cells from non-ABX, ABX, or FT mice 7 days p.i., Gene expression represents relative Ct value compared to *Hprt* Ct value (ΔCt) and the mean of ΔCt values in the control group (ΔΔCt). Right panel, cytometry analysis of the percentage of TNFα-producing $MerTK^+CD64^+SiglecF^+CD11c^+$ alveolar macrophages following *in vitro* stimulation by LPS. Data from 1–2 independent experiments ($n = 4$–5 mice/group/experiment) were pooled and the graphs show median with interquartile range of the pooled data and were analyzed using the Mann-Whitney test $^*p < 0.05$.

(**Figures 4B,C**) and was no longer observed at later time points p.i. (**Figure 4B**), in line with our findings for lung bacterial loads (**Figures 2A,B**). Altogether, these data suggest that the host microbiota promotes early resistance to *M. tuberculosis* infection through sustaining the accumulation of MAIT cells in the lungs.

We next sought to assess whether the decreased number of MAIT cells observed in the lungs of ABX mice reflected a diminished recruitment and/or proliferation of these cells following *M. tuberculosis* infection. We found that MAIT cells from ABX mice expressed less Ki67, an intracellular marker of cell cycling and proliferation, than their control or FT counterparts (**Figure 4D**). The tissue-homing chemokine receptor CCR6 and the chemokine CCL20 (referred to as the CCR6/CCL20 axis) are known to be involved in the recruitment of leukocytes, including T lymphocytes, from the intestine and other organs to the lungs (37, 38), and were found to be

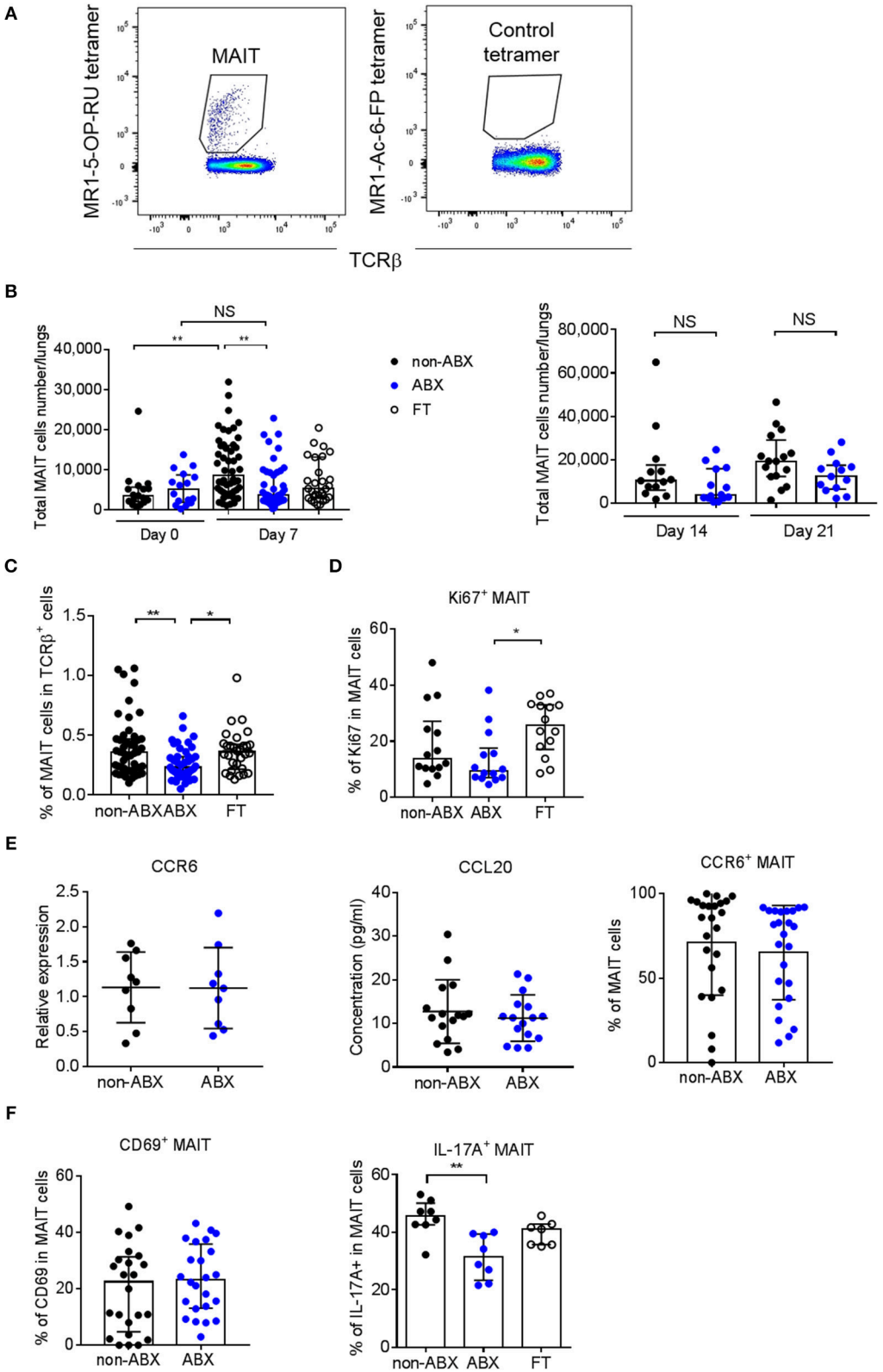


**FIGURE 4 |** MAIT cell accumulation in the lungs and IL-17A-production are decreased early after *M. tuberculosis* infection in microbiota-altered mice. **(A)** Gating strategy to analyze MAIT cells (MR1-5-OP-RU tetramer$^{+}$TCRβ$^{+}$) by flow cytometry. A MR1–Ac-6-FP tetramer$^{+}$TCRβ$^{+}$ staining was used as a control. **(B,C)** Total number **(B)** and percentage **(C)** of MAIT cells in the lungs of control (non-ABX) ABX, and FT mice 0 and 7 (**B**, left panel), 14 and 21 (**B**, right panel), and 7 **(C)** days p.i., *(Continued)*

**FIGURE 4 |** Data from 2–6 independent experiments ($n$ = 4–8 mice/group/experiment) were pooled and the graphs show median with interquartile range of the pooled data. Data were analyzed using the Kruskal-Wallis test. **(D)** Intracellular analysis of Ki67 expression in MAIT cells from non-ABX, ABX, and FT mice 7 days p.i., by flow cytometry. Data from 2 independent experiments ($n$ = 4–8 mice/group/experiment) were pooled and the graphs show the median with interquartile range of the pooled data. Data were analyzed using the Kruskal-Wallis test. **(E)** The expression of CCR6 (left) in whole lung homogenates or in MAIT cells (right), and the production of CCL20 in lung supernatant (middle), were measured by RT-qPCR (left), flow cytometry (right) and ELISA (middle) in ABX and control (non-ABX) mice 7 days p.i., RT-qPCR data were analyzed as in **Supplementary Figure 2**. Data from 3 (left) or 4 (middle and right) independent experiments ($n$ = 3–7 mice/group/experiment) were pooled and the graphs show mean ± SD of the pooled data. Data were analyzed using the Student's $t$-test. **(F)** Cytometry analysis of percentage of activated (MR1-5-OP-RU tetramer$^+$TCRβ$^+$CD69$^+$) MAIT cells (left), and IL-17A-producing (MR1-5-OP-RU tetramer$^+$TCRβ$^+$IL-17A$^+$) MAIT cells (right) in lungs of control (non-ABX), ABX and FT mice 7 days p.i., Data from 4 (left) or 1 representative of 3 (right) independent experiments ($n$ = 5–8 mice/group/experiment) were pooled and the graphs show mean ± SD of the pooled data. (left) or median with interquartile range (right) of the pooled data. Data were analyzed using the unpaired Student's $t$-test (left) or the Kruskal-Wallis test (right). NS, not significant; $^*p < 0.05$; $^{**}p < 0.01$.

altered during microbiota dysbiosis in response to pulmonary infection (39). Because MAIT cells express CCR6 (40), we next determined if the CCR6/CCL20 axis was perturbed in ABX mice. We found no difference in global expression of the CCR6 transcript and production of the CCL20 protein in the lungs between *M. tuberculosis*-infected ABX and control mice 7 days p.i. (**Figure 4E**). Flow cytometry analysis revealed no difference in CCR6 expression in MAIT cells between the two groups of mice (**Figure 4E**). These data indicate that if microbiota dysbiosis impairs the early recruitment of MAIT cells to the lungs following *M. tuberculosis* infection, this does not rely on the CCR6/CCL20 axis. Moreover, they reveal that the proliferation of MAIT cells is reduced in the lungs of ABX mice.

Finally, we sought to evaluate whether the activation and effector functions of MAIT cells could be altered in the lungs of *M. tuberculosis*-infected ABX mice. Activated MAIT cells are known to express CD69 (25) and to produce various cytokines, including IL-17A (24, 41). In the mouse, lung MAIT cells mostly produce IL-17A (41). We found that although CD69 expression by MAIT cells was similar in ABX and control mice, MAIT cells from ABX animals produced less IL-17A, which was partially restored in FT mice 7 days p.i. (**Figure 4F**). The number of, and Ki67 expression by MAIT cells in the spleen did not differ between ABX and control animals. Altogether, our data reveal that a healthy microbiota is required for pulmonary MAIT cell functions, which correlates with an improved early control of *M. tuberculosis* growth in the lungs.

## DISCUSSION

Our results demonstrate that the microbiota contributes to early host defense against lung colonization by *M. tuberculosis*, possibly *via* effector functions MAIT lymphocytes. Several previous studies reported that *M. tuberculosis* infection results in a perturbation of the composition of the host microbiota in humans, as well as, in experimentally infected animals (18, 20, 42). However, the functional impact of the microbiota on host susceptibility or resistance to TB remains to be fully understood.

In a previous study using a different ABX model, Khan and colleagues reported that antibiotics-treated mice were more susceptible to *M. tuberculosis*, with a higher bacillary load in the lungs 21 days after infection, compared to control mice (43). This correlated with a decrease in IFNγ- and TNFα-producing CD4 T lymphocytes and an increase in FoxP3$^+$ Tregs in the spleen of the infected ABX animals, compared to controls. At first glance, these results would appear to contradict our data since we did not observe any difference in lung bacterial load in ABX vs. control animals at 14 or 21 days p.i., However, in our study antibiotic treatment was stopped 2 days before *M. tuberculosis* infection, while in the study by Khan and colleagues antibiotics were maintained in the drinking water throughout the infection period. It is likely that in our experiments, animals became progressively recolonized following recovery of the endogenous microflora in an uncontrolled manner, and this may explain why we only observed an increased susceptibility of ABX mice at early time-points during infection. Thus, our study and that by Khan et al. do not appear to be contradictory but rather complementary, illustrating the overall protective function of the host microbiota against *M. tuberculosis* infection at early vs. late time points post-exposure. Altogether, these two studies might reveal a previously underappreciated influence of the gut microbiota on host resistance to the TB bacillus through the so-called "gut-lung axis" (44), as observed in other lung infections (11, 45). Another, yet non-mutually exclusive possibility, is that local commensals of the airways (15, 16), which are probably also affected by antibiotics treatment (46), might influence host resistance to TB, which will need further investigation (47). A limitation in our study is that the composition of the microbiota in ABX mice was not monitored over time and that animals were infected only 2 days after cessation of the antibiotics treatment. The microbial composition in ABX mice in the weeks following cessation of antibiotics treatment is unlikely to be similar to that of control animals, and monitoring the microbial composition over time following cessation of antibiotics treatment would be an important study to perform. Similarly, assessing the impact of various microbiota, in particular of a "healthy microbiota," on susceptibility to TB is an important issue that is not addressed here and would require developing different animal models.

We found that unconventional T cells, namely MAIT cells, might be involved in microbiota-mediated early control of *M. tuberculosis* infection, e.g., through the production of IL-17A. MAIT cells form a population of innate-like lymphocytes expressing a semi-invariant T cell antigen receptor (TCR), activated by the MHCI–related molecule MR1 presenting microbial-derived metabolites (48, 49). MAIT cells preferentially reside in mucosal tissues, such as the lungs and the gut, and their development and maturation depend on the presence of the host microbiota (26, 50). This explains why the MAIT cell compartment is disturbed in several inflammatory disorders

in which the gut microbiota is altered, such as inflammatory bowel disease and obesity (51, 52). MAIT cells were found to be involved in host protection from a variety of pathogens, including respiratory pathogens such as *Klebsiella pneumoniae*, and *Francisella tularensis* (24, 53). Reminiscent of our study, mice lacking MAIT cells also display an increased early susceptibility to mycobacterial infections (25, 26, 54). In humans, TB patients display a functionally impaired MAIT cell compartment (55, 56). In our study, we found that MAIT cells tend to proliferate less in ABX mice, which may rely on a reduced availability of MAIT ligands in this setting (49, 57, 58). Although we cannot exclude that the recruitment of these cells to the lung is also diminished in ABX mice, we provide evidence that the reduced accumulation of MAIT cells in the lungs of ABX mice is independent of the CCR6/CCL20 axis. Yet, MAIT cells express other receptors involved in cell migration, namely CCR5, CCR9, CXCR4, and CXCR6, at least in humans (59). The role played by these receptors, and possibly others, in microbiota-mediated recruitment of MAIT cells to the lungs during *M. tuberculosis* infection, will need to be investigated. We found that MAIT cells produce less IL-17A in ABX mice. The role of IL-17 in TB is still unclear, possibly depending on the cell type producing this cytokine and the time point at which it is produced during infection (33). Whether the host microbiota influences early control of *M. tuberculosis* through the modulation of the production of IL-17, and possibly other cytokines, and how it does so, will need to be addressed. In the mouse, MAIT cells mostly produce IL-17A (41) but their ability to control *M. tuberculosis* in infected macrophages does not seem to depend on this cytokine (54), suggesting that the possible protective effect of IL-17A we observed is not direct. The development of MAIT cells is known to depend on the microbiota, particularly on microbes synthesizing riboflavin (26, 60). Addressing the role of specific microbiota species in the MAIT-mediated early protection against *M. tuberculosis* infection would require recolonizing ABX mice with riboflavin-producers (e.g., *Escherichia coli*) and -non producers (e.g., *Enterococcus faecalis*), which will be of interest for future studies.

In conclusion, we show here that the host microbiota contributes to early resistance to *M. tuberculosis* infection in mice, possibly through stimulating the accumulation and effector functions of MAIT cells in the lungs. Whether these findings apply to humans will need further investigation. A significant fraction of those individuals in close contact to TB cases are known to remain tuberculin skin test (TST)-negative, and are thus thought to represent "innate controllers" (61). It is tempting to speculate that in addition to other factors, the host microbiota might contribute to the ability to control *M. tuberculosis* infection in an innate fashion, *via* MAIT cells and possibly other innate cells (62).

## ETHICS STATEMENT

Animal care and experimentation were performed following the French regulation. All procedures were approved by the Ministry of Higher Education and Research (Agreement APAFIS 6497).

