

ANTIGEN SPECIFIC CD4⁺ AND CD8⁺ T CELL RECOGNITION DURING
MYCOBACTERIUM TUBERCULOSIS INFECTION

A Dissertation Presented

By

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Abstract

Mycobacterium tuberculosis (Mtb) causes human tuberculosis, and more people die of it than of any other pathogen in the world. Immunodominant antigens elicit the large majority of T cells during an infection, making them logical vaccine candidates. Yet, it is still unknown whether these immunodominant antigen-specific T cells recognize Mtb-infected cells. Two immunodominant antigens, TB10.4 and Ag85b, have been incorporated into vaccine strategies. Surprisingly, mice vaccinated with TB10.4 generate TB10.4-specific memory CD8⁺ T cells but do not lead to additional protection compared to unvaccinated mice during TB. Ag85b-specific CD4⁺ T cells are also generated during vaccination, but the literature on whether these cells recognize Mtb-infected cells is also inconsistent.

We demonstrate that TB10.4-specific CD8⁺ T cells do not recognize Mtb-infected cells. However, under the same conditions, Ag85b-specific CD4⁺ T cells recognize Mtb-infected macrophages and inhibit bacterial growth. In contrast, polyclonal CD4⁺ and CD8⁺ T cells from the lungs of infected mice can specifically recognize Mtb-infected macrophages, suggesting macrophages present antigens other than the immunodominant TB10.4. The antigen location may also be critical for presentation to CD8⁺ T cells, and live Mtb may inhibit antigen presentation of TB10.4. Finally, we propose that TB10.4 is a decoy antigen as it elicits a robust CD8⁺ T cell response that poorly recognizes Mtb-infected macrophages, allowing Mtb to evade host immunity.

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List of Symbols, Abbreviations or Nomenclature

APC: Antigen presenting cell
 β 2m: β -2 microglobulin
 BAL: Bronchoalveolar lavage
 BCG: Bacillus Calmette-Guérin
 BMDC: Bone marrow derived dendritic cells
 BMDM: Bone marrow derived macrophages
 CTL: Cytolytic
 DC: Dendritic cell
 DOTS: Directly-observed-treatments
 ESAT-6: Early secreted antigenic target of 6-kDa protein
 FAP: Fibronectin attachment protein
 HSP: Heat shock protein
 iNK: Invariant natural killer cells
 IV: Intravenous
 LXA₄: Lipoxin A₄
 mDC: Myeloid DCs
 MDDC: Monocyte derived dendritic cells
 MDR: Multi-drug-resistant
 Mtb: Mycobacterium tuberculosis
 NK: Natural killer cells
 NO: Nitric oxide
 OVA: Ovalbumin
 RNI: Reactive nitrogen intermediates
 ROI: Reactive oxygen intermediates
 SNARE: Soluble NSF attachment protein receptor
 TB: Tuberculosis
 TLR: Toll-like receptor
 TNF: Tumor necrosis factor
 Treg: T-regulatory cells
 TST: Tuberculin skin test
 XDR: Extensively-drug-resistant
 WHO: World Health Organization
 WT: Wild type

CHAPTER I. INTRODUCTION

A well-documented past

Mycobacterium tuberculosis (Mtb) causes human tuberculosis (TB), killing more people than any other infection in the world [1]. Robert Koch first isolated and identified the disease-causing bacterium in 1882, though the human struggle against TB can be found speckled throughout history, beginning as early as 8000 BCE [2]. Of note, Homer, in *Odyssey*, described it as a “grievous consumption which took the soul from the body” in 800 BCE [2]. Hippocrates called a disease with similar symptoms “phthisis,” Greek for “to waste away,” in 400 BCE [2]. From the 14th to the 19th century, it took on the names of the “King’s evil,” “consumption,” and “the great white plague” [2]. Yet, despite its long and storied existence, we have yet to eradicate it.

1.7 billion people in the world is infected with Mtb [3]. Most of the new cases occur in South-East Asia and Africa, and the total global count is 10.4 million new cases per year [1]. Of those new cases, about 20% are due to malnourishment, 10% due to HIV co-infection, and 1% due to diabetes. From 2000 to 2016, the mortality rate of TB has declined by 37%; nonetheless, the current mortality rate translates to 1.6 million people dying each year [1]. The mortality rate is higher among those co-infected with HIV or those with diabetes. The WHO estimates the global economic burden due to tuberculosis to be \$12 billion annually. Since the large majority of tuberculosis endemic regions are in developing nations, this

disease burden poses a significant threat to the future development of those countries [1].

Given the huge disease burden, antibiotics and vaccines are logical choices in curtailing the spread of TB. The only clinically approved vaccine, Bacillus Calmette-Guerin (BCG) vaccine, is derived from an attenuated strain of *Mycobacterium bovis*, which causes tuberculosis in cattle. It has been used since 1921, and many endemic countries have compulsory vaccination [1]. However, while earlier studies of the BCG vaccination show risk reduction of contracting TB as much as 50% [4], later studies demonstrate that the protection can be variable in adults [5]. Depending on the geographical location and in vitro culturing conditions of the BCG, a large heterogeneity, ranging from 0% to 80%, in its protectiveness exists [5]. Nonetheless, the protection is more consistent in children, when given at infancy and for preventing tuberculosis-induced meningitis [6, 7]. For patients with TB, antibiotic treatment requires a cocktail of drugs taken over a long period of time. In 1952, the first combination therapy including streptomycin, aminosalicylic acid and isoniazid was used in a 2-year treatment course [8]. Today, we not only have first-line treatment, but also, due to rising multi-drug-resistant (MDR) and extensively-drug-resistant (XDR) Mtb, second-line treatment with an urgent need for new antibiotics against future drug resistance [8]. Despite large public health efforts and directly-observed-treatment (DOTS) programs, lack of compliance, or non-adherence, to medication regimen remains a challenge and adds to the complexity of curing TB patients [9].

These facts may present a discouraging view on our likelihood of eradicating TB. However, it is not due to a lack of trying that we do not have a short, effective treatment or a consistently protective vaccine. Antibiotic and vaccine development remain high priorities in the research community, with 17 TB drugs and 12 TB vaccines in various stages of clinical trial and development [1, 8, 10]. Instead, one may infer that the long history shared by humans and Mtb has resulted in a bacterium that is extremely well adapted to survive and thrive within its host. Genetic analysis of 259 strains of Mtb point to a co-evolution process for over 70,000 years [11]. There is also hyperconservation of epitopes recognized by human T cells across different Mtb strains in the world, leading to the theory that Mtb has evolved to allow certain immune responses to occur that may benefit its survival [12, 13]. Not surprisingly, Mtb clinical isolates that have faster mutation rates lead to higher likelihood of being multi-drug-resistant in infected patients [14]. These studies reflect the extent of which Mtb has co-evolved with humans. They also highlight the importance and necessity of understanding how Mtb interacts with the host, especially host immunity, if we want to discover new therapies and vaccines.

A cough and a life-long partner

Mtb is an acid-fast, rod-shaped, aerobic, intracellular facultative bacterium. Its thick, “waxy,” mycolic acid cell wall makes it tough for antibiotics to penetrate through [15]. Indeed, a key difference between the virulent laboratory strain of Mtb,

H37Rv, and its attenuated, non-virulent derivative, H37Ra, is a mutation in *hadC*, which plays a role in the synthesis of mycolic acid [16]. *Mtb* is a slow growing microorganism, having a doubling time of roughly 24 hours in vitro [17, 18]. In vivo doubling time of *Mtb* is complex, given that *Mtb* responds and changes its growth rates in the presence of host immunity. In high dose aerosol infections (i.e. ~200 colony forming units (CFU) initial dose) of C57BL/6 mice, the in vivo growth rate shows a doubling time of 24.6 hours during the first 2 weeks of infection and 1,676 hours during week 4 through 16 post-infection [19]. The slow doubling time may allow *Mtb* to reduce the expression of its proteins in order to evade host immunity.

TB pathogenesis is just as complex as *Mtb* itself. *Mtb* is transmitted via aerosolized droplets containing the bacterium from an infected person's cough. Once in the airway, the droplets travel down into the distal parts of the lung. Here, inside the alveolar spaces, phagocytic cells such as alveolar macrophages and dendritic cells will engulf the free *Mtb*, just as it would for any other foreign pathogen [20]. Rather than being eliminated by the macrophages' innate antimicrobial mechanisms, however, *Mtb* survives and replicates within the macrophages [21, 22]. Over time, recruitment of other immune cells leads to the containment of infection, often via the formation of granulomas [23]. Of all people infected with TB, 90-95% of the patients will contain the infection and live without any symptoms, or asymptotically [22]. These patients have "latent TB" and do not transmit the disease. However, the other 5-10% of the patients, often those who are immunocompromised, will develop symptoms, such as fever, coughing

with sputum production, coughing up blood (or hemoptysis), weight loss, and night sweats. These patients have “active disease” and will transmit TB to others via coughing. Though it is a primarily pulmonary infection, TB can disseminate to and cause disease in other areas of the body, including scrofula in the cervical lymph node, meningitis, Pott’s disease in the spine, cutaneous tuberculosis such as lupus vulgaris, and disseminating to the gastrointestinal and genitourinary tracts [24-26]. Of the 90-95% of patients who have latent tuberculosis, there is also a 10% chance of them progressing to active disease. Immunocompromised patients, such as those with HIV/AIDS or uncontrolled diabetes, are much more likely to develop active disease than their immunocompetent, latently infected counterparts [27, 28]. The pathogenesis of TB indicates 2 important observations: 1) Mtb can live a dormant lifestyle in order to persist within the host, and 2) our immune responses are sufficient to contain, though not sterilize, the infection.

Innate immune response to *Mycobacterium tuberculosis*

Both the innate and adaptive immune responses help the host contain Mtb. The importance of innate immune response has been shown by observing a difference in the bacterial burden between susceptible and resistant rabbits as early as 7 days post infection, before the initiation of adaptive immunity [29]. BCG vaccination has effectively prevented disseminated disease, such as meningitis, in children but not primary disease [6, 7], supporting the argument that the adaptive immune response does not prevent initial infection [29]. While most Mtb-infected

people either contain the infection or have active disease, there is a third population who presumably can clear the infection [23]. The best evidence of this population comes from studying household contacts in endemic regions. In households where there was at least one case of TB, epidemiologists followed others living in the same households and recorded the status of their tuberculin skin test (TST) [30]. The TST is based on a type IV hypersensitivity reaction, and a positive TST means that individual has a memory T cell response to the tuberculin antigen, indicating prior exposure or infection. Over the 2 years of follow up, while some converted their TST from negative to positive, others, named “persistent TST negatives,” never converted [30]. While there has been controversy over the specificity of the test [31], this data supports the notion that innate immune response is responsible for these resisters in clearing the infection.