## AUTHOR CONTRIBUTIONS

AD, DC, DH, YP, and ON designed research. AD, DC, FL, AC, and AP performed research. AD, DC, FL, AP, DH, YP, and ON analyzed data. AD and ON wrote the paper with editorial help from DC, DH, and YP.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02656/full#supplementary-material

**Supplementary Figure 1 |** Microbiota dysbiosis does not alter immune cell populations in the lungs. **(A)** The total numbers of F4/80$^+$CD11c$^+$ macrophages, CD11c$^+$MHCII$^+$ dendritic cells, CD11b$^+$GR1$^{hi}$ neutrophils, CD3$^-$NK1.1$^+$ NK cells, CD3$^+$CD4$^+$ T lymphocytes and CD19$^+$ B cells were quantified by flow cytometry in lung homogenates from uninfected ABX vs. non-ABX mice. **(B)** Cytometry analysis of percentage of TNFα-producing F4/80$^+$CD11c$^+$ macrophages, TNFα-producing CD3$^+$CD4$^+$ T lymphocytes and IL-17A-producing CD3$^+$CD4$^+$ T lymphocytes in lungs from uninfected ABX vs. non-ABX mice. Data from 1–3 independent experiments ($n$ = 4–5 mice/group/experiment) were pooled and the graphs show median with interquartile range of the pooled data (B cells, TNFα$^+$ macrophages, TNFα$^+$ CD4 T cells, IL-17A$^+$ CD4 T cells) or mean ± SD (macrophages, dendritic cells, neutrophils, NK cells, CD4 T cells). Data were analyzed using the Mann-Whitney test (B cells, TNFα$^+$ macrophages, TNFα$^+$ CD4 T cells, IL-17A$^+$ CD4 T cells) or using the unpaired Student's $t$-test (macrophages, dendritic cells, neutrophils, NK cells, CD4 T cells).

**Supplementary Figure 2 |** Microbiota dysbiosis does not impact early inflammatory response to *M. tuberculosis* infection in lungs. **(A)** The expression of inflammatory cytokines and the antimicrobial peptide CRAMP was measured by RT-qPCR in the lungs of non-ABX vs. ABX mice 7 days p.i., Gene expression represents relative Ct value compared to *Hprt* Ct value (ΔCt) and the mean of ΔCt values in the control group (ΔΔCt). **(B)** The production of TNFα, IFNγ, and IL-12(p70) was measured in lung homogenates from control (non-ABX) and ABX mice 7 days p.i., Data from 2–3 independent experiments ($n$ = 2–3

mice/group/experiment) were pooled and the graphs show mean ± SD (TNFα, TGFβ, CRAMP in **A**) or median with interquartile range (IL-1β, IL-6, IFNγ, IL-17A in **A**; TNFα, IFNγ, IL-12 in **B**) of the pooled data. Data were analyzed using the Student's *t*-test (TNFα, TGFβ, CRAMP in **A**) or the Mann-Whitney test (IL-1β, IL-6, IFNγ, IL-17A in **A**; TNFα, IFNγ, IL-12 in **B**).

**Supplementary Figure 3 |** Microbiota dysbiosis does not alter innate myeloid and lymphoid cell populations during early lung colonization by *M. tuberculosis*. The total number of F4/80$^{+}$CD11c$^{+}$ macrophages, CD11c$^{+}$MHCII$^{+}$ dendritic cells, CD11b$^{+}$GR1$^{hi}$ neutrophils, and CD3$^{-}$NK1.1$^{+}$ NK cells were quantified by flow-cytometry in lung homogenates from *M. tuberculosis*-infected ABX vs. non-ABX mice 7 days p.i., Data from 4 independent experiments ($n$ = 6–8 mice/group/experiment) were pooled and the graphs show median with interquartile range of the pooled data (macrophages, neutrophils) or mean ± SD (dendritic cells, NK cells). Data were analyzed using the Mann-Whitney test (macrophages, neutrophils) or using the unpaired Student's *t*-test (dendritic cells, NK cells).

**Supplementary Figure 4 |** NKT and γ/δ T cells are not modified in infected microbiota-altered mice. **(A)** Gating strategy to analyze unconventional innate-like lymphocytes, namely NKT cells (CD3$^{+}$NK1.1$^{+}$) and γ/δ T cells (CD3$^{+}$TCRγ/δ$^{+}$) by flow cytometry. **(B,C) (B)** Total NKT cells (left) and γ/δ T cells (right) and **(C)** percentages in the lungs of control (non-ABX) and ABX mice 7 days p.i., Data from 2 independent experiments ($n$ = 8 mice/group/experiment) were pooled and the graphs show the mean ± SD of the pooled data. Data were analyzed using the unpaired Student's *t*-test.

**Supplementary Table 1 |** Primers used in the study for RT-qPCR.

**Supplementary Table 2 |** Antibodies used in the study for flow cytometry.

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REVIEW
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# Neutrophils: Innate Effectors of TB Resistance?

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Certain individuals are able to resist *Mycobacterium tuberculosis* infection despite persistent and intense exposure. These persons do not exhibit adaptive immune priming as measured by tuberculin skin test (TST) and interferon-γ (IFN-γ) release assay (IGRA) responses, nor do they develop active tuberculosis (TB). Genetic investigation of individuals who are able to resist *M. tuberculosis* infection shows there are likely a combination of genetic variants that contribute to the phenotype. The contribution of the innate immune system and the exact cells involved in this phenotype remain incompletely elucidated. Neutrophils are prominent candidates for possible involvement as primers for microbial clearance. Significant variability is observed in neutrophil gene expression and DNA methylation. Furthermore, inter-individual variability is seen between the mycobactericidal capacities of donor neutrophils. Clearance of *M. tuberculosis* infection is favored by the mycobactericidal activity of neutrophils, apoptosis, effective clearance of cells by macrophages, and resolution of inflammation. In this review we will discuss the different mechanisms neutrophils utilize to clear *M. tuberculosis* infection. We discuss the duality between neutrophils' ability to clear infection and how increasing numbers of neutrophils contribute to active TB severity and mortality. Further investigation into the potential role of neutrophils in innate immune-mediated *M. tuberculosis* infection resistance is warranted since it may reveal clinically important activities for prevention as well as vaccine and treatment development.

**Keywords: *Mycobacterium*, tuberculosis, inflammation, NETs, antimicrobial, protection, necrosis**

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## INTRODUCTION

Not all individuals exposed to *Mycobacterium tuberculosis* become infected as inferred by a lack of T cell memory response to *M. tuberculosis* antigens. Moreover, these individuals do not develop signs and symptoms suggestive of 'active tuberculosis' (TB). The majority of *M. tuberculosis* infected individuals remain asymptomatic with what is known as latent tuberculosis infection (LTBI).

Only 5–15% of those infected will progress to active TB disease, given they have no underlying co-morbidity which would increase their risk further (1, 2). This resulted in an estimated 10.4 million new cases and 1,674 million TB deaths reported in 2016 (3). The remaining 85–95% of persons with LTBI who do not develop disease indicates that the majority of those infected have a natural immunity to prevent the progression from infection to disease. Similarly, certain individuals who are highly exposed, never develop evidence of infection. This suggests that they are naturally resistant to *M. tuberculosis* and can prevent infection via an innate immune response prior to adaptive immune cell priming, and are known as "innate resisters" (4). The mechanisms that underlie the resistance to infection in persons of the "innate resister" phenotype are not fully known. In the present article, we explore the possible contribution of neutrophils to innate infection resistance.

## Evidence of *M. tuberculosis* Infection

LTBI is defined as the presence of *M. tuberculosis*-specific T-cell sensitization in the absence of clinical signs and symptoms of TB. Host sensitization is used as a proxy for this assumed latent *M. tuberculosis* infection in human hosts and is measured by reactivity to mycobacterial antigens using the tuberculin skin test (TST) or interferon-γ (IFN-γ) release assays (IGRAs). The TST is performed by injecting purified protein derivate (PPD) intradermally (5). A delayed-type hypersensitivity reaction occurs if the host is reactive to *M. tuberculosis* antigens. Due to the limited *M. tuberculosis* specificity of TST, more specific *in vitro* blood-based assays (T-SPOT.*TB* and QuantiFERON-TB Gold) were developed using early secretory antigen target-6 (ESAT-6), culture filtrate protein 10 (CFP-10), and TB-7.7 as *M. tuberculosis* antigens. These assays measure the *ex-vivo* IFN-γ release by T cells in response to the aforementioned *M. tuberculosis* peptide antigens (6). A disadvantage of TST and IGRA for the diagnosis of infection is that they are unable to distinguish between an amnestic response and persistent infection. It is therefore possible that an unknown proportion of persons who test positive in the immune assays are no longer infected with *M. tuberculosis*. Conversely, persons who test negative in the immune assays may be (i) not sufficiently exposed to *M. tuberculosis*, (ii) anergic to *M. tuberculosis* antigens used in the assays, or (iii) exposed but able to clear *M. tuberculosis* infection without triggering the onset of acquired anti-*M. tuberculosis* immunity.

## Natural Immunity Against *M. tuberculosis*

While the lack of a direct assay for the determination of current infection complicates studying resistance to infection, multiple lines of evidence support human variability in resistance to infection with *M. tuberculosis*. Historical epidemiological studies have long supported the concept of infection resistance as a *bona-fide* biological phenotype. During an outbreak on a US naval ship, 66 sailors shared a cabin with 7 sailors who had active TB. Of the 66 sailors, 13 (20%) remained TST negative after 6 months (7). Fifty-seven (55%) of 104 elderly residents with a previously TST negative result remained uninfected after being exposed for at least 12 months to a fellow resident with sputum positive TB (8). An average of 50% of close contacts of TB patients develop positive TST or IGRA tests in overcrowded living conditions or household contact studies (9, 10). In Uganda only 4.1% of adults (age > 15 years old) with close household contacts remained PPD negative (<10 mm for HIV- adults, <5 mm for all HIV+) over a 2 year follow up period (11). Other studies done in individuals in environments with high exposure to *M. tuberculosis*, show that 10–20% do not become TST/IGRA positive (12–14). In South African goldminers who have a documented high exposure to *M. tuberculosis* and an estimated LTBI prevalence of 89% in 2006, 13% of the HIV-negative participants had a TST = 0 mm response (15). Together, these studies suggest that 5–20% of the population may possess resistance to *M. tuberculosis* infection.

Molecular genetics studies support the concept of resistance to *M. tuberculosis* infection. In a highly TB endemic area in South Africa 20% of the highly exposed population remained TST negative which was stringently defined as TST = 0 mm. This phenotype is linked to a major locus, *TST1*, which represents T cell-independent *M. tuberculosis* infection resistance (13). A genome-wide association study in HIV-infected persons identified a locus on chromosome region 5q31.1 in proximity of *IL9* which significantly associates with TST positivity (16). In addition, the study replicated associations in the region of *TST1* as well as on chromosome regions 2q21-2q24 and 5p13-5q22 that had been identified by genome-wide linkage analysis of Ugandan families (13, 16, 17). Current genetic evidence suggests that the resistance phenotype is likely due to a combination of genetic variants synergistically contributing to the phenotype rather than a single genetic variant.

# THE HETEROGENEOUS NATURE OF NEUTROPHILS

It is tempting to speculate that neutrophils of individuals who exhibit *M. tuberculosis* infection resistance are a unique subset of cells genetically or epigenetically programmed to control infection and inflammation. Epigenetic reprogramming of neutrophils offers an attractive avenue of investigation as neutrophils show increased variability in both gene expression and DNA methylation compared to phenotypically naïve T-cells and classic monocytes (18). This observation supports the concept of physiologically distinct inter-individual neutrophil populations.

Different intra-individual neutrophil subsets have also been defined in multiple studies investigating various diseases including cancer, systemic lupus erythematosus (SLE), TB, and HIV-1 (19–23). However, the heterogeneous nature of neutrophils with subsets displaying functional as well as phenotypic differences is still under debate and most subsets remain incompletely defined and phenotyped (20, 24–29).

Genetic variants, which underlie epigenetic and transcriptional variability, also contribute to differences in neutrophil activity. For example, 21 neutrophil genes showed significant differences in expression levels between males and females while a SNP in *SELL*, which encodes the CD62L receptor, strongly influenced expression levels of CD62L cell

receptors on neutrophils (18, 30). Not surprisingly, genes of the inflammasome pathway are significantly enriched in neutrophils and play an important role in the regulation of interleukin 1 (IL-1)-dependent cytokine production (31). In murine studies, IL-1 deficiency predisposes to a lack of *M. tuberculosis* infection control and non-resolving inflammation (32). During persistent infections, such as active TB, inflammasome activation correlates with pathology (33, 34). Taken together, these data suggest that if neutrophils contribute to *M. tuberculosis* infection resistance the effector mechanisms involved are likely to be under both genetic and epigenetic regulation. However, at least some of the underlying variability may be ascribed to the inherent difficulties in working with these cells since they cannot be cryopreserved, are easily activated and are short-lived (35, 36). Possible genetic variability is further highlighted by the conflicting results published around the role of neutrophils in *M. tuberculosis* infection.

## NEUTROPHILS IN *M. TUBERCULOSIS* INFECTION AND DISEASE

*M. tuberculosis* is an airborne pathogen and is transmitted via the aerosol inhalation of transmitted droplets containing the bacteria from an infected individual. *M. tuberculosis* enters the airways and reaches the pulmonary alveolus where some of the first cells encountered are resident alveolar macrophages (AM) (37) which release pro-inflammatory cytokines tumor necrosis factor (TNF), IL-6, IL-1α, and IL-1β (38). If this first line of defense fails, *M. tuberculosis* enters the pulmonary interstitial tissue by either using the infected AM as a host vehicle to migrate or by infecting the epithelium or pneumocytes (2). Acute inflammatory signals are released and the other phagocytes are recruited to the site of infection. Local tissue macrophages recognize *M. tuberculosis* by Toll-like receptors (TLR) and are also activated to release pro-inflammatory cytokines including TNF, IL-6, and IL-1β (39, 40) (**Figure 1A**).

Neutrophils are some of the first phagocytes recruited from the pulmonary vasculature to the pulmonary interstitium (41). Multiple receptors (including TLRs and C-type lectins receptors (CLRs) and cytokine receptors) have been implicated in the interaction between neutrophils, *M. tuberculosis* and pro-inflammatory cytokines (42–46). Upon exposure to *M. tuberculosis* neutrophil blood counts in human pulmonary TB (PTB) contacts are initially higher than in unexposed control subjects and subside after 6 weeks (47). Interestingly, low neutrophil counts are associated with IGRA positivity in TB contacts (47). The initial neutrophil peak seen in TB contacts, implicates neutrophils in the acute inflammatory response to *M. tuberculosis.*

Individuals in contact with patients with pulmonary TB are less likely to be infected with *M. tuberculosis* if they have higher peripheral blood neutrophil counts (47). One hour after *in vitro* infection with virulent *M. tuberculosis* and stimulation with TNF, neutrophils suppressed the growth of the inoculum by 50–95% (48). Unstimulated neutrophils inhibit on average 40.6% of the growth of the *M. tuberculosis* inoculum. Interestingly, there was significant variability in this mycobactericidal capacity between donor neutrophils. Neutrophils from some donors were capable of inhibiting *M. tuberculosis* growth spontaneously while, despite the addition of TNF or IFN-γ, others were not. Neutrophil-depleted whole blood had a 3.1 fold decreased capacity to control *M. tuberculosis* infection *ex vivo* (47). This finding was recently confirmed and highlights the importance of neutrophils in *M. tuberculosis* infection (49). Granulocyte (CD15+) depleted blood does not control *M. tuberculosis* infection as efficiently as blood depleted of CD4+, CD8+, or CD14+ cells. Addition of viable CD15+ granulocytes significantly improved *M. tuberculosis* control (49).

However, infection in highly susceptible strains of mice shows the detrimental effect of uncontrolled neutrophil recruitment on TB infection and inflammation control and eventually an increase in TB disease severity (50). Most studies concur that neutrophils are final mediators of lung damage and disease (51–53). C57BL/6 mice with neutrophil and monocyte derived-cells lacking *Atg5* succumb after 30–40 days post *M. tuberculosis* infection due to a massive influx of neutrophils, and increased lesion number and bacterial load, that is not observed in wild type mice (54, 55). Whilst ATG5 is normally associated with autophagy, the neutrophilic influx associated with premature death was independent of any autophagic response. Granulomas of various susceptible mouse strains contain a substantial number of necrotic neutrophils (53, 56–58) in comparison to more "resistant" mouse strains showing only scattered neutrophils and little or no necrosis (59).

In humans, as in the mouse model, necrotic neutrophils are unable to control *M. tuberculosis* infection (49). Phagocytosis of *M. tuberculosis*-induced necrotic neutrophils by macrophages promotes bacterial growth (60, 61). *M. tuberculosis* mostly remains encapsulated in apoptotic neutrophils (60). This enables fusion of neutrophil granular contents with macrophage lysosomes after efferocytosis of the apoptotic neutrophil by the macrophage (60). The neutrophil membranes surrounding *M. tuberculosis* prevent direct contact between the bacillus and the macrophage phagosomal membrane thus preventing *M. tuberculosis* inhibition of phagolysosome maturation (60). However, during neutrophil necrosis, *M. tuberculosis* is released from the disintegrated phagosome and enters the phagocytosing macrophage as extracellular bacteria (60). Once phagocytosed by a macrophage, the bacillus is able to evade phagolysosomal fusion in the macrophage and mycobacterial growth is promoted (60, 61).

Necrotic neutrophils added to whole blood increased the metabolism of *M. tuberculosis,* as measured by mycobacterial luminescence, and released IL-10 as well as growth factors, granulocyte- and granulocyte macrophage-colony-stimulating factors (G-CSF and GM-CSF), and the monocyte chemotactic protein chemokine ligand 2 (CCL2) (49). The predominant role of these molecules is to attract and prime more cells (49). G-CSF supports the growth and proliferation of neutrophils and their precursors (62). GM-CSF has the potential to act on earlier progenitor cells than G-CSF and therefore neutrophil progenitors as well as monocytes proliferate (63). G-CSF and GM-CSF not only drive the increased production of neutrophils

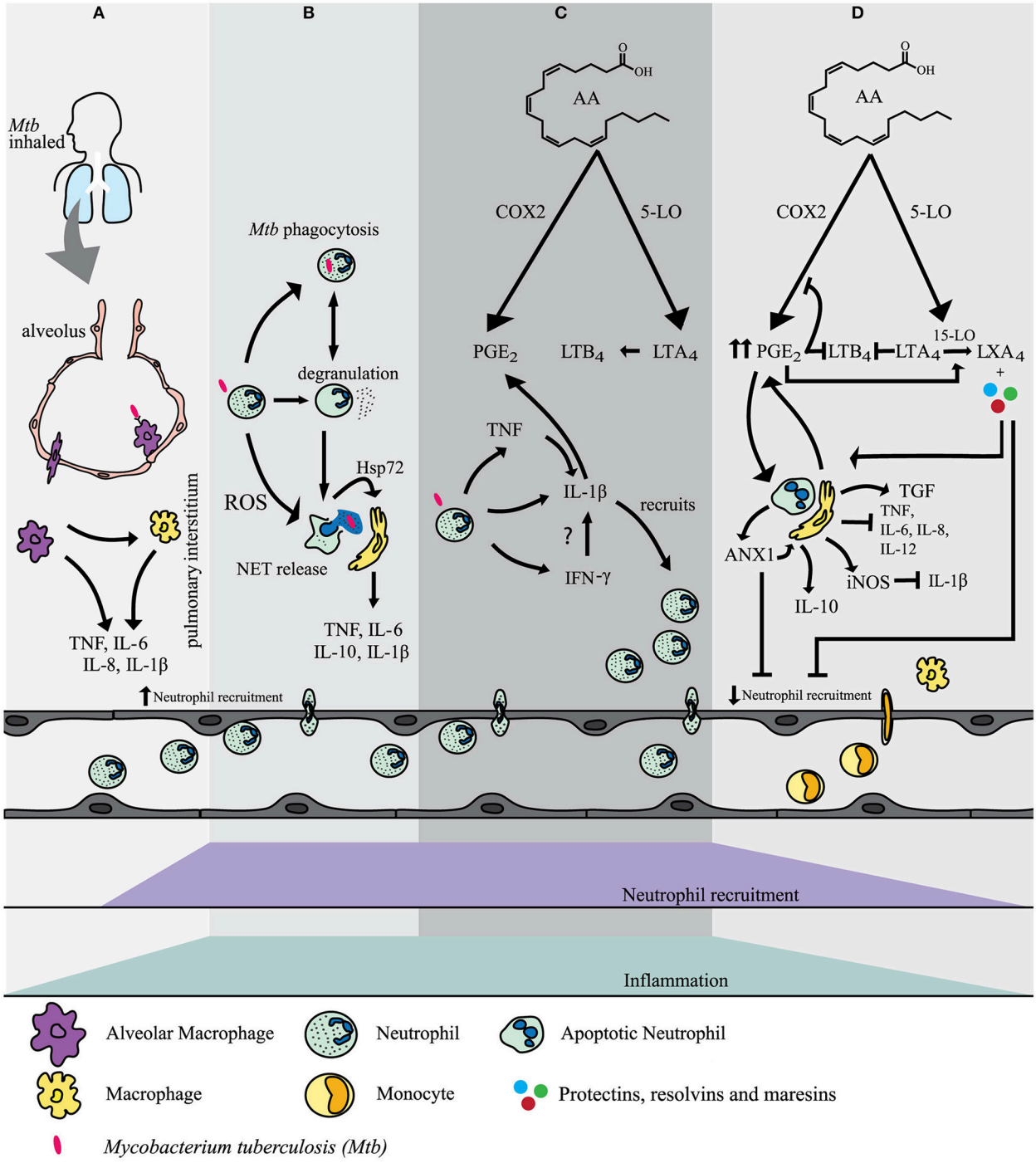


**FIGURE 1 | (A)** Alveolar macrophages (AM) are the first cells to encounter *M. tuberculosis* after inhalation of the bacillus. Acute pro-inflammatory signals are released by AM and local tissue macrophages to recruit neutrophils to the site of infection.**(B)** Neutrophils use a variety of mechanisms to mediate *M. tuberculosis* infection. These included phagocytosis, degranulation, ROS formation and NET release. NETs transfer Hsp72 to adjacent macrophages inducing a pro-inflammatory response.**(C)** Interaction of recruited neutrophils with *M. tuberculosis* mediates the activation of several pathways which contribute to inflammation and clearance of *M. tuberculosis* infection. Interleukin-1β (IL-1β) release is mostly mediated in an inflammasome dependent manner. Tumour necrosis factor (TNF) induces NF-κB which mediates the induction of gene expression of IL-1β in neutrophils. Interferon-γ (IFN-γ) may also regulate the release of IL-1β. IL-1β is a key player in mediating the release of prostaglandin $E_2$ ($PGE_2$) and leukotriene $B_4$ ($LTB_4$) both of which contribute to inflammation and the recruitment of neutrophils. **(D)** $PGE_2$ eventually becomes a stop signal and has a negative feedback on cyclo-oxygenase-2 (COX-2) and 5-lipoxygenase (5-LO). The production of lipoxin A4 (LXA4) is favoured. In addition, AnnexinA1 (ANX1) stimulates IL-10 release by macrophages. Neutrophils express inducible nitric oxide synthase (iNOS) which has a further negative feedback on IL-1β release. The net effect is an increase in neutrophil apoptosis and clearance by tissue macrophages. More macrophages are recruited and further neutrophil recruitment is inhibited and inflammation is resolved.

and monocytes but also have the ability to indirectly affect neutrophil function and phenotype (64–68). Both of these growth factors delay neutrophil apoptosis and "prime" neutrophils for enhanced oxidative effects that can lead to tissue destruction (67, 69). In a setting where *M. tuberculosis* induces necrotic cell death, the newly released cells would undergo the same cycle of necrosis, release tissue damaging substances, recruit more cells, and contribute to continuous inflammation as seen in TB disease, with neutrophilia being an independent predictor of TB mortality (70).

## Neutrophil Mechanisms to Clear *M. tuberculosis* Infection

Despite the involvement of neutrophils in tissue damage in late stage clinical TB, they display an inter-individual ability to control *M. tuberculosis* infection. Neutrophils can use oxidative and non-oxidative mechanisms to clear *M. tuberculosis* infection. Both mechanisms are involved in either the direct clearance of *M. tuberculosis* or in the mediation thereof.

### Oxidative Mechanisms

Neutrophils are primed or activated by *M. tuberculosis* and pro-inflammatory cytokines, which in turn triggers degranulation and respiratory burst (71–79). Proteases (e.g., elastase, cathepsin G, and protease 3), hydrolyses, antimicrobial peptides and oxidants are released. The oxidants mediate tissue breakdown by activating matrix metalloproteinases (MMPs) (80, 81). These effectors do not discriminate between pathogen and host tissue and collateral damage is inevitable.

Neutrophil-produced reactive oxygen species (ROS) have been shown to drive *M. tuberculosis*-induced necrosis (60). Inhibiting myeloperoxidase (MPO) derived ROS prevents neutrophil necrosis and improves efferocytosis of these cells by macrophages and therein the control of *M. tuberculosis* growth (60). Similarly, chronic granulomatous disease (CGD) neutrophils are protected from necrosis after infection with *M. tuberculosis* (79). *One* would therefore expect an improved control of *M. tuberculosis* infection in CGD patients who are characterized by an inability to produce ROS but this does not always seem to be the case (82). Indeed, CGD patients are more susceptible to active TB supporting the possible role of neutrophils in mediating *M. tuberculosis* infection resistance (83–86). This view is supported by multiple studies that have shown neutrophils to be protective in control of early infection (47–49).

The NOX2 complex is an isoform of the large family of NAPDH oxidases (NOX) and is found in phagocytes including neutrophils (87, 88). It is an enzyme that is involved in infection and inflammation control and is activated by neutrophil chemotactic factors such as IL-8 and leukotriene B4 (LTB4) (88, 89). Hydrogen peroxide ($H_2O_2$) that is produced during respiratory burst contributes to neutrophil migration and subsequently retention at the site of infection (89). CGD patients have impaired neutrophil accumulation, in contrast to the increase in granuloma formation seen in CGD (89). Inflammatory leukotrienes are released by neutrophils in CGD patients but due to a lack of ROS there is a lack of degradation of these leukotrienes and delayed clearance of inflammation (44, 89, 90).

Reactive oxygen species have been shown to affect transcription factors such as NF-κβ (91, 92) which mediates the induction of IL-1β and IL-8 expression. However, CGD shows that NF-κβ activation is independent of ROS and is also mediated by TNF and IL-1 (93, 94) and so neutrophils in these individuals are still able to release these pro-inflammatory factors and uncontrolled chronic inflammation ensues (95, 96). Pro-inflammatory mediators alone, such as leukotrienes and IL-1β, are not enough to control infection and it is likely that the overproduction thereof augments the lack of *M. tuberculosis* infection control in CGD patients (44).

*M. tuberculosis* is relatively resistant to the bactericidal effects of $H_2O_2$ mediated by DNA damage (97). However, even if ROS does not have a direct bactericidal effect on *M. tuberculosis*, it still amplifies the neutrophil antimicrobial response. It does this by activating the formation of neutrophil extracellular traps (NET, discussed in 3.1.3), stimulating the release of pro-inflammatory cytokines such as TNF and macrophage inflammatory protein 2 (MIP-2), as well as decondensed DNA to which the contents of cytoplasmic granules adhere in a net-like structure (98–100). This is extensively reviewed by Deffert et al. (44).

### Non Oxidative Mechanisms

Neutrophil granules can fuse with the phagolysosome, degranulate and release antimicrobial peptides (AMPs) (**Figure 1B**). Antimicrobial peptides (AMPs) are classified according to their amino acid motif and structure. Three classes are found in humans: defensins, cathelicidins, and histatins (101–103). Neutrophils contain α-defensins in azurophilic granules and cathelicidin LL-37 in specific granules, as well as other neutrophil specific AMPs as will be discussed below (101, 104). Macrophages can traffic phagocytosed apoptotic neutrophil debris, including neutrophil granules, to endosomes. The purified neutrophil granules in the endosomes fuse with the macrophage phagosome in which the *M. tuberculosis* bacillus resides. This mechanism of cell-cell cooperation provides an effective antimicrobial response to *M. tuberculosis* (105). Although this efferocytosis occurs between macrophages and apoptotic neutrophil debris, it is not known whether alveolar macrophages do the same. AMPs can also be associated extracellularly with NETs and facilitate in the clearing of microbial infection.

a. AMPs in azurophilic granules

Azurophilic granules are poorly mobilized in response to *M. tuberculosis* infection. Pathogenic mycobacteria block the fusion of azurophil granules with the phagosome and consequentially unlike specific granules they are unable to release their contents in to the phagosome for antimicrobial effect (106). However, azurophilic proteins obtained from apoptotic neutrophil debris, increase macrophage ability to restrict *M. tuberculosis* growth either by direct action or by lysosome fusion with the maturation-arrested mycobacterial phagosome in the macrophage (107).

-Defensins: Human neutrophil peptide 1 (HNP-1), one of four α-defensins found in the primary or azurophilic granules of neutrophils (101) has the ability *in vitro* to reduce the growth

of *M. tuberculosis* in culture as well as within macrophages (105, 108, 109). Furthermore, HNP-1 also shows *in vivo* antimycobacterial activity in mice (110).

-Azurocidin: Defensin depleted azurophilic granules at 100 μg/ml were shown to restrict the growth of 55% of *M. tuberculosis* in culture after 24 h of incubation. However, the specific role of azurocidin in *M. tuberculosis* infection remains unclear (107).

-Cathepsins: *M. tuberculosis* infection decreases cathepsin gene expression in macrophages, with a parallel decrease in cathepsin protein levels (111). Genetic linkage and association studies have previously implicated cathepsin Z in susceptibility to TB (112, 113). A likely alternative source of cathepsin for macrophages is through the phagocytosis of apoptotic neutrophil material. Uptake of liposomal encapsulated cathepsin G and neutrophil elastase (NE) by alveolar macrophages in mice improves antimicrobial activity against *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (114).

b. Specific granules

-Cathelicidin: Neutrophils produce LL-37, the 37 amino acid biologically active C-terminal domain cleaved from the human cathelicidin propeptide (hCAP18) by proteinase 3, when infected with *M. tuberculosis* (115). LL-37 has been shown to restrict growth of *M. tuberculosis* in neutrophils (47). Similarly it restricts growth of *M. tuberculosis* in infected macrophages when hCAP18 is exogenously activated by neutrophil proteinase 3, which has only a low level of constitutive expression in macrophages (107, 116).

c. Gelatinase granules

-Lipocalin 2: Lipocalin 2 binds mycobacterial siderophores which scavenge iron for the bacillus in iron-limiting conditions (47). Lipocalin 2 has a greater mycobacterial suppressive effect (60%) in an iron-depleted broth (10 nM iron) compared to iron-replete broth (150 μM Fe) of 45%. It may be more effective in the phagolysosome where the molar ratio to siderophores would be higher.

d. Neutrophil cytoplasmic proteins

Calprotectin (S100A8/S110A9): Calprotectin is known as a damage-associated molecular pattern (DAMP) molecule and is a heterodimer of S100A8/A9. It sequesters free zinc and limits mycobacterial growth (107, 117). *M. tuberculosis* infection induces S100A8/A9 proteins. This is associated with neutrophil accumulation and exacerbated inflammation (52, 118).