Before arriving at the distal airways where the macrophages await, *Mtb* first encounters numerous airway epithelial cells that line the respiratory tract [32]. The epithelial cells form a physical barrier to foreign pathogens by having tight junctions and adherens junctions, and damage to that barrier leads to more successful invasion by *Mtb* [32-34]. Using human tissues and organ structures to mimic the respiratory tract, Middleton et al find that *Mtb* do not attach to healthy areas of the mucosa but preferentially attach to damaged mucosa where the underlying extracellular matrix is exposed [34]. Interestingly, the adherence to these surfaces decrease significantly when the human tissues are pre-treated with fibronectin

attachment protein (FAP) or Ag85b protein, confirming that Mtb uses Ag85b to attach to the fibronectin in order to infect cells [34].

After invading and translocating across the respiratory tract epithelium [35], Mtb can be phagocytosed by resident alveolar macrophages, dendritic cells, and monocyte-derived macrophages via complement receptors (for opsonized bacteria) and via mannose and scavenger receptors (for nonopsonized bacteria) [36]. Macrophage recognition of Mtb occurs via Toll-like receptors (TLR) such as TLR2 as well as TLR9 and also via C-type lectin receptor [22, 37]. The result is the production of pro-inflammatory cytokines, including $\text{TNF}\alpha$, IL-1B, IL-6, IL-12, IL-15 and IL-18, as well as anti-inflammatory cytokines, including IL-10, TGFB, and IL-4 [38, 39]. Of note, tumor necrosis factor (TNF) is important in human TB patients because latently infected patients have an increased risk of developing active TB after using anti-TNF treatments, often prescribed to manage symptoms of Crohn's disease and rheumatoid arthritis [40]. Together, these cytokines can activate macrophages and also help differentiate T cells, though a disruption in the fine balance between the pro- and anti-inflammatory cytokines can lead to detrimental effects on the host [41].

Macrophages kill Mtb with a variety of mechanisms. $\text{IFN}\gamma$ production by T cells can activate infected macrophages to kill Mtb, using a process dependent on vitamin D [42]. Two important mechanisms for macrophage killing are the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) [43]. Activated macrophages can undergo apoptosis to kill

Mtb, and the process depends on both caspase and nitric oxide (NO) [44]. Whether cells die of apoptosis or necrosis also has consequences. Apoptotic vesicles that bleb off from the dying macrophage may contain live Mtb, and these vesicles are eliminated by uninfected macrophages that engulf them [45]. This mechanism, termed efferocytosis, allows the uninfected macrophage to phagocytose the dying cells and form a phagosome around the previous phagosome that contain the live Mtb. This double phagosome, or efferosome, allows for the efficient recruitment and fusion of lysosomes and subsequent elimination of the Mtb within [46]. Furthermore, mice with a more susceptible allele in the *ss11* (supersusceptibility to tuberculosis 1) locus led to increased necrosis of infected macrophages and higher bacterial burden compared to mice with a more resistant allele that led to more apoptosis of infected macrophages and lower bacterial burden [47] [48].

Neutrophils and natural killer (NK) cells are also important innate immune cells during TB. Neutrophils are recruited to the lung after infection [49]. In patients with active TB, neutrophils outnumber macrophages in both sputum and bronchoalveolar lavage (BAL) fluid, and they also contain more Mtb than macrophages and epithelial cells [50]. The role of neutrophils during infection remains incompletely understood. There is both evidence that shows neutrophils as protective [51] and as harmful to the host [52] [32]. Neutrophil influx may correlate with increased severity of disease and inflammation [49]. Regulation of neutrophil recruitment, through the inhibition of CD4⁺ T cells' production of IL-17, is necessary to prevent pathogenic neutrophil accumulation and detrimental

inflammation in the lung [53]. Natural killer (NK) cells also contribute host defense. During co-culture with Mtb-infected monocytes, NK cells have been shown to upregulate activating receptors NKp46 and NKG2D, both of which are required for successful lysis of Mtb-infected monocytes [54]. Interestingly, invariant NK (iNK) T cells, which do not form memory cells, can be rapidly activated by IL-12 and IL-18, cytokines produced by macrophages. They secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), which is critical for the suppression of Mtb growth in infected cells [55].

Ultimately, the innate immune response does not clear the infection, but it does initiate the formation of granulomas to contain the infection [56]. Granulomas are collections of foamy macrophages and multinucleated giant cells, and, in the context of TB, these cells often contain live bacteria [57]. In the zebra fish model, which lacks an adaptive immune response, granulomas form after infection with *Mycobacterium marinum*, supporting that notion that innate immunity initiates granuloma formation [58]. The maintenance of the granuloma and subsequent control of the infection, however, requires the adaptive immune response [23].