### NET Formation

During NETosis, neutrophils release their DNA contents coated in cytoplasmic and granular proteins to trap and possibly clear invading pathogens (119, 120). NETosis is an alternative form of cell death, different to apoptosis and necrosis, and mediated by phagocytosis and the generation of ROS by NADPH oxidase in *M. tuberculosis* infection (121, 122). Once activated, neutrophils lose their lobulated morphology (123). The nuclear membrane initially remains intact whilst the chromatin (histones and DNA) starts to decondense. Once the nuclear and granular membranes rupture, the decondensed chromatin comes into contact with the granular as well as cytoplasmic components of the cell. The NET components are released extracellularly when the cell membrane breaks (122). The most abundant non-histone protein in NETs is NE (124). In addition to this, NETs contain myeoloperoxidase (MPO) as well as other proteins from intracellular neutrophil organelles. These include substances from the primary neutrophil granule (cathepsin G, defensins, BPI-bactericidal substance), the secondary neutrophil granule (alcaic phosphatase, lactoferrins, lysozyme, cathelicidins, collagenase), tertiary granules [gelatinase, matrix metalloproteinase 9 (MMP-9)]; and catalase from peroxisomes (125–128). Other components include calprotectin, constituents of the neutrophil cytoskeleton and glycolytic enzymes (125, 128).

Although *M. tuberculosis* has been shown to induce NETosis, no experimental evidence exists that NET formation improves resolution of *M. tuberculosis* infection (129). However, the AMP NET components have been shown to restrict *M. tuberculosis* growth as discussed earlier. Also neutrophils can assist macrophages to clear *M. tuberculosis* infection. During infection, NET formation and *M. tuberculosis*-induced apoptosis occur independently. *M. tuberculosis*-induced NETs transfer the danger signal heat shock protein 72 (Hsp72) to adjacent macrophages (121). This interaction induces a pro-inflammatory response in macrophages leading to the release of IL-6, TNF, IL-1β, IL-10. In addition to these cytokines, calprotectin is released from the neutrophil cytoplasm into NETs (130). IL-10 is also released as part of the anti-inflammatory regulatory response via inhibiting IFN-γ and TNF production and downstream Th1 responses (121). It is possible that NETs play a role in trapping and localizing the infection. The sequestration of AMPs in the NET structures may also increase their effective concentrations. Furthermore; NETs contain the release of cellular contents to prevent distal tissue destruction (121, 123). Hence, NETs are potentially an effective defense mechanism that neutrophils could use to mediate *M. tuberculosis* infection resistance (**Figure 1B**).

## NEUTROPHILS AND THE ROLE OF CYTOKINES AND CHEMOKINES IN INFLAMMATION IN *M. TUBERCULOSIS* INFECTION RESISTANCE

### Initial Inflammation

*M. tuberculosis* infection triggers TLR signaling and induces NF-κB which mediates the induction of gene expression of pro-inflammatory cytokines such as IL-1β and TNF in neutrophils (42, 131). Inflammasomes are multimeric protein complexes and play a key role in the activation of IL-1α, and IL-1β (132). Neutrophils express components of the NOD-like receptor protein 3 (NLRP3) and absent in melanoma 2 (AIM2) inflammasomes (133). The latter are found in the cytoplasm as well as secretory and tertiary granule compartments (133). Neutrophils release IL-1β mostly in an inflammasome-dependent manner and do not release IL-1α (133). The inflammasome subunit caspase-1 activates pro-IL-1β to form IL-1β (132, 133). IL-1β activation can also occur in a caspase-1 independent manner via neutrophil proteases; NE, and proteinase 3 (PR3) (133). Furthermore, it is of interest that inflammasome components are found in neutrophil secretory vesicles. The components may play a role in phagosomal functionality or may

be released into the extracellular environment and utilized by other phagocytes, but this remains to be proven in neutrophils (133).

One of the key roles of IL-1β is to mediate the release of prostaglandin E2 (PGE2), an eicosanoid. Eicosanoids are important lipid mediators derived from arachidonic acid (AA) and are rapidly synthesized by phagocytes after acute challenge with *M. tuberculosis* (134, 135). Cyclo-oxygenase-2 (COX-2) competes with 5-Lipoxygenase (5-LO) or 15-lipoxygenase (15-LO) for the generation of each of the different eicosanoids. During inflammation macrophages and other cells, including neutrophils, can produce COX-2, which converts AA to PGE2. 5-Lipoxygenase (5-LO) converts AA to LTB4 from leukotriene A4 (LTA4). PGE2 and LTB4 mainly have proinflammatory effects and mediate the rapid recruitment of neutrophils to the site of infection and inflammation (136, 137). LTB4 promotes phagocytosis and the bactericidal activity of neutrophils (136, 138, 139) (**Figure 1C**).

Furthermore, neutrophils are a possible source of IL-12 mediated IFN-γ release (140). However, whether this occurs through direct *M. tuberculosis* stimulation is unknown. Neutrophils release IFN-γ after stimulation by degranulating agents which is due to an available small storage of IFN-γ (140). In addition, neutrophil stimulation by IL-12 alone or in combinations with lipopolysaccharide (LPS), IL-2, IL-18, or IL-15, induces IFN-γ synthesis by neutrophils (140).

Neutrophils matured with IFN-γ have marked upregulation of multiple transcripts where Guanylate Binding Protein (GBP) showed the highest changes. GBPs are a subfamily of the IFN inducible GTPase superfamily (141, 142). GBP-5, in particular, is strongly upregulated in transcriptomes from an immature myeloid cell line (PLB-985) matured in the presence of IFN-γ (143). PLB-985 cells can differentiate into terminally mature neutrophils and have the ability to mimic the physiological conditions of stimulation (144). The exact role of GBP-5 has not been described in neutrophils yet, but it is possible that it enhances the NLRP3 inflammasome and IL-1β production, as in macrophages (143) (**Figure 1C**).

IFN-γ may increase the half-life of neutrophils in culture by being anti-apoptotic (143) and in this manner contributes to the pro-inflammatory state. Pathology in pulmonary tuberculosis is associated with neutrophils expressing IFN-γ and type I IFNs (145). This transcriptional signature is found in patients with active TB but infrequently in healthy individuals or those with latent TB (145). Type 1 IFNs may contribute to disease progression but the pro-inflammatory effect of IFN-γ from a neutrophil perspective may be effective for short bursts and in a setting where *M. tuberculosis* is effectively killed. The promotion of this initial pro-inflammatory state and release of TNF and IFN-γ by neutrophils is essential to effectively clear *M. tuberculosis* infection (48, 143).

## The Resolution of Inflammation in *M. tuberculosis* Infection

Apoptosis represents a pivotal point in the control of inflammation as well as in the control of the cellular immune response (146). A delicate balance exists between apoptotic cell death, clearance of apoptotic cells and ongoing inflammatory responses (80, 147, 148). Not only does the efferocytosis of apoptotic neutrophils by tissue resident macrophages prevent spillage of neutrophil content into surrounding tissue (80, 147, 149, 150), but it also decreases pro-inflammatory mediators (148). Clearance of infection without a significant acquired immune response is favored by early killing of *M. tuberculosis* by neutrophils, followed by apoptotic neutrophil death, and an anti-inflammatory response in the phagocytosing macrophage (35, 105).

A hallmark of the anti-inflammatory response is the production of TGF-β and PGE2, and the inhibition of IL-6, IL-8, IL-12, and TNF release by the phagocytosing macrophages (151). Studies have shown that cAMP-elevating agents such as PGE2 result in increased levels of AnnexinA1 (ANXA1) (152). ANXA1, a protein found in neutrophils, stimulates release of the anti-inflammatory cytokine, IL-10, by macrophages, and inhibits neutrophil migration (153). In addition, ANXA1 promotes efferocytosis of apoptotic cells (154, 155) (**Figure 1D**).

In addition to the release of endogenous anti-inflammatory mediators, pro-resolution action is also required. Lipoxins, protectins, resolvins and macrophage mediator in resolving inflammation (maresins) are unique mediators fulfilling this duality (137, 156, 157). Rising PGE2 levels eventually act as a "lipid mediated class switch" by transcriptionally inducing 15-LO in neutrophils and shifting the production of $PGE_2$ and LTB4 in favor of lipoxin A4 (LXA4) (158). LXA4 decreases neutrophil-mediated tissue damage, neutrophil proliferation, and adhesion, and increases efferocytosis of apoptotic neutrophils and IL-10 production by macrophages (159). Resolvins, protectins and maresins are oxygenated metabolites derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that is biosynthesized from omega-3 essential polyunsaturated fatty acids (137, 160). Collectively resolvins, protectins, and maresins regulate neutrophil apoptosis, efferocytosis by macrophages, inhibition of pro-inflammatory cytokines, release of IL-10 by local macrophages and tissue regeneration (159) (**Figure 1D**).

Finally, neutrophils express inducible nitric oxide synthase (iNOS) which converts the amino acid L-arginine to L-citrulline and nitric oxide (NO). iNOS/NO limits the production of IL-1β and therefore limits further recruitment of neutrophils (34, 161, 162). It is not known to what extent these neutrophil anti-inflammatory mechanisms are at play during early encounters of PMNs with *M. tuberculosis* in the lung (**Figure 1D**).

## CONCLUSION

At first glance, the association of uncontrolled neutrophil recruitment and pathology in TB would argue against a role of these cells in *M. tuberculosis* infection resistance.

However, neutrophils are multi-functional cells with variable roles in host defense. For example, there is documented inter-individual variability in the ability of neutrophils to kill *M. tuberculosis* suggesting that the role of neutrophils in an early encounter with *M. tuberculosis* may differ from the more integrated role in the presence of a strongly developed acquired immune response to the bacillus. As reviewed, the neutrophil has a large armamentarium of highly effective anti-microbial effector mechanisms that may come into play during the early stage of *M. tuberculosis* infection. Investigating the possible role of neutrophils in persons who remain free of *M. tuberculosis* infection despite documented high exposure to the bacillus offer an interesting opportunity. It may be that resisters possess a different ratio of neutrophil subpopulations, predominated by effective killers with a propensity to undergo apoptosis, compared to those who develop TB, predominated by inflammatory necrotising damage causing neutrophils. By comparing neutrophils and their anti-microbial responses from "innate resisters" with those from *M. tuberculosis* infection susceptible persons might illuminate if and how neutrophils play a protective role in the very stage of *M. tuberculosis* infection. Experiments along these lines will not only provide a better understanding of TB pathogenesis but also contribute to a better understanding of neutrophil biology in general.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# The Wonder Years: What Can Primary School Children Teach Us About Immunity to *Mycobacterium tuberculosis*?

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In high burden settings, the risk of infection with *Mycobacterium tuberculosis* increases throughout childhood due to cumulative exposure. However, the risk of progressing from tuberculosis (TB) infection to disease varies by age. Young children (<5 years) have high risk of disease progression following infection. The risk falls in primary school children (5 to <10 years), but rises again during puberty. TB disease phenotype also varies by age: generally, young children have intrathoracic lymph node disease or disseminated disease, while adolescents (10 to <20 years) have adult-type pulmonary disease. TB risk also exhibits a gender difference: compared to adolescent boys, adolescent girls have an earlier rise in disease progression risk and higher TB incidence until early adulthood. Understanding why primary school children, during what we term the "Wonder Years," have low TB risk has implications for vaccine development, therapeutic interventions, and diagnostics. To understand why this group is at low risk, we need a better comprehension of why younger children and adolescents have higher risks, and why risk varies by gender. Immunological response to *M. tuberculosis* is central to these issues. Host response at key stages in the immunopathological interaction with *M. tuberculosis* influences risk and disease phenotype. Cell numbers and function change dramatically with age and sexual maturation. Young children have poorly functioning innate cells and a Th2 skew. During the "Wonder Years," there is a lymphocyte predominance and a Th1 skew. During puberty, neutrophils become more central to host response, and CD4+ T cells increase in number. Sex hormones (dehydroepiandrosterone, adiponectin, leptin, oestradiol, progesterone, and testosterone) profoundly affect immunity. Compared to girls, boys have a stronger Th1 profile and increased numbers of CD8+ T cells and NK cells. Girls are more Th2-skewed and elicit more enhanced inflammatory responses. Non-immunological factors (including exposure intensity, behavior, and co-infections)

may impact disease. However, given the consistent patterns seen across time and geography, these factors likely are less central. Strategies to protect children and adolescents from TB may need to differ by age and sex. Further work is required to better understand the contribution of age and sex to *M. tuberculosis* immunity.

**Keywords: tuberculosis, children, adolescence, *Mycobacterium tuberculosis,* vaccination, infection, immunity, protection**

## INTRODUCTION

About a quarter of the global population (1), including nearly 70 million children and adolescents <15 years of age (2), is infected with *Mycobacterium tuberculosis*. Many infected individuals are able to contain *M. tuberculosis* without the organism ever causing pathology. However, in a subset, the intricate immunological response necessary to contain bacterial proliferation is lost. Of the nearly 70 million children and adolescents <15 years of age with TB infection, about 1 million develop TB disease each year (3). Young children, especially those <2 years of age, have an extremely high risk of developing TB disease after becoming infected. The risk then falls to a nadir in primary school children before rising during adolescence (4). In fact, the primary school years could be considered the "Wonder Years" of TB protective immunity: even when infected with *M. tuberculosis*, primary school children have the lowest risk, of any age throughout life, of progressing to TB disease.

Primary school children also have the most benign clinical manifestations of TB disease: classically, this age group has paucibacillary, intra-thoracic disease with greater involvement of the mediastinal lymph nodes than the lung parenchyma. Young children (<5 years of age) also have this type of intrathoracic disease but are additionally at high risk of disseminated TB, which has a high mortality. Largely for this reason, of the nearly 250,000 individuals <15 years of age who die from TB each year, most are <5 years of age (5). Around the time of puberty, mediastinal lymph node disease and disseminated TB become uncommon. Pulmonary TB begins to present as destructive lesions of the lung parenchyma, frequently in the upper lobes and with cavitation (6–9); TB-related mortality rises again. Taken together, these observations suggest that primary school children are protected from the two extremes of TB disease: the disseminated pathology commoner in young children and the destructive pulmonary disease commoner in adolescents and adults (**Figure 1**). The first might be considered a failure of control of infection and the latter a failure to control disease, with primary school children possessing a balanced inflammatory response capable of both.

In this article, we present the data on age-related variations in TB progression risk and disease forms. Although no studies have directly examined the reasons for these age-based differences, we discuss possible underlying immunological mechanisms, in an attempt to garner a greater understanding of the immunological correlates of protective immunity which exist during the Wonder Years of childhood protection immunity. We will make the case that young children <5 years have deficiencies in the initial response to *M. tuberculosis* infection, which reduce the chance of successful containment of the organism. These deficiencies increase the risk of progression to disseminated disease. In contrast, adolescents develop an excessively inflammatory response in the early stages of disease, which leads to tissue damage and favors localized replication of *M. tuberculosis*. We will also highlight divergences in disease risk between adolescent boys and girls, and explore the possible impact of sex hormones on host response to *M. tuberculosis*. Finally, we discuss other potential factors underlying these age- and gender-associated differences in TB risk. Understanding why TB risk changes with age may provide insight into correlates of protection—which, in turn, may lead to the development of TB vaccines, immunomodulatory therapies, and diagnostic assays that utilize host immunological profiles.