Adaptive immune response to Mtb

The adaptive immune response is required for the successful containment of Mtb [41, 59, 60]. T cell priming during TB requires dendritic cells (DCs) [61] and occurs in the mediastinal lymph node [62]. However, a unique characteristic of the adaptive immune response during TB is its delay in onset. In acute infections such

as influenza or LCMV, the initiation of adaptive immunity occurs within 3 to 5 days post infection. Yet the adaptive immune response to TB is not detected until 42 days post exposure in humans [63]. In mice, the initiation of antigen-specific T cell responses does not occur until day 11 post aerosol infection and depends more on the bacterial burden in the lymph node where priming occurs rather than the lung where the infection is [64]. In both Mtb-infected C57BL/6 and C3H mice, Mtb must disseminate to the lymph node prior to T cell priming, though the dissemination and T cell responses occur faster in the more resistant C57BL/6 mice [65]. Interestingly, Griffiths et al have shown that delivering DCs primed with Mtb antigens to vaccinated mice at the time of aerosol infection bypasses this “bottleneck” and accelerates the initiation of the T cell responses [66]. This data also argues that Mtb antigen load during early stages of infection may not be sufficient to initiate prompt T cell priming.

Despite the delay, once T cell immunity is initiated, CD4⁺ and CD8⁺ T cells play a crucial role in controlling the bacterium. Monoclonal antibody depletion of T cells in thymectomized, Mtb-infected mice leads to higher bacterial burden and shorter survival time compared to control mice [67, 68]. Adoptive transfer of T cells into sub-lethally irradiated, Mtb-infected mice leads to significant protection, and it is the splenic T cells that are responsible for this protection [69]. Mtb-infected, TCR $\alpha\beta^{-/-}$ mice, which lack $\alpha\beta$ T cells, has 2-log higher bacterial burden and live 210 days shorter than the Mtb-infected, wild type mice [70]. These data show that,

after T cells appear in the lungs of infected mice, the bacterial burden can be controlled throughout infection [60].

The role of CD4⁺ T cells

Th1 CD4⁺ T cells have long been thought of as the most important part of the adaptive immune response, based on their ability to produce IFN γ , TNF and IL-2 during infection [23]. T cells from pleural effusion exudate of TB patients produce high amounts of IFN γ when co-cultured with purified protein derivative (PPD), which is made from sterilized Mtb cultures, and this response diminishes when antibodies against CD4⁺, but not CD8⁺, T cells are added [71]. As described to above, IFN γ is important in activating macrophages to kill intracellular bacteria. In Mtb-infected IFN γ ^{-/-} mice, there is a much higher bacterial burden in the lung, spleen, and liver as well as worsened pathology [72]. These mice also succumb to Mtb much earlier compared to WT mice, around 50 days post aerosol infection vs. over 300 days [70]. Patients with Mendelian susceptibility to mycobacterial diseases (MSMD), who may have mutations in the IFNGR1 or IFNGR2 genes that normally encode part of the IFN γ receptors, show increased susceptibility to mycobacterial diseases [73, 74]. Several studies have demonstrated that CD4⁺ T cells are an importance source of IFN γ during infection [75-77]. However, there is controversy as to how protective IFN γ is during infection. CD4⁺ T cells isolated from Mtb-infected mice suppress Mtb growth in Mtb-infected, IFN γ R^{-/-} macrophages, indicating that IFN γ may be dispensable [78]. More recently,

adoptive transfer of either WT or IFN γ ^{-/-} CD4⁺ T cells into Mtb-infected, RAG1^{-/-} mice demonstrated that the IFN γ secreted by the WT CD4⁺ T cells contributed only 30% of the total bacterial burden reduction by the CD4⁺ T cells [79]. Increasing the production of IFN γ from CD4⁺ T cells actually accelerated the demise of the infected mice, suggesting that there is a balance of IFN γ production in order to achieve optimal control [79]. Interestingly, the importance of TNF has also come under question after discovering that TNF^{-/-} CD4⁺ T cells can afford protection at a similar level as WT CD4⁺ T cells [79]. A subtlety in these studies is that the contribution of CD4⁺ T cells is more important during early stages of the infection, and other immune cells play an important role in sustaining control throughout later stages of infection [80]. While these studies suggest an incomplete understanding of the contribution made by Th1-CD4⁺ T cell cytokines during infection, it is well appreciated that CD4⁺ T cells are required for optimal protection against Mtb [77]. In humans, CD4⁺ T cells are indispensable, as evidenced by the increased risk of getting active TB disease in a co-infected HIV patient whose CD4⁺ T cells have decreased significantly [81].