## DEFINITIONS

It is important to define key terms that will appear throughout this article. We use "infants" to refer to children <12 months of age. For the purposes of this article, "young children" refers to those who are <5 years of age and includes infants (<1 year); "early childhood" is the corresponding time period. "Primary school children" are 5- to <10-year-olds, and "adolescents" are 10- to <20-year-olds. Early adulthood refers to the third decade of life (10). Although stages of childhood encapsulate much more than chronological age, we use these definitions because most epidemiologic data are available in 5-year age bands. As puberty varies in age of onset, these definitions do not fully capture differences in physiological stages of development. Therefore, we also employ the terms "pre-pubescent," "pubescent," and "post-pubescent" to differentiate children and adolescents with respect to their physical features.

Second, we apply the terms "sex" and "gender" according to their standard definitions. "Sex" refers to the physiological distinction associated with sex chromosomes, sex steroids, and reproductive organs. In contrast, "gender" comprises the social and cultural constructs of males and females, as well as one's self-identity (11). We do not consider intersex or transgender individuals in this article due to the lack of data on these groups. We have elected to use "gender" when reporting epidemiological data because of the influence of both physiology and social behavior on such measures.

Next, we differentiate "TB infection" and "TB disease." "TB infection," or "*M. tuberculosis* infection," refers to an asymptomatic state evidenced only by immune sensitization to *M. tuberculosis*, as diagnosed by a tuberculin skin test (TST) or interferon gamma release assay (IGRA). These tests are limited

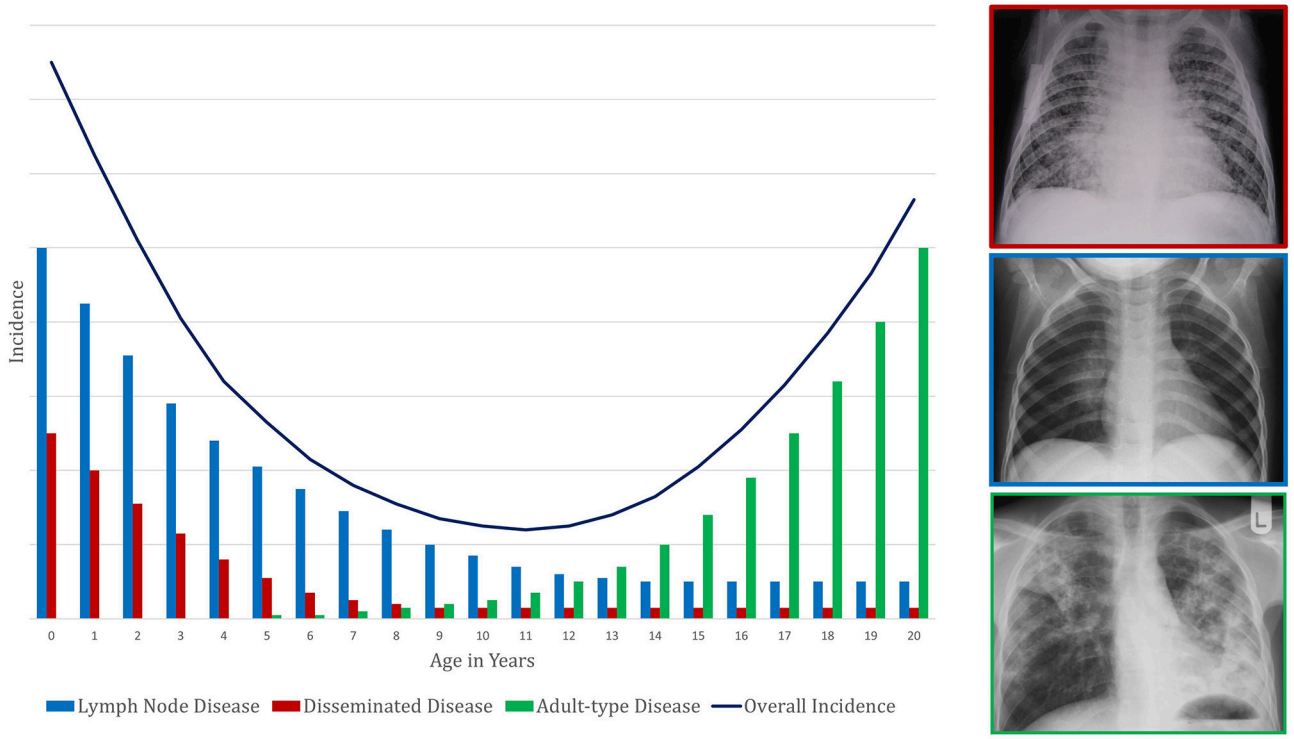


**FIGURE 1 |** Conceptual framework to demonstrate the pattern of change in tuberculosis incidence with age. This represents a composite of risk of infection and risk of subsequent disease progression. The presentation of disease is demonstrated by a representative X-ray in a box colored according to the disease phenotype legend.

in not being able to distinguish the timing, nor current state, of infection. The most commonly used term for this state is "latent TB infection (LTBI)," but "LTBI" is a suboptimal term because it inaccurately implies both the metabolic dormancy of the mycobacteria and its persistence in the host (12). "TB disease" refers to the wide continuum of radiologically and/or clinically apparent abnormalities caused by the host response to *M. tuberculosis*. Just as "TB infection" often is called "LTBI," "TB disease" commonly is referred to as "active TB."

Finally, we define pathologically distinct clinical phenotypes of TB disease (**Figure 1**). "Intrathoracic lymph node TB" is generally paucibacillary and results due to spread from the site of the initial mycobacterial infection (the Ghon focus) to the regional lymph nodes. This manifestation historically was encompassed within "primary TB," a term now less commonly used. The "Ghon complex" is the name for the triad of the Ghon focus, surrounding lymphangitis, and regional intrathoracic lymphadenopathy. "Adult-type pulmonary TB" refers to disease that spreads bronchogenically within the lungs and may occur following prolonged initial control of infection. It typically initiates within the apico-posterior segments of the upper lobes or superior segment of the lower lobes; it appears as parenchymal infiltrates, often with cavitation, which facilitates contained expansion of bacillary numbers accompanying extracellular replication. Other terms for this clinical phenotype are "post-primary TB" and "reactivation TB," now less commonly in use. While 60% of adult TB is pulmonary, localized disease can occur in other organs and is termed "extra-pulmonary" TB. "Disseminated TB," also known as "miliary TB," refers to the clinical manifestations of unrestrained haematogenous spread of mycobacteria (13, 14).

## CHANGES IN TUBERCULOSIS DISEASE PRESENTATION BY AGE

As described above, age impacts the clinical phenotype of TB disease during childhood and adolescence (**Figure 1**). Pre-pubertal children, including both young children and primary school children, generally develop intrathoracic lymph node TB (15), which can have complications. If regional lymph nodes enlarge substantially, they can compress airways. If the node ulcerates into the bronchi and deposits caseous material into the airway, then inhalation will lead to dispersal of the mycobacteria throughout the portion of the lung supplied by that airway. This dispersal can lead to inflammation caused by acute hypersensitivity or segmental/lobar bronchopneumonic disease.

Young children, particularly those <2 years of age, also have a high risk of disseminated TB and/or TB meningitis. Disseminated TB can present with lung pathology (including the classic miliary picture seen on chest radiograph) and/or disseminated lesions throughout the body, including liver, spleen, gut, bone, and kidney. TB meningitis usually starts with an insidious clinical picture that rapidly progresses to neurological deficit. If untreated, both disseminated TB and TB meningitis almost universally lead to death (16, 17). During the primary

school years, the risk of these disease forms is minimal. As children become older and approach puberty, they increasingly tend to develop adult-type pulmonary TB (6, 9, 18–20). This clinical phenotype also leads to disease transmission (21).

## AGE-RELATED RISK OF TUBERCULOSIS PROGRESSION AND MORTALITY

Our understanding of the impact of age on risk of progression from TB infection to disease comes from observational studies from the pre-chemotherapy era, many of which were summarized in a review by Marais et al. (4). This review included seven studies, which were conducted in Europe and North America after the advent of the TST and chest radiography, but prior to the discovery of anti-TB drugs and the HIV epidemic. The studies, which had a combined sample size of over 10,000 children and adolescents, evaluated the risk of progression to TB disease for TB-infected individuals of different ages. **Table 1** summarizes these studies, as well as other relevant reports, including a trial of the Bacillus Calmette-Guérin (BCG) vaccine with over 82,000 TB-infected children and adolescents in the control arm (18).

The risks of disease progression described in these studies should be interpreted with caution. First, to define the age of acquisition of TB infection, some studies used baseline TST positivity and/or identification of the child or adolescent as a contact of a TB case (**Table 1**). Using these methods, it is difficult to establish when an individual was infected. Even if a source case is identified in the household and the child or adolescent screened soon thereafter, it is often unclear how long he or she had been exposed. It may also be unclear if the child or adolescent had prior exposure to another infectious TB case. Second, the TST has limited sensitivity and specificity to diagnose TB infection in young children. Third, the diagnosis of TB disease in children can be challenging. A clinical diagnosis can lack specificity due to the overlap in symptoms between TB and other conditions in children. Microbiological confirmation lacks sensitivity due to the paucibacillary nature of most forms of childhood TB disease, as well as the challenges in obtaining respiratory specimens from children. Finally, these studies were conducted in different settings, including wartime conditions, which likely impacted rates of progression to TB disease.

The widely varying risks of TB disease progression described in these studies reflect the limitations described above. Other reasons for the wide variation include the different quantities and varieties of tuberculin used across studies, as well as inconsistent definitions of TST positivity and TB disease. Additionally, the studies calculate risk of disease progression over different follow-up periods. Because risk of disease progression is greatest in the first year after acquisition of *M. tuberculosis* infection and then declines over time (36, 37), risks that are given per person-year inversely correlate with the length of follow-up. Not all the studies cited in **Table 1** include risk of disease progression over the full spectrum of ages from infancy through late adolescence, and the studies use different age groupings. Nonetheless, as **Table 1** illustrates, there is a general pattern of high risk during early childhood (particularly the first 2 years of life), a nadir during the primary school years or early adolescence (most likely occurring right before the onset of puberty), and a second peak during late adolescence. This overall picture reflects the different types of childhood TB, with falling rates of disseminated and lymph node TB in early childhood, superimposed on a rise in adult-type TB coinciding with puberty onset. Given that, in high burden settings, the risk of being infected with *M. tuberculosis* increases throughout childhood due to cumulative exposure (38), the resulting TB disease incidence seen in a community is a composite of risk of infection combined with risk of disease progression following infection. The TB incidence in high burden settings reflects the risk of disease progression, namely a U-shaped pattern, only shifted a few years older so that the nadir is at about the age of 10 years (**Figure 1**).

The data in **Table 1** show that mortality from TB disease follows the same pattern of age-related risk. Again, despite this consistent pattern, the rates themselves vary considerably, reflecting the different conditions in which the studies were conducted and the different denominators (e.g., whole populations of geographic areas vs. children and adolescents hospitalized for TB). A recent systematic review and meta-analysis that evaluated the risk of death in children and adolescents <15 years of age with TB disease found that mortality from untreated TB disease (in the pre-chemotherapy era) was 44% in children <5 years but only 15% in 5 to <15-year-olds. Mortality in individuals with TB who were diagnosed and treated was <1% (39).

## DIFFERENCES IN TUBERCULOSIS RISK BY GENDER

Although this article focuses on age-related differences in TB risk, it is worth noting that multiple studies have documented gender-based differences in TB risk, emerging coincident with the adolescent rise in TB risk and persists during adulthood. Overall, more males globally develop TB each year; compared to males, females tend to have higher risks of progression from TB infection to disease during adolescence. In a cohort of over 400,000 school children aged 6–19 years in Massachusetts, U.S.A., the incidence of TB disease among female TST reactors was approximately double that of male reactors (25). A TB vaccine trial that followed over 54,000 14- and 15-year-olds reported a 20% higher incidence of TB disease for girls than for boys (40). In a cohort of TB-infected children treated at Bellevue Hospital in New York City, twice as many girls as boys developed pulmonary infiltrates, and in more than a quarter of cases, adult-type pulmonary TB developed within a year of menarche (41). A study of over 82,000 children and adolescents in Puerto Rico, a trial of isoniazid prophylaxis in Alaskan Inuits, and an observational cohort in Ontario, Canada, all found that compared to males, females had higher rates of progression to TB disease in adolescence and early adulthood (18, 42, 43).

Similar patterns have been observed with respect to mortality. From 1880 to 1930 in Massachusetts, girls between the ages of 10 and 20 years had nearly twice the risk of death from TB

**TABLE 1 |** Age-related risks of progression to and mortality from tuberculosis disease.

| Location | Timeframe | Type of study | Sample size (denominator) from which risk was calculated[a] | Inclusion criteria | Definition/measurement of endpoint (TB disease or death)[b] | Risk per 1,000 person-years | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Ages 0 to <5 | Ages 5 to <10 | Ages 10 to <15 | Age 15 to <20 |
| **STUDIES REPORTING RISK OF PROGRESSION TO TB DISEASE, STRATIFIED BY AGE (IN YEARS) AT TIME OF DIAGNOSIS OF TB INFECTION** | | | | | | | | | |
| Minneapolis, MN, U.S.A. (22–24) | 1921–1941 | Cohort study | 3,612 | TST positivity at enrollment or TST-conversion during follow-up; TST positivity defined as induration of ≥5 mm to 0.1 or 1.0 mg of old tuberculin | "Clinical TB," not including "primary pulmonary infiltrates" on CXR in the absence of symptoms | 257 (age 0 to <1); 160 (age 1 to <2); 143 (age 2 to <3); 50 (age 3 to <4); 46 (age 4 to <5)[b] | 48 (age 5 to <6); 38 (age 6 to <7); 45 (age 7 to <8); 78 (age 8 to <9); 49 (age 9 to <10)[b] | 49 (age 10 to <11); 40 (age 11 to <12); 59 (age 12 to <13); 77 (age 13 to <14); 82 (age 14 to <15)[b] | 50 (age 15 to <16); 108 (age 16 to <17); 52 (age 17 to <18)[b] |
| Massachusetts, U.S.A. (25) | 1924–1934; f/u period 1–12 (mean 11.4) years | Cohort study | 64,834 | Positive reaction (not further defined) to the von Pirquet tuberculin test | (1) All cases and deaths that were reported in Massachusetts from 1924 to 1936 and matched one of the individuals in the cohort, or (2) radiographic and clinical diagnosis of TB in a subset with follow-up exams | n/a | 0.4 | 1.7 | 3.7 |
| London, U.K. (26) | 1930–1954; f/u period 2–25 (mean 9) years | Cohort study | 1,567 | History of contact with TB case; no TST required for study entry | Children developing tuberculous lesions, further divided into intrathoracic and extrathoracic | 608 [b] | 432 [b] | 409 [b] | n/a |
| Kinn Administrative District, Norway (27) | 1937–1944; f/u period through 1945 | Survey | 152 | ≥3 mm induration to the von Pirquet tuberculin test | TB disease diagnosed through clinical symptoms, signs, and radiology | 778 (age 0 to <7) | 778 (age 0 to <7); 603 (age 7 to <15) | 603 (age 7 to <15) | 583 (age 15 to <20) |
| Newcastle-upon Tyne-and Northumberland, U.K. (28) | 1941–1961; f/u period 1–10 years | Cohort study and literature review | 2,376 | 8 different studies included; each study used slightly different entry criteria | Diagnoses of TB meningitis, miliary TB, pleural TB, skeletal TB, or pulmonary TB | 447 (0 to <1); 48 (0 to <2); 68 (0 to <5); 265 (0 to <7)[b] | 265 (0 to <7)[b] | n/a | n/a |
| Brentwood, Essex, U.K. (29) | 1942–1953; f/u period 5–10 years | Cohort study | 317 | Diagnosis of simple primary TB | Complications, including pulmonary TB, grave haematogenous TB, and other extrapulmonary disease | 150 (age <2); 139 (age 2 to <5)[b] | 144[b] | 177[b] | n/a |
| Puerto Rico, U.S.A. (18) | 1949–1969; f/u period 18–20 years | Control arm of BCG vaccine trial | 82,269 | No receipt of BCG vaccine and ≥6 mm induration to 1 or 10 units of PPD | TB disease confirmed by death certificates, case reports, and reports of admission to TB hospitals and clinics | 1.648 (age 1 to <7) | 1.648 (age 1 to <7); 0.77 (age 7 to <13) | 0.77 (age 7 to <13); 0.946 (age 13 to <19) | 0.946 (age 13 to <19) |