Other subsets of CD4⁺ T cells also play a role during Mtb infection. Th2 CD4⁺ T cells, which produce IL-4 and IL-10, arise during TB. However, studies suggest that they may not be beneficial to the host. Th2 responses may be induced by Mtb virulence factors in order to dampen the Th1 response in favor of Mtb survival [82]. In mice, antibody blockade of IL-10R during BCG vaccination increased protection against subsequent Mtb infection and led to increased Th1

and Th17 responses [83]. In patients with active disease, cytokines made by Th2 cells can be detected, and this has been associated with active, progressive disease [84, 85]. Another subset of CD4⁺ T cells made during TB is Th17 cells. They produce IL-17, which is important in recruiting neutrophils to the lung environment and may contribute to protective immunity to Mtb [86, 87]. IL-17⁺CD4⁺ T cells have been identified from peripheral blood mononuclear cells (PBMCs) of TB patients, with the frequency being higher in patients with latent disease, leading to the idea that IL-17 producers may help control Mtb [88]. T regulatory (Treg) cells proliferate and accumulate at the site of infection, but they have been implicated in the delay of CD4⁺ and CD8⁺ T cell priming [89]. Interestingly, depletion of Tregs before aerosol infection led to suppression of bacterial growth in infected mice, supporting the idea that Tregs do not promote a protective response [90].

The role of CD8⁺ T cells

The requirement of CD8⁺ T cells for optimal control of Mtb has only recently been fully appreciated. Early studies involving adoptive transfer of splenic immune cells to Mtb-infected mice showed reduced bacterial burden in sub-lethally irradiated mice, and antibody depletion confirms that the Ly-2⁺ (i.e., CD8⁺) T cells are responsible for the reduction [91]. A classic study shows that infection of mice deficient in β 2-microglobulin (β 2m), a key component of the MHC class I molecule, results in greater bacterial burden, shorter survival, and more necrotic granulomas, all signs of uncontrolled disease, when compared to WT mice [92]. However, β 2m⁻

$I^{-/-}$ mice also results in an iron-rich environment that promotes Mtb growth, suggesting that the $\beta 2m^{-/-}$ susceptibility phenotype may be due to bacterial growth in a nutrient rich environment rather than a lack of effective MHC class I presentation [93]. Nonetheless, other studies examining the MHC class I pathway have shown the importance of CD8⁺ T cells during TB. Double knockout of H-2K^b and H-2D^b in mice increased their susceptibility to Mtb, both through the IV and aerosol infection route [94]. In another classic study, IV infection of TAP $^{-/-}$ (transporter associated with antigen presentation) mice, which lack of MHC class I presentation, succumbed to Mtb much earlier than their wild type counterparts [95].

The role that CD8⁺ T cells play during TB has often been overshadowed by the perceived importance of the CD4⁺ T cells. Using high dose IV infection ($\sim 5 \times 10^6$ H37Rv), Leveton et al demonstrate that antibody depletion of CD4⁺ T cells results in disease outcomes similar to the depletion of both CD4⁺ and CD8⁺ T cells, while depletion of CD8⁺ T cells alone shows similar survival as a non-depleted mouse [67]. Other antibody depletion studies, where depletion of CD4⁺ T cells leads to higher bacterial burden compared to depletion of CD8⁺ T cells, have confirmed this finding as well [68]. Mogue et al show that MHC class II $^{-/-}$ mice died about 160 days earlier than MHC class I $^{-/-}$ mice [70]. At first glance, these data suggest that the contribution by the CD4⁺ T cells may be more important than that of CD8⁺ T cells. However, the use of antibody depletion and genetic knock out mice only allows for studying each cell subset individually and misses the relative

contribution of each subset throughout the entire infection. It is well known that CD4⁺ T cell help is required for successful generation of effector and memory CD8⁺ T cells [96-100]. Therefore, depleting CD4⁺ T cells when assessing the function of CD8⁺ T cells may disrupt the development of fully functional CD8⁺ T cells. In Mtb-infected mice without CD4⁺ T cells, effector CD8⁺ T cell activation and subsequent IFN γ production [101] as well as cytotoxic activity is diminished significantly [102]. During infection, IFN γ ^{-/-} CD4⁺ T cells result in a decrease in CD8⁺ T cell recruitment to the lung and also fewer IFN γ - or TNF-producing CD8⁺ T cells [76]. While data suggests that a small primary CD8⁺ T cell response may occur without CD4⁺ T cell help, CD4⁺ T cells are definitely required during secondary response [100]. Together, these data suggest that assessing the effect of CD8⁺ T cells by depleting CD4⁺ T cells may inadvertently harm the development of CD8⁺ T cells, thus giving the illusion that CD8⁺ T cells make relatively small contribution to the overall immunity against Mtb.

Recent studies have confirmed the importance of CD8⁺ T cells during TB. Transfer of antigen-specific CD8⁺ T cells cloned from vaccinated mice confer protection to Mtb-infected, unvaccinated mice [103]. Memory CD8⁺ T cells have also been shown to be important during TB [104, 105]. Transfer of a CD4⁺, PPD- (Purified Protein Derivative)-specific T cell line did not control Mtb in thymectomized CBA mice, suggesting that CD8⁺ T cells are necessary for optimal control of Mtb [67]. In the above section, the importance of IFN γ , and especially IFN γ from CD4⁺ T cells, has been described. A study examining CD4⁺ KO or MHC

class II^{-/-} mice show that IFN γ production in the lungs does not diminish after infection, and that IFN γ production comes from CD8⁺ T cells [106]. In chronic infection, CD8⁺ T cells increase in their frequency of IFN γ producers [107]. By using a latent TB model where aerosol-infected mice were first treated with antibiotics before allowing the bacterial burden to rise, monoclonal antibody depletion of CD8⁺ T cells during chronic infection led to a significant increase in the bacterial burden, whereas depletion of CD4⁺ T cells did not [108]. In non-human primate models, depletion of CD8⁺ T cells diminished the protection against Mtb in BCG-vaccinated rhesus macaques [109]. These data show that CD8⁺ T cells are important in animal models of TB, and their contribution may be better appreciated during the chronic phase of infection.

How CD8⁺ T cells carry out their effector functions during TB is also of great importance to the immune response. Cytotoxic CD8⁺ T cells carry out their effector functions by secreting cytokines such as IFN γ and TNF or through T cell receptor (TCR) dependent cytotoxicity, including the delivery of perforin and granzymes as well as Fas-FasL interactions [23]. During TB, CD8⁺ T cells can make both TNF and IFN γ [60], which are important for controlling Mtb [70, 110]. Two studies show that cytokine production is the main mechanism of protection by CD8⁺ T cells [111, 112], however, mounting evidence suggests an equally important role for cytolytic mechanisms. CD8⁺ T cells in the lungs of Mtb-infected or vaccinated mice can lyse Mtb-infected macrophages [72, 103, 113, 114]. These CD8⁺ T cells express perforin in vivo [113], and the killing depends on the delivery of cytotoxic

granzymes and perforin to the target cells [115-117]. Furthermore, CD95/CD95L deficient mice, lacking the Fas-FasL killing mechanism, exhibit greater bacterial burden during the chronic phase of infection [118]. In humans, patients with active TB often have CD8⁺ T cells with lowered expression of granzyme B compared to patients with latent TB or healthy individuals, hinting at the importance of granzyme B in control of TB [119]. Moreover, CD8⁺ T cells' delivery of granulysin is an important effector function during human TB control [120]. A lack of the granulysin homolog in mice has made it challenging to study its functions, but it does not exclude the idea that there exists an undiscovered molecule delivered by CD8⁺ T cells that mediate the same effects during murine TB infection [121].

The adaptive immune response is essential in controlling TB and maintaining latency in chronically infected people and mice. The role of CD8⁺ T cells during TB is complicated, but emerging evidence over the past twenty years have reinforced the idea that CD8⁺ T cells contribute to the optimal control of TB. Despite their many abilities, both CD4⁺ and CD8⁺ T cells can only contain and not eradicate Mtb from the host. This may reflect yet another consequence of the co-evolution of Mtb and humans. Indeed, a body of evidence exists showing that Mtb has evolved to have sophisticated immune evasion mechanisms.

Immune evasion and subversion by *Mycobacterium tuberculosis*

Mtb persists in humans because of its highly evolved ability to evade and subvert the host immunity [122]. Macrophages engulf Mtb via receptor mediated

phagocytosis, but the phagosomes cannot undergo successful acidification once the Mtb is inside [123, 124]. Acidification is one of the important mechanisms for macrophages to defeat engulfed pathogens [43]. Mtb can survive quite well in a slightly acidified environment, around pH 5.0-5.5, however, it does not survive in pH < 4.0 [125]. Studies show that vacuole containing *Mycobacterium avium* and phagosomes containing *Mycobacterium bovis* BCG have a pH of 6.3 to 6.5 [126, 127]. The Mtb phagosomes appear immature as they display more early-endosomal markers, and they have limited ability to fuse with lysosomes [123, 128]. The failure of phagolysosomal fusion is a key mechanism that allows for Mtb to survive intracellularly [129]. Mtb subverts vesicular trafficking by interfering with SNARE (soluble NSF attachment protein receptor) proteins and Rab family proteins, specifically preventing the recruitment of Rab7 [122, 130]. Mtb also inhibits innate immunity through its protein PtpA, which binds to ubiquitin in the host cells and impairs signaling pathways involved in Jnk, p38 and NF- κ B [131]. Mtb genes such as noxR1 has been implicated in helping the bacterium become resistant to RNI and ROI antimicrobial mechanisms [132].

Cell death modality plays an important role in defense against Mtb. Apoptosis, where the cell undergoes programmed cell death that leaves the cell membrane intact, is beneficial to the host during TB [133]. In human macrophages infected with Mtb, both TNF and Fas-FasL pathways promotes apoptosis and limits Mtb growth, whereas nonapoptotic cell death has no beneficial effects [134]. Mtb can disrupt the plasma membrane of the infected cell and prevent prostaglandin

E₂ (PGE₂)-dependent plasma membrane repair by manipulating the macrophage to upregulate lipoxin A₄ (LXA₄) production [135]. Then, the damaged macrophage undergoes necrosis rather than apoptosis. Whereas apoptotic cells can be easily engulfed by uninfected cells, allowing the clearance of Mtb via efferocytosis, necrotic cells lack the receptors that allow for efferocytosis [45, 46]. Necrosis of the infected macrophages allows escape of the Mtb to infect other cells, propagating the infection [136]. These data show that Mtb subverts macrophage defenses to avoid elimination.