*(Continued)*

**TABLE 1** | Continued

| Location | Timeframe | Type of study | Sample size (denominator) from which risk was calculated[a] | Inclusion criteria | Definition/measurement of endpoint (TB disease or death) [b] | Risk per 1,000 person-years | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Ages 0 to <5 | Ages 5 to <10 | Ages 10 to <15 | Age 15 to <20 |
| **STUDIES REPORTING RISK OF MORTALITY FROM TB DISEASE, STRATIFIED BY AGE (IN YEARS) AT TIME OF DEATH** | | | | | | | | | |
| Philadelphia, PA, U.S.A. (30) | 1920 | Epidemiologic survey | Not specified | All white children in Philadelphia (including children without TB disease) | Death from all forms of TB, data source not specified | 0.33 (age <1), 0.14 (age 1 to <5)[c] | 0.07[c] | 0.138[c] | 0.763[c] |
| Baltimore, MD, U.S.A. (31) | 1928–1937; f/u period 1–10 years | Cohort study | 1,117 | Child contacts of TB cases (including children without TB disease) | Mortality from TB, data source not specified | Caucasians: 11.86 (age <1), 4.3 (age 1 to <5); African-Americans: 59.68 (age <1), 18.55 (age 1 to <5) | Caucasians: 0.88; African-Americans: 3.8 | Caucasians: 0; African-Americans: 2.77 | Caucasians: 7.54; African-Americans: 21.62 |
| Massachusetts, U.S.A. (32) | 1930 | Epidemiologic survey | Not specified | All children in Massachusetts (including children without TB disease) | Deaths from all forms of TB, from U.S. Mortality Statistics | Males: 0.41; Females: 0.27 | Males: 0.11; Females: 0.13 | Males: 0.21; Females: 0.37 | |
| Kingsport, TN, U.S.A. (33) | 1930–1931 | Epidemiologic survey | Not specified | All children (including healthy children) from 132 African-American households with TB | TB-related deaths reported from interviews with family members | 2.9 (age <1), 0.8 (age 1 to <5)[b] | 1.6[b] | 4.2[b] | |
| Stockholm, Sweden (34) | 1930–1938 | Cohort study | 453 | Diagnosis of primary TB | Death, data source not specified | 359 (age <1); 156 (age 1 to <3); 44 (age 3 to <7)[c] | 44 (age 3 to <7); 8 (age 7 to <16)[c] | 8 (age 7 to <16) [c] | n/a |
| New York, NY, U.S.A. (35) | 1930–1947 | Cohort study | 964 | Radiologic evidence of primary TB | Deaths from TB meningitis and other complications of primary TB, including disseminated forms and local progression of primary forms | 475 (age <0.5); 360 (age 0.5 to <1); 230 (age 1 to <2); 280 (age 2 to <3); 150 (age 3 to <5)[b,c] | 150[b,c] | 210[b,c] | n/a |
| Brentwood, Essex, U.K. (29) | 1942–1953; f/u period 5-10 years | Cohort study | 712 | Diagnosis of TB disease of any severity, including simple primary TB | Deaths from TB, data source not specified | 60 (age <2); 1 (age 2 to <5)[b] | 1[b] | 40[b] | n/a |

[a] *Whereas, Marais and colleagues reported the entire study population in their 2004 review article, we are reporting the population from which the risk was calculated.*

[b] *Risks given as per 1,000 persons rather than per 1,000 person-years.*

[c] *These risks were extrapolated from a line graph in the original report, and age was defined at time of diagnosis of TB disease.*

*BCG, Bacillus Calmette-Guérin; CXR, chest radiography; f/u, follow-up; PPD, purified protein derivative; TB, tuberculosis; TST, tuberculin skin test.*

compared to boys in the same age range (32). More recent surveys conducted in India and China also showed that females during adolescence had higher rates of mortality due to TB than males (44).

It is difficult to establish whether these data reflect a greater risk of disease progression in post-pubertal females compared to post-pubertal males on an individual level, or whether females as a group have a greater risk of disease progression because they enter puberty sooner and, thus, have longer post-pubertal periods during adolescence.

## KEY STAGES IN THE IMMUNOPATHOLOGY OF TUBERCULOSIS

There are a number of key stages in the host response to *M. tuberculosis* in humans, each governed by particular immune mechanisms, many of which remain incompletely understood. Age-related differences in risk and presentation may be explained by differences in the immune response at each of these key stages (**Figure 2**).

Exposure to infectious cases of TB can lead to inhalation of droplet nuclei containing viable *M. tuberculosis*. These infectious droplets may be cleared by the physical structures of the lung or by the innate immune system, but if they overcome these primary barriers and sensitize the adaptive component of the immune system without effective killing, the individual becomes infected with *M. tuberculosis*. Upon initial inhalation, bacilli are phagocytosed by the alveolar macrophage, which recruits neutrophils and other innate responders as a first line of defense. However, the ability of the innate immune response to control infection may be inhibited by bacterial-mediated mechanisms, particularly inhibition of phagolysosome fusion (the key bacterial killing mechanism of these phagocytes), resulting in persistence

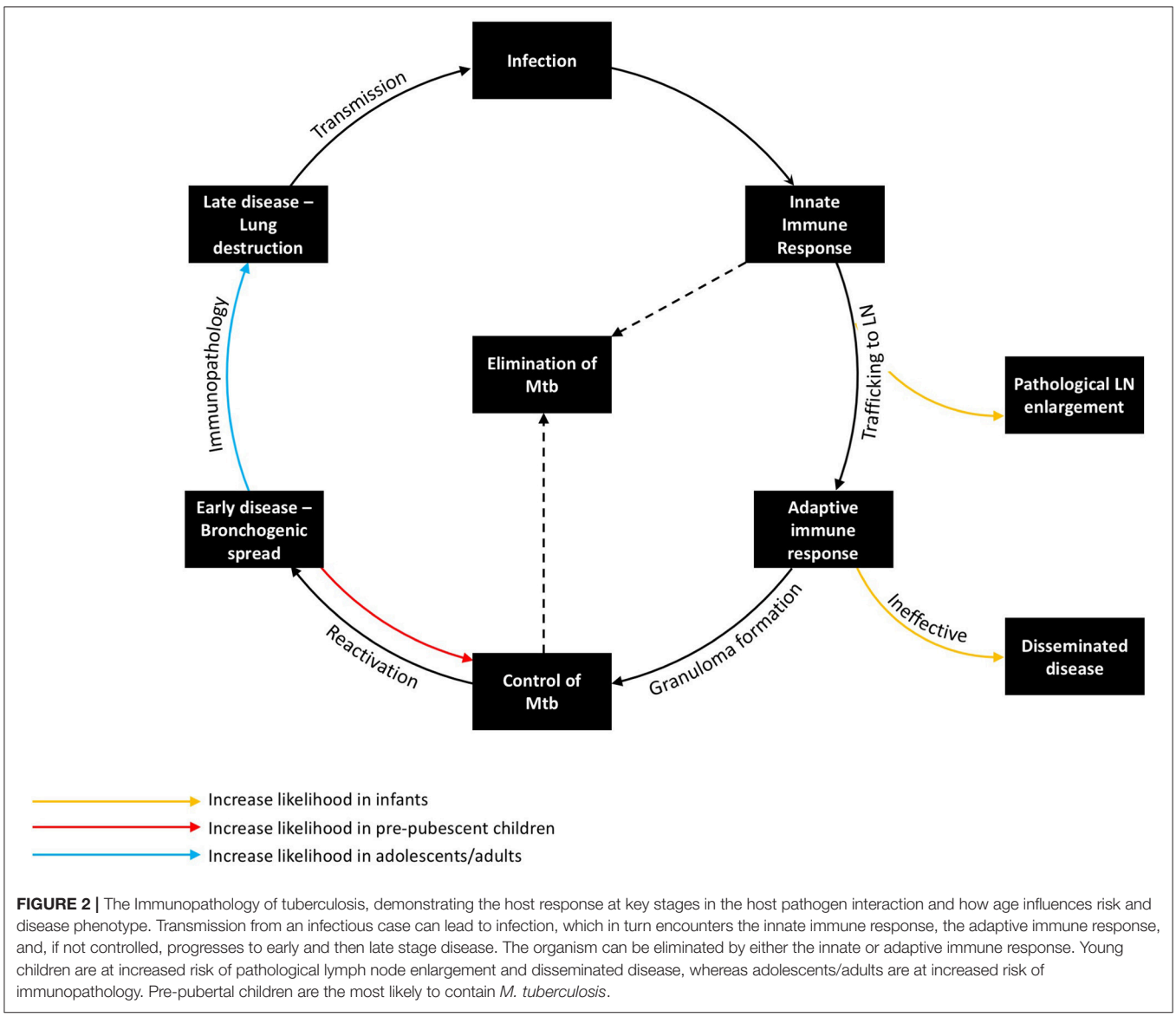


**FIGURE 2 |** The Immunopathology of tuberculosis, demonstrating the host response at key stages in the host pathogen interaction and how age influences risk and disease phenotype. Transmission from an infectious case can lead to infection, which in turn encounters the innate immune response, the adaptive immune response, and, if not controlled, progresses to early and then late stage disease. The organism can be eliminated by either the innate or adaptive immune response. Young children are at increased risk of pathological lymph node enlargement and disseminated disease, whereas adolescents/adults are at increased risk of immunopathology. Pre-pubertal children are the most likely to contain *M. tuberculosis*.

of bacilli intracellularly. It is during this period that initial antigen trafficking to the lymph nodes by dendritic cells (DCs) is felt to occur. The acquired immune response to *M. tuberculosis* usually develops 1–3 months after initial infection (as evidenced by TST or IGRA immune sensitization), with antigen-specific lymphocytes trafficking back into the lung, facilitating activation of macrophages and granuloma formation (45). At the center of the granuloma, macrophages may fuse together, forming multinucleated giant cells or differentiating into lipid-rich foam cells (46, 47). Meanwhile, the neutrophils, which are short lived, undergo necrosis, contributing to a caseous center. This granulomatous response, if able to activate macrophages sufficiently to control bacterial replication, is thought to aid in containment of bacterial spread and reduce bacillary numbers, hence controlling or potentially eliminating *M. tuberculosis*.

Deficiencies in the antigen-specific Th1 immune response, which have been well-described in children <2 years-old, likely contribute to the presentation of locally progressive and disseminated TB in this age group (48, 49). BCG vaccination boosts mycobacterium-specific cell mediated immunity, which is associated with a reduced incidence of disseminated disease (50). Following early childhood, the initial granulomatous control of infection likely is effective (51). If infection is not eliminated by this stage, there is a risk of reactivation. Factors impeding effective T-cell-macrophage interaction within the granuloma increase the likelihood of disease progression. Some of these factors are well-known causes of systemic immunosuppression, such as HIV, malnutrition, and anti-tumor necrosis factor (TNF) therapy (12). While many of the factors that precipitate reactivation are poorly understood, seasonal changes in vitamin D levels or concurrent viral infection (e.g., influenza) may be involved (52, 53). Following failure of granulomatous control of *M. tuberculosis,* a pathologically distinct phase ensues in the lung. During this phase, which typically is characterized by a pneumonic process with bronchogenic spread, the number of organisms initially is low, although abundant antigen may be detected in uninfected cells and tissue (54). Progression of this early stage of reactivation TB is not linear; regression and self-healing of lesions are common (55). A critical event during this phase appears to be lung necrosis, which is likely caused by a number of pro-inflammatory mechanisms. Following lung necrosis, an increase in bacillary number is observed (55). Recently, necrotic cells themselves have been shown to be a niche for bacillary replication (56). Contributors to this inflammatory pathology may include immune complex deposition; complement activation; neutrophil recruitment; cell-mediated cytotoxicity directed against infected and uninfected antigen-containing cells; and tissue-degrading enzymes, which have the role of facilitating cell recruitment and vascularization, but result in the breakdown of tissue architecture (57–59). The late stage of adult-type disease is then characterized by lung destruction, cavitation, and a localized exponential growth of extracellular organisms that is most commonly seen in immunocompetent adults, who are the main contributors to TB transmission (21). The lower rate of disease seen in primary school children may relate to increased likelihood of a favorable outcome during the early stage of reactivation TB, resulting in its resolution (**Figure 2**). It is possible that changes in the immune response that occur during puberty increase the likelihood of necrosis and disease progression.

## IMPACT OF AGE ON THE HOST RESPONSE TO *M. tuberculosis*

To understand the potential mediators of protective immunity which exists during the pre-pubertal "Wonder Years," this unique age needs to be studied relative to the changes that occur as the immune system develops in infants and young children, and the further changes that occur during puberty. The primary driver of these developmental changes are sex hormones. Whilst the primary role of these hormones is development of reproductive organ function, they have wide-ranging effects on the immune system [for a detailed review see (60–62)]. Sex steroid receptors are found in the cytoplasm of the majority of immune cells, including T cells, B cells, DCs, natural killer (NK) cells, neutrophils, and macrophages (63). Once internalized in the cell, sex hormones bind their respective steroid receptors, inducing translocation to the nucleus and regulation of gene transcription via a variety of mechanisms. These mechanisms include direct binding to hormone response elements in DNA promoters to activate transcription; complexing with other transcription factors, such as AP-1, SP1, C/EBPβ, and NFκB; or indirectly binding DNA via chromatin-modifying co-regulators (64, 65).

Multiple fluctuations in hormonal exposure occur throughout life, but the greatest changes occur during *in utero* development, puberty, and menopause (**Figure 3**). *In utero*, maintenance of foeto-maternal immune tolerance is critical to enable full term pregnancy. Fetal and maternal Th1 responses are harmful to the pregnancy and associated with pre-term labor and spontaneous abortion (66). High progesterone levels contribute to polarizing maternal and fetal immunity toward a more tolerant Th2 response (67, 68). The progesterone receptor (PR) is expressed by NK, DC, macrophages, and T cells (69). Activation of PR (a) downregulates TNF, IL-1β, IL-6, and IL-23 from DC (70–72); (b) decreases microbial activity of macrophages as well as downregulating inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production and polarizing macrophages to an M2 alternative-type (73); (c) decreases cytotoxicity and interferon-gamma (IFNγ) production from NK and CD8 cells (62, 74); and (d) increases expression of anti-inflammatory transcription factor FOXP3 in regulatory T cells (Tregs) and reducing IL-17 production (75). The high number of Tregs in the fetal circulation contribute to fetal tolerance of maternal alloantigen. This Th2 bias and increased number of peripheral Tregs persist into the neonatal period and affect responses to foreign antigen (49). This tolerant phenotype, along with the reduced antimicrobial activity and poor antigen presentation of innate immune cells, would be expected to contribute to impaired granulomatous control of intracellular *M. tuberculosis* infection in infants, resulting in high risks of disease progression and extrapulmonary dissemination (**Figures 1**, **2**).

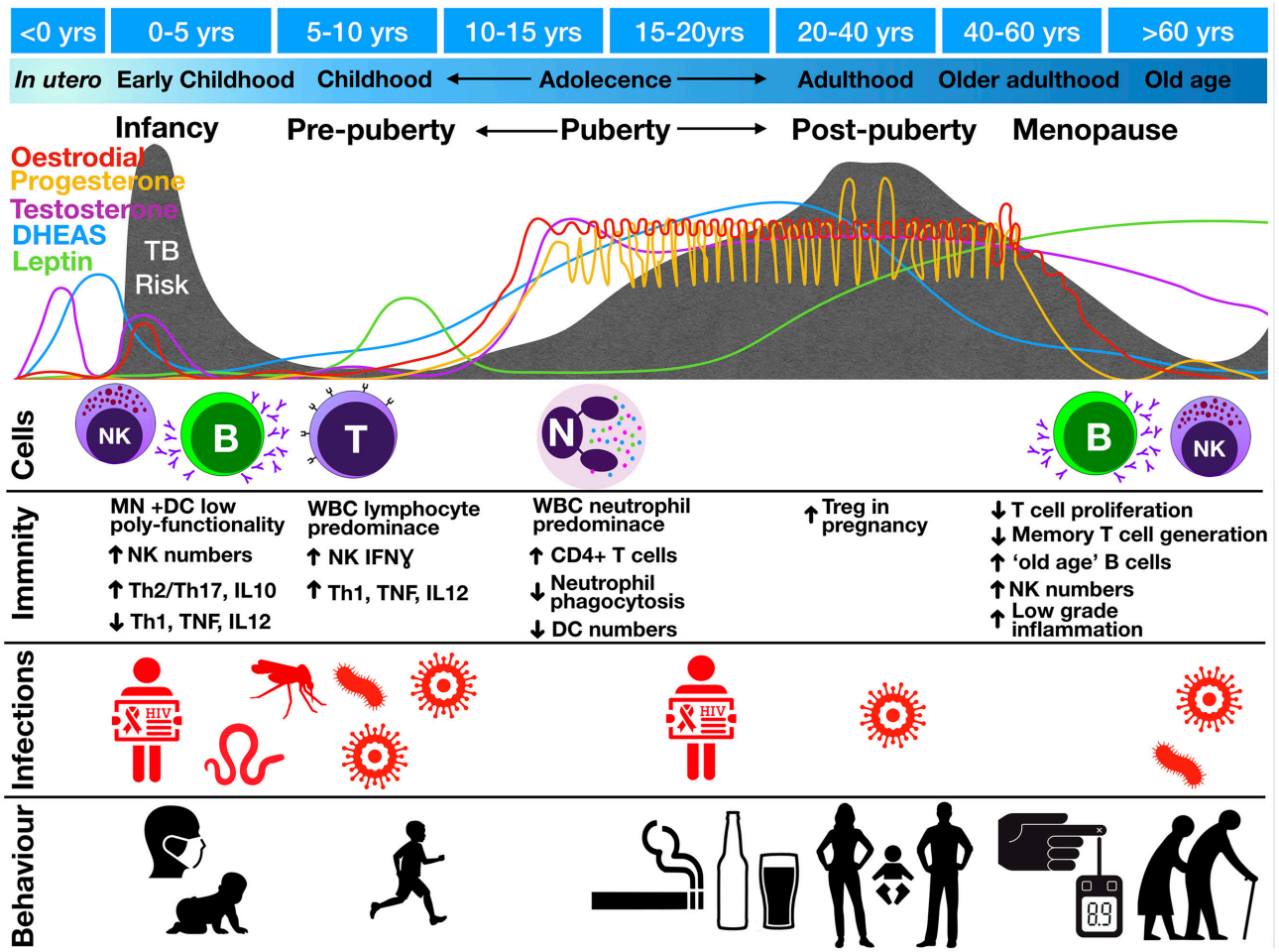


**FIGURE 3 |** Change in tuberculosis risk throughout life stages compared to fluctuations in hormone levels, circulating cell populations ("Cells"), immune functions ("Immunity"), co-infections ("Infections"), and social interactions and behaviors ("Behavior"). TB risk is indicated by the gray shading with peaks of risk indicated underneath various life course stages. Images represent infections: mosquito, malaria; worm, helminths; virus, EBV, CMV, influenza; and behaviors: infant exposures to adult TB, children playing outdoors, smoking, drinking, sexual activity, pregnancy, Diabetes Mellites and old age. B, B cells; DC, dendritic cells; IFN, interferon; MN, monocytes; N, neutrophils; NK, natural killer cells; T, T lymphocytes; Th, T helper; Treg, regulatory T cells; WBC, white blood cells.

Whilst babies are born with an "underdeveloped" immune system due to low antigen exposure *in utero*, they experience rapid antigen exposure immediately following birth. During this period, both arms of the innate and adaptive immune system mature. Monocyte and DC maturation occurs during the 1st year of life (76), whilst NK cell function normalizes around the age of 5 years (77). NK cell function also changes as children progress into adolescence, switching from IFNγ-producing to more cytotoxic (77, 78). Infants have impaired type I IFN and Th1 (TNF, IFNγ, IL-12) responses and higher Th2/Th17 and IL-10 production (79). Early monocytes and DCs also show less polyfunctionality in cytokine production (80). This underdeveloped innate and adaptive immune system, with suboptimal innate cell-mediated killing and suppressed effector cell responses, is hypothesized to lead to poor outcome following *M. tuberculosis* infection, including widespread dissemination.

Puberty is defined by adrenarche and gonadarche, two temporally correlated but physiologically distinct events. Adrenarche, which begins around age 6, is marked by the onset of production of dehydroepiandrosterone (DHEA), the primary adrenarche hormone, and its storage form, DHEA-sulfate (DHEAS), hereafter collectively referred to as DHEA(S). DHEA(S), which leads to the development of axillary and pubic hair and sweat glands, is the most abundant steroid hormone in the body (81). In gonadarche, production of oestradiol or testosterone signals the maturation of reproductive organs and secondary sex characteristics. In addition, leptin—an adipocyte-produced hormone that regulates energy expenditure and signals the body is prepared for sexual maturation—increases at the onset of puberty (82).

In both sexes, the number of circulating leukocytes changes during the ages of 12–18 years, predominated by a drop in the high number of B cells found in young and primary school children and a rise in helper T lymphocytes (83). The functional requirement of these T cells for TB immunity was demonstrated in a recent study investigating the whole blood transcriptional signature of children and adolescents with pulmonary and extra-pulmonary TB. It identified a decline in T cell transcripts with

increasing disease severity, with lower transcript levels found in those with TB meningitis compared to those with pulmonary TB. Moreover, this decline was associated with a functional defect in T cell proliferation following broad T cell receptor stimulation, which recovered after treatment (84).

The lymphocyte predominance seen in pre-pubertal years is overtaken by a predominance of neutrophils during adolescence and into adulthood (83), with neutrophils being considered a driver of cavitation and immunopathology in adult pulmonary TB (85). Early studies also show that the phagocytic capacity of neutrophils, at least to *Staphylococcus aureus,* decreases significantly during adolescence, with maximal kill peaking at 14 years of age (86). A decline in neutrophil phagocytic ability after the age of 14 would thereafter correspond to the rise in TB risk observed in late adolescence. The decreased phagocytic activity may be hypothesized to lead to increased necrosis and more tissue damage synonymous with adult pulmonary TB.

## IMPACT OF SEX ON THE HOST RESPONSE TO *M. tuberculosis*

### Sex Differences in Inflammatory Responses

In general, it is considered that of the sex hormones, oestrogens have immune-enhancing effects, such that the level of inflammatory response is generally higher in females, whilst progesterone and androgens, such as testosterone, exert mainly immunosuppressive properties (**Table 2**) (87). Moreover, males generally have a Th1 skewed response, and females, Th2 (63). Thus, females are thought to have a stronger innate and adaptive immune response, although overall skewed to a Th2 state (**Figure 4**) (89).

Androgens, including dihydrotestosterone (DHT) and testosterone, suppress Th2/Th17 responses and decrease antibody responses and B cell proliferation (87, 88, 129, 138). With regard to innate cells, testosterone treatment increases IL-12, and IL-1β production in monocytes, whilst reducing IL-6 (99, 100). In DCs it reduces Th2 responses including suppressing IL-4, IL-10, and IL-13 production (129). Conversely, following TLR stimulation, estrogen induces the genes *TLR7*, *MYD88*, *RIG1*, *IRF7*, *IFNB*, *AJK2*, *STAT3*, *NFKB*, *IFNG,* and *TNF*—many of which have estrogen response elements in their promoters (62, 139). Consequently, these genes have higher expression in females after vaccination (139). Estrogens also elicit rapid non-nuclear responses via binding estrogen receptors associated with the inner plasma membrane. This activates NO and cyclic AMP production, the mobilization of intracellular calcium and the stimulation of protein kinase pathways, such as PI3K/AKT and ERK (112).

With respect to changes in lymphocyte populations as children progress into adolescence, as sex hormones begin to exert their effects on cell development, differences in the frequency of different cell populations begin to emerge. Males have been shown to have a higher number of circulating CD8 and NK cells, indicating a higher frequency of cytotoxic cells (62, 133). Males develop a more robust innate immune response early in development (140), which may contribute to early infection control and slower disease progression. However, once disease develops, these features may contribute to greater immunopathology and severity of disease in males. Conversely, females develop higher circulating levels of immunoglobulins (Igs) and B cell receptor expression (136); a lower number of B cells; a higher number of CD4+ T cells; and a higher CD4:CD8 ratio (86, 141, 142). Given the recent support of Fc-mediated antibody protection in latently infected individuals, including enhanced phagolysosomal maturation and antimycobacterial activity of macrophages (143), the decreased antibody production and B cell proliferation in males may contribute to gender differences in TB prevalence and presentation.

The difference in TB case prevalence between genders begins to appear in mid-adolescence (144). This timing corresponds to the steep rise in production of adrenarchal and gonadarchal hormones, which decline in the fourth or fifth decade of life (**Figure 3**) (81, 145–147). Levels of leptin, also follow the same pattern (148). These hormonal declines correspond temporally to the fall in TB rates, which occurs earlier and faster in women and corresponds to the transition into menopause, which generally begins in the late 30s or early 40s (62, 144). The association of a rise and decline in TB risk with the rise and decline in sex hormones supports a potential role for these hormones in controlling the inflammatory imbalance that leads to TB disease progression.

Sex differences in immune responses have been identified for the "type" of inflammatory response produced, i.e., Th1 vs. Th2; the level of regulation governed by transcriptional and post-transcriptional modifications; and differences in the absolute number of different circulating cell populations (**Table 2** and **Figure 4**). These differences can be due to differential exposure to sex hormones, as well as genetic and epigenetic differences impacting gene expression.

As the outcome of infection depends on the immune pathways activated by the pathogen and the pre-existing environment and propensity of the host to respond to those stimuli (140, 149), the sexually distinct activation pathways likely impact infection outcome. It has been hypothesized that even though females elicit more immune-enhancing effects, due to their inherent Th2 skewing, they have a more controlled response to Th1-inducing pathogens (140). Males, although generally immune-suppressive, are Th1-skewed, and thus are likely to elicit an exaggerated response to Th1-inducing pathogens, such a *M. tuberculosis* (140). Conversely, the strong Th2 and humoral response of females is hypothesized to be the underlying cause of higher rates of autoimmune diseases experienced by females (89, 150).

This gender difference in inflammatory response may explain the difference in *Mtb* lineages identified in TB patients due to an interaction between the inherent host inflammatory response and inflammatory pathways activated by different *Mtb* lineages (151). A recent large population study in Vietnam tracing 1635 *Mtb* strains by whole genome sequencing found that young people and females in particular are more susceptible to TB caused by the Beijing 2.2 lineage whilst males and the elderly are more susceptible to Lineage 1 strains (152).

**TABLE 2 |** Impact of hormones on components of the immune system implicated in the immune response to *Mycobacterium tuberculosis*, as reported in humans, animals, and *in vitro* models.

| General response | DHEA(S) | Adiponectin | Leptin | Oestradiol | Progesterone | Testosterone |
|---|---|---|---|---|---|---|
| Immune phenotype (87, 88) | Enhancing | Suppressive | Enhancing | Enhancing | Suppressive | Suppressive |
| T cell polarization (63, 67, 68, 87, 89) | Th1 | | Th1 | Th2 | Th2 | Th1 |
| **MACROPHAGE ACTIVITY** | | | | | | |
| TNF expression[§] (64, 70–72, 90–97) | ↑↓ | ↓ | ↑ | ↑ at low levels, ↓ at high levels[#] | ↓ | ↑↓ |
| IL-12 expression[§] (63, 64, 90, 91, 97, 98) | ↑ | ↓ | ↑ | ↑ | ↓ | |
| IL-1β, IL-6 expression (70, 71, 99–101) | | | | ↑ at low levels, ↓ at high levels[#] | ↓ | ↑ |
| IFNα expression (102) | | | | ↑ | | |
| Foam cell differentiation (103–109) | ↑ | ↓ | ↑ | ↓ | ↓ | ↑ |
| Phagocytic activity (97, 110, 111) | | | ↑ | ↑ | ↓ | ↓ |
| Microbial activity (iNOS, NO) (73, 112–115) | | | ↑ | ↑ | ↓ | ↑↓ |
| Autophagy of *Mtb*-infected macrophages (116) | ↑ | | | | | |
| Lung granuloma formation[§] (97, 114, 117) | | | ↑ | ↑ | | ↓ |
| **DENDRITIC CELL ACTIVITY** | | | | | | |
| MHC expression (70, 90, 91, 118) | ↑ | ↓ | | ↑ | ↓ | |
| $CD1a^+$ expression (119) | | ↓ | | | | |
| **NEUTROPHIL ACTIVITY** | | | | | | |
| Numbers (62, 120) | | | | ↑ | | ↑ |
| Degranulation (62) | | | | ↑ | | |
| Apoptosis (121) | | | | ↓ | ↓ | |
| Phagocytosis (122–124) | | | | ↑ | | ↑↓ |
| **LYMPHOCYTE ACTIVITY** | | | | | | |
| IFNγ expression (63, 64, 90, 91, 97, 117, 125, 126) | ↑ | ↓ | ↑ | ↑ | ↓ | ↓ |
| Th1 differentiation (87, 91, 127, 128) | ↑ | | ↑ | ↑ at low levels, ↓ at high levels[#] | ↓ | ↑ |
| Th2 (63, 67, 68, 87, 89, 129) | | | | ↑ | ↑ | ↓ |
| Th17 (75, 87, 130) | | | | ↓ | ↓ | ↓ |
| FOXP3, Treg (49, 131, 132) | | | | ↑ | ↑ | ↑ |
| Cytotoxicity of NK and CD8 (62, 74, 98, 133, 134) | | | | ↓ | ↓ | ↑ |
| IFNγ expression from NK and CD8 (124, 126, 135) | | | | ↑ | ↓ | ↓ |
| B cell proliferation, Antibody production (87, 89, 134, 136, 137) | | | | ↑ | ↓ | ↓ |