Mtb evades and resists T cell responses as well [137]. Mtb delays the initiation and recruitment of T cell immunity to the lung, promoting the establishment of a persistent infection [60]. Mtb can subvert the eicosanoid pathways to delay priming of T cell immunity. Mtb has been shown to induce the production of lipoxin A₄ (LXA₄), which inhibits prostaglandin E₂-dependent plasma membrane repair and leads to more necrosis of the infected cell [135]. Infection of *Alox5*^{-/-} mice, which cannot make LXA₄, leads to faster generation of antigen specific CD8⁺ T cells during priming and also enhanced CD4⁺ and CD8⁺ T cell responses, suggesting a mechanism by which Mtb prevents the initiation of T cell priming [138]. A proapoptotic mutant of Mtb, the *nuoG* mutant [139], leads to faster appearance of Mtb in the mediastinal lymph node and subsequent earlier priming of antigen specific CD4⁺ T cells, compared to the wild type H37Rv [140]. Studies in both mouse models and T cells isolated from TB patients have also shown that Mtb infection leads to high IL-10 production from the T cells, which impairs priming

and worsens disease outcome [141, 142]. Antibody blockade of IL-10 during BCG vaccination enhanced adaptive immune response and decreased bacterial burden for both the resistant C57BL/6 and the susceptible CBA/J mice [83]. Infection of mice with recombinant ovalbumin (rOVA)-expressing BCG reduced CD8⁺ T cell priming compared to when the mice were infected with rOVA-expressing *Listeria monocytogenes*; but increasing the amount of OVA being expressed corrected the defect [143]. Ag85b, an important protein made by Mtb that helps the bacteria attach to fibronectin during infection, is transiently expressed during TB [144]. Indeed, the lowered expression of Ag85b by 3 weeks post infection has been attributed as the reason for a lack of effective response by Ag85b-specific CD4⁺ T cells [144]. Moreover, correcting this phenotype by having a Mtb strain constitutively expressing Ag85b led to better CD4⁺ T cell responses [145]. These data show that Mtb turns down its expression of antigens in order to evade adaptive immunity. Human monocyte derived DCs, when matured with the early secreted antigenic target of 6-kDa (ESAT-6) protein from Mtb, induce a Th17 T cell response and inhibited Th1 T cell response [146]. ESAT-6-treated, Mtb-infected PBMCs decrease IFN γ production by T cells [147]. The Mtb 19-kDa lipoprotein interferes with MHC class II antigen presentation [122]. In humans studies, Mtb can infect fibroblast cells and downregulate IFN γ -induced MHC class II expression, leading to defective antigen presentation to the CD4⁺ T cells [148]. In mice, inhibition of IFN γ -induced MHC class II expression has also been documented [149]. Mtb-infected macrophages produce membrane vesicles containing Mtb

byproducts and proteins, and these vesicles can impair the activation and priming of naïve CD4⁺ T cells during infection [150]. Mtb-infected DCs can undergo an active, kinesin-2 dependent export of Mtb antigens out of the infected cells, allowing the infected cells evade host T cell responses [151].

The above data show that Mtb has an arsenal of immune evasion and subversion mechanisms to thrive within the host. Some of the mechanisms allude to Mtb's ability to evade immune recognition. While Mtb-specific CD4⁺ and CD8⁺ T cells are generated during infection, whether they can recognize Mtb-infected cells remain inconclusive.

Recognition of *Mycobacterium tuberculosis*-infected cells

T cell recognition defined

In order for CD4⁺ and CD8⁺ T cells to carry out their functions, they must first be activated, and this activation is often referred to as recognition of cognate peptides by the T cells. Professional APCs present both foreign and self-peptides on their cell surfaces, and the T cells sample the APCs to see if they may recognize their peptides. CD4⁺ and CD8⁺ T cells differ in their recognition pathways. MHC class I molecules are responsible for presenting to CD8⁺ T cells, whereas MHC class II molecules present to CD4⁺ T cells [152]. MHC class I presentation focuses mainly on proteins generated inside a cell, where the proteins are degraded by the proteasome and loaded for presentation [153, 154]. However, MHC class I presentation can also involve “cross-presentation,” where antigens of exogenous

nature can be taken up by the APC and presented onto MHC class I molecules [155, 156]. Cross-presentation, commonly defined using DCs, can occur via the cytosolic pathway, where phagocytosed antigens are exported out into the cytosol first to be processed by the proteasome before loaded onto MHC class I [157]. Cross-presentation can also occur via the vacuolar pathway, where the phagocytosed antigen is degraded in the phagosome and loaded directly onto MHC class I molecules on the phagosomal membrane [158]. The MHC class II pathway mainly presents extracellular antigens phagocytosed by the APCs, though they can also present self-proteins degraded in the endosomes [152, 159]. T cell recognition requires 1) T cell receptors to interact with the peptide-loaded MHC molecules, 2) costimulatory molecule interaction between CD28 (on the T cells) and B7 (on the APCs), and, for optimal priming and function, 3) cytokines such as IL-12, type I IFNs, and, in the context of TB, IL-27 [60, 160].