*DHEA(S), dehydroepiandrosterone (sulfate); IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; Mtb, Mycobacterium tuberculosis; NK, natural killer; NOS, nitric oxide synthase; Th, T helper; TNF, tumor necrosis factor; Treg, regulatory T cell. Red arrow indicates higher and blue arrow indicates lower, a blank box indicates no reported significant difference.*

[§]*Also expressed by or involves dendritic cells and Th1 cells.*

[#]*High levels of estrogen occur during the follicular phase of the menstrual cycle and during pregnancy.*

## Genetic and Epigenetic Causes of Differential Sexual Responses to Infection

Differential expression of genes on the X and Y chromosomes defines sexual development. Male development is mediated by the SRY gene on the Y chromosome, whilst females regulate X chromosome expression via X-linked gene inactivation. The differential level of X inactivation that occurs between males and females can impact the level of expression of X-linked genes between sexes [reviewed in Markle and Fish (60)]. The X chromosome also contains ∼10% of all 800 microRNA produced by humans, whilst the Y chromosome accounts for only 2% (153). Incomplete X-inactivation in females therefore increases the expression of regulatory miRNA, the expression of which can also be under hormone control, further increasing gene regulation differences between sexes. Toll-like receptor 7 (TLR7) which is activated by viral cytosolic nucleic acids, has higher expression in females than males (102). Subsequently, plasmacytoid dendritic cells (pDC) of females make double the amount of IFNα to

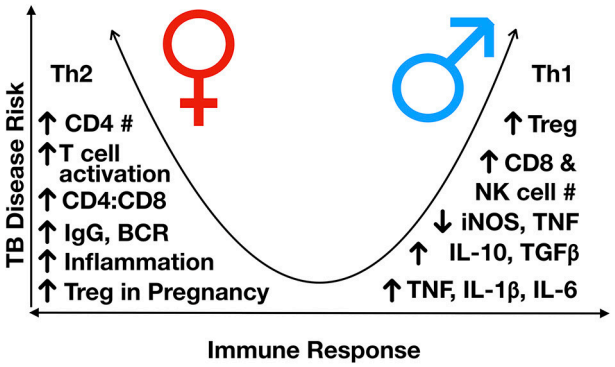


**FIGURE 4 |** Immunological differences observed between males and females, post-puberty. TB disease risk increases as the immune response more heavily favors either Th2 or Th1 skewing, with a more balanced Th1/Th2 response having the lowest risk of disease progression. In general, males are Th1 skews and females Th2 skewed, although females have a higher inflammatory response to an exogenous stimulus, partly mediated by variable X-inactivation and the presence of estrogen response elements in many immune response genes, leading to higher responses once activated. BCR, B cell receptor; Ig, immunoglobulin; IL, interleukin; NK, natural killer; iNOS, inducible nitric oxide synthase; Th, T helper; TGFß, transforming growth factor-beta; TNF, tumor necrosis factor; #, number.

TLR7 ligands, such as HIV (154). Type 1 interferons, IFNα and IFNβ, have recently been shown to have detrimental effects on *M. tuberculosis* control depending on their context of activation (155, 156); this finding suggests this difference in females may contribute to exacerbating an inflammatory imbalance, skewed to *M. tuberculosis* survival and proliferation. Conversely, in response to TLR8 and TLR9 agonists, male androgens suppress macrophage phagocytic activity, and correlate to higher IL-10 secretion (157). In addition, males have higher pro-inflammatory production following LPS stimulation of TLR4, including higher TNF production from neutrophils (158). Thus, viral and bacterial responses in males and females may also differ according to TLR expression and activation in each sex. Of particular relevance to TB pathology, compared to monocytes and T cells, neutrophils have greater differences in global methylation and gene expression profiles between males and females (159). Twenty-one genes expressed from neutrophils were identified to be differentially expressed between sexes. Meanwhile, one of the top two differentially expressed genes between male and female neutrophils, SEPT4, is often found in the neutrophil-driven whole blood signatures of TB (160–162).

## Sex Hormone Impact on Response to *M. tuberculosis*

As shown in **Table 2**, hormones may impact adolescent TB through multiple plausible mechanisms and potentially in a multifactorial manner. Increased DHEA(S) and leptin levels may promote foam cell differentiation (103–106), leading to disease progression and lung cavitation. DHEA(S), estradiol, and leptin may contribute to disease severity by promoting aggressive Th1 responses (63, 90, 91, 118, 127, 128). A study comparing TST response after Bacillus Calmette-Guérin (BCG) vaccination gives clinical support to the association between DHEA(S) and Th1 responses; in this study, pubertal subjects had higher DHEAS levels and larger TST reactions than pre-pubertal subjects (163). Likewise, the link between gonadarche and disease severity is strengthened by a guinea pig model that demonstrated associations between increased exposure to oestradiol and testosterone and greater mortality and more extensive, caseous lung lesions (164). Suppression of TNF by DHEA(S) may also contribute to disease progression (92–96).

The greater resistance of female mice to mycobacterium species has been demonstrated in a number of studies (113, 114, 165, 166). In a C57BL/6 mouse model of TB, male mice have accelerated disease and increased mortality compared to females. The male mice had increased lung bacterial loads, increased iNOS, IFNγ, IL-1α/β, and IL-6 production early and late in infection, and higher levels of various inflammatory chemokines, all of which correlated with increased bacterial burden (114). Castration of male BALB/c mice reduced mortality and increased levels of inflammation to those seen in female mice (113). However, converse to what was seen in C57BL/6 mice, during early infection, female and castrated BALB/c expressed higher lungs levels of *TNF*, *IFNG*, *IL12*, *iNOS*, and *IL17* than non-castrated males. Moreover, castration 60 days after infection increased the survival of male mice, decreasing bacterial load and increasing *TNF*, *IFNG*, and *IL12*. Interestingly, whilst female castration resulted in declines in bacterial numbers early during infection, they rose at day 60 compared to non-castrated females. Testosterone treatment of female mice also increases susceptibility to *M. marinum* (165), whilst oestradiol treatment of ovariectomised mice increases mycobacterial killing, synergistically with IFNγ (167). Testosterone treatment of rats increased recruitment of inefficient neutrophils following LPS treatment, with impaired bacterial activity decreased MPO activity and increased IL-10 and TGFβ expression (168). Together, these studies suggest that high levels of testosterone can contribute to disease progression in males, whilst estrogen elicits a more protective response. However, the difference in genetic backgrounds of the animals infected also creates different inflammatory processes correlated to disease. Thus, the effect of hormones will be governed by the overall inflammatory state of the individual, which may be further governed genetically, epigenetically or environmentally by other co-morbidities, including co-infections.

# BEYOND AGE- AND SEX-RELATED IMMUNOLOGICAL CHANGES IN CHILDREN AND ADOLESCENTS

Although age- and sex-related changes in the immune response to *M. tuberculosis* are a critical factor in the relative reduction in TB disease prevalence in primary school children and increase in adolescence, it is important to consider other contributing factors (**Figure 3**). The degree of TB exposure is one alternative explanation for and/or contributing factor to different risks of disease progression. A study from Canada found that among recently infected individuals, those who developed TB disease

had exposure to a higher number of infectious TB cases than those who did not (169). At the same time, a recent animal study suggested that repetitive aerosol exposure with *M. tuberculosis* drives greater lung tissue destruction, including cavitation, than a single exposure (170). This explanation fits with the increased socialization in adolescence that is assumed to occur in many societies, and may contribute to increased risk of TB progression (171). This generalization about adolescent social mixing is supported by data from various settings, including eight European countries, rural Andean communities in Peru, a South African township, and a city in Siberia, Russia. These cross-sectional surveys all found that on a daily basis, adolescents come into contact with 1.5- to 3-times as many individuals as young children do (172–176). However, the hypothesis that a higher number of TB exposures increases the probability of TB disease progression does not account for the higher risks observed in young children vis-à-vis primary school children, or adolescent girls vis-à-vis adolescent boys. Young children come into contact with fewer individuals on a daily basis than primary school children (172–176), and to our knowledge, there are no data to suggest that adolescent girls have more frequent exposure to infectious TB cases compared to adolescent boys. The social mixing studies showed no difference in mean number of contacts for females vs. males, but these data were not age-disaggregated (172, 175).

On the other hand, if one of the drivers of risk of disease progression is the intensity of exposure (i.e., the size of the bacillary load), then the increased risk during early childhood makes more sense. Data from various settings support the idea that young children, particularly those <2 years of age, spend more time with household contacts than primary school children and adolescents (173, 175, 176); thus, they are exposed to a larger inoculum of *M. tuberculosis* from caregivers with TB disease. For this reason, young children are more likely than older children and adolescents to become TB-infected from a household exposure (177, 178). However, when comparing primary school children and adolescents, social mixing studies did not find significant differences in the amount of time spent with individual contacts (173–175).

Initiation of substance use and sexual activity also may contribute to the increase in TB disease progression and mortality in adolescents. Recreational drugs, alcohol, and smoking all are associated with increased risk of TB disease (179–182). Sexual activity may lead to pregnancy, which can predispose a woman to TB disease progression due to a wide range of immunosuppressive effects (183, 184). Moreover, sexual activity increases the risk of acquiring transmissible infections, including HIV.

Among co-infections that may contribute to the age- and sex-related changes seen in TB risk, HIV is likely the most significant. As individuals enter adolescence and commence sexual activity, girls are more likely than boys to acquire HIV (185). Globally, adolescent females have higher HIV seroprevalence than adolescent males (186). Because HIV dramatically increases the risk of TB disease progression following infection (187, 188), this difference in seroprevalence may contribute to the higher TB disease risk in adolescent girls. It is possible that HIV prevalence is lower in the primary school years compared to both early childhood and adolescence, since untreated vertically transmitted HIV leads to death in ∼60% of children before age 2 years (189) and primary school children have minimal risk of horizontal transmission. However, this possibility is difficult to confirm because, to our knowledge, global childhood HIV prevalence has not been disaggregated into 5-year age bands (190). The data on age-based differences in risk of TB disease progression were collected prior to the HIV epidemic and exist in regions with little HIV; thus, HIV epidemiology cannot be the sole reason for age- and sex-based discrepancies in TB risk.

Other pathogens also may alter an individual's susceptibility to TB disease. Cytomegalovirus (CMV) has been implicated in TB disease pathogenesis (191); it exerts profound immunodysfunction on infants, and has been associated with increased TB risk in this age group (192, 193). Moreover, increases in CMV seroprevalence are highest during infancy and adolescence, and the acceleration of CMV seroprevalence is steeper in adolescent girls than boys (191).

TB epidemics demonstrate a seasonal pattern and follow influenza outbreaks (194–196). A study conducted in a Danish TB sanatorium in the mid-twentieth century found that a strong association between influenza and clinical exacerbation of TB (197). Other studies have found associations between influenza and excess TB mortality (198–200). It is possible that influenza—and perhaps other viruses affecting the lower respiratory tract—may predispose to TB disease progression and mortality by disrupting mucosal integrity and altering host immunology. This link could partially explain the increased TB risks experienced by young children, who are vulnerable to severe lower respiratory disease due to influenza and other respiratory viruses. As children reach primary school age, they become less vulnerable to lung disease from common respiratory viruses (201). With the notable exception of the 1918–1919 influenza pandemic, adolescents do not experience increased morbidity and mortality from respiratory viruses (200). Therefore, this hypothesis does not explain the rise in TB risk during adolescence.

Finally, helminths and malaria infections are both common in young children in high TB-burden settings and cause immune dysregulation that could impact TB risk (202–205). However, the incidence of these infections do not rise again during adolescence, so they do not explain the adolescents' elevated TB risk. It is therefore possible that different etiologies contribute to the elevated risk in young children and adolescents. Further research is needed to disentangle the contribution of exposure intensity and frequency, substance use, and co-infections to the U-shaped pattern of TB risk during childhood and adolescence.

## IMPLICATIONS

The Wonder Years offer a unique insight into the immunological protection against TB disease progression. We have made the case that if infected during this period the host response is effective in containing *M. tuberculosis* within granuloma in contrast to younger children, who more commonly progress to disseminated disease. In addition, primary school children

are also less likely to mount a tissue damaging, inflammatory response to *M. tuberculosis*. They frequently experience localized bacillary replication within the lung and are less likely than post-pubescent adolescents and young adults to develop cavitation. Thus, comparing age-related immunological changes that occur before, during, and after puberty may reveal immunological pathways that could be targeted to promote balanced protective immunity.

Development of an effective TB vaccine has been hampered by insufficient understanding of protective immunity. Given that the presentation of disease and underlying immunological responses change during different risk periods, the approach to inducing an optimal vaccine response in young children may differ to the approach needed in adolescents and adults. Strategies in infants or young children could be aimed at either preventing the establishment of infection by priming the innate immune system or promoting cell-mediated immunity that provides superior protection from dissemination than BCG. Strategies for adolescents could aim to: clear mycobacteria before the stage of increased progression risk; enhance containment within the granuloma; or attenuate the excessive inflammatory response should containment break down. It may be appropriate to revaccinate children prior to adolescence (potentially with a vaccine of different mechanism) or provide host-directed therapies following exposure. Furthermore, the difference in host response of adolescent boys and girls may need to be modulated by vaccines and immunotherapies in different ways. This is supported by the first successful outcome of the new M72 TB vaccine, which demonstrated 57% efficacy in the population, but 75% efficacy in males and 84% efficacy in those $\leq$25 years, although this secondary analysis is confounded by the enrolled population being skewed toward young males (206). Future vaccine studies must be designed with sufficient power to rigorously test the effect of age and gender on trial outcomes.

In addition, approaches to diagnostics may have to factor in age and sex. Recently, there has been great interest in whole blood transcriptional signatures as potential diagnostics and prognostics for TB infection and disease. These biomarkers identify a characteristic host response to a particular disease process; however, in TB, pathogenesis of disease may vary by age and, to a lesser extent, by sex. As a result, a transcriptional signature that performs well in diagnosing adult-type pulmonary disease may not capture disease in children, and vice versa.

Ultimately, more research is required to better understand how immunological responses to *M. tuberculosis* change with age and sex. Animal studies, including the use of juvenile animals, could assist in delineating the impact of age and sex on host response to *M. tuberculosis,* as well as the changing disease phenotypes that result. It would also be possible to evaluate different vaccination strategies and host-directed therapies to prevent infection and/or disease progression in these animal models. Longitudinal cohorts of young children either prior to TB exposure, or following known exposure, could contribute vital information. In these cohorts, blood samples could be taken at regular intervals to document cell phenotypes and functions and to understand immunological risk factors for disseminated disease. For adolescents, longitudinal cohorts would also be informative, with children identified prior to puberty and with an evaluation of how host immunological responses change with age, puberty, and with sex. Specific attention to methylation profiles of boys and girls at different ages may identify the changes induced by puberty which could impact disease risk and protective immunity. Impact of co-infections in both young children and adolescents would be important to consider. Although longitudinal cohorts are expensive to conduct, it is often strategic and efficient to undertake such studies within the framework of therapeutic clinical trials or vaccine trials.

## CONCLUSIONS

Primary school children teach us that there is still much about TB that we do not understand. They sit within the flexion point where TB pathology and risk changes; the immunological changes that occur during early childhood and puberty are likely to impact the response to infection and risk of disease. The difference in TB risk that emerges after puberty also indicates that diagnostics and strategies for prevention and treatment may need to be targeted according age and sex.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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