Differentiating cognate from non-cognate recognition remains problematic when assessing recognition. This challenge arises from the observation that T cell IFN γ production, a common readout of recognition, can be non-specifically induced by IL-12 and IL-18 secreted by infected cells, in both *Mtb* and other infections [55, 161-163]. This means that, rather than TCR-dependent activation or recognition, these cytokines can activate T cells independent of TCR interacting with peptide loaded MHC molecules. Human CD4⁺ T cells recognize *Mtb*-infected monocyte derived dendritic cells (MDDC), but that recognition, assessed via IFN γ production, largely derives from the activation effects of the IL-12 and IL-18 secreted by the

infected MDDCs [164]. Detecting TCR-dependent, cognate recognition is important because this leads to delivery of cytotoxic granules such as granzyme through the pore forming actions of perforin [165]. On the other hand, cytokine-activated T cells can produce IFN γ [166] but cannot carry out the full spectrum of effector functions [167-169].

CD4⁺ T cell recognition of Mtb-infected cells

Given the importance of CD4⁺ T cells during Mtb infection, it is logical to assume that the CD4⁺ T cells recognize Mtb-infected cells. Indeed, experiments have shown that CD4⁺ T cells must recognize infected cells in order to reduce bacterial burden [170].

Studies supporting CD4⁺ T cell recognition often examines IFN γ , IL-2, proliferation, and/or bacterial burden control. Bone marrow derived macrophages (BMDM) infected with a virulent strain of Mtb, H37Rv, trigger Ag85b-specific hybridoma BB7 T cells to produce small amount of IL-2, and this production increases in the presence of autophagy-inducing agent rapamycin [171, 172]. Co-culture of BB7 T cells with either untreated or IFN γ -activated BMDMs that are infected with H37Ra or BCG also leads to IL-2 production [173-175]. Polyclonal CD4⁺ T cells from infected mice produce IFN γ and reduce bacterial growth upon co-culture with BMDMs and BMDCs infected with Erdman, another virulent Mtb strain [76, 78, 80]. Both naïve polyclonal CD4⁺ T cells and polyclonal CD4⁺ T cells

isolated from the lungs of infected mice produce IL-2 and IFN γ when co-cultured with CD11c⁺I-Ab⁺ lung DCs isolated from infected mice [176].

In human studies, CD4⁺ T cells also recognize Mtb-infected macrophages. Human CD4⁺ T cells isolated from the PBMCs of PPD⁺ individuals, stimulated in vitro with Mtb crude cell wall extract, can secrete IFN γ and reduce intracellular bacterial burden upon co-culture with H37Rv-infected APCs, and this effect is restricted to MHC class II [177]. CD4⁺ T cell specific to the antigen peptide CFP10₇₆₋₈₅, which is derived from a dominant Mtb antigen CFP10, reduce intracellular bacteria in Mtb-infected cells, and this killing depends on granulysin and perforin [178]. However, as mentioned above, studies have shown that the production of IFN γ may be largely driven by IL-12 and IL-18 produced by the infected cells [148, 164]. TCR-dependent T cell activation is a requirement for T cell proliferation [179, 180]. Since human CD4⁺ T cells proliferate when co-cultured with Mtb-infected cells, we can infer that these CD4⁺ T cells have cognate recognition of Mtb-infected cells [181, 182].

There is also evidence that CD4⁺ T cells do not recognize Mtb-infected cells, especially when examining Mtb antigen-specific CD4⁺ T cells. While there is consensus that Mtb-infected cells present ESAT-6, the data concerning Ag85b presentation is more complicated [144, 145, 151, 183]. Early after infection, Ag85b₂₄₁₋₂₅₆ elicits a strong CD4⁺ T cell response, yet this response diminishes after 3 weeks post infection, mostly due to a decreased production of Ag85b by Mtb [144]. Thus, while Ag85b₂₄₁₋₂₅₆-specific CD4⁺ T cells recognize DCs from

infected mice 14 days post infection [184], there is little recognition of Mtb-infected cells in vivo by day 21 [144]. Intravital imaging of liver granulomas in infected mice shows that antigen-specific CD4⁺ T cells do not interact with the granulomas, suggesting a lack of recognition during later stages of infection [185]. Mtb infection of BALB/c mice lowers expression of the costimulatory molecule M150 on splenic macrophages, and Th1 T cells co-cultured with Mtb-infected BALB/c macrophages express lower IL-2 and proliferate less, and the phenotype is reversed when M150 is added to the co-culture [186]. In human T cells, Mtb-infected monocytes have reduced DR expression, and T cells proliferate less when co-cultured with either Mtb-infected APCs or APCs treated with heat killed bacteria, again arguing for a lack of recognition [187]. Several studies further report that antigen-specific CD4⁺ T cell recognition does not occur efficiently during Mtb infection, and that this is an active mechanism by which Mtb evades host immunity [151, 188, 189].

Despite the data arguing against recognition by the CD4⁺ T cells, cognate contact is important for CD4⁺ T cells to carry out their effector functions. Srivastava et al elegantly show this by using mixed bone marrow (BM) chimeric mice made from WT and MHC class II deficient BM [170]. Following infection, polyclonal CD4⁺ T cells suppress Mtb growth more efficiently in MHC class II-expressing cells than in MHC class II-deficient cells. This evidence convincingly argues that cognate recognition of infected cells by polyclonal CD4⁺ T cells limits bacterial growth.

All these data present the reality that we do not fully understand how CD4⁺ T cells recognize Mtb-infected cells. The recognition may be dependent on the

antigen as well as the timing during infection. Almost all the studies mentioned above do not assess cognate vs. noncognate recognition, making it challenging to interpret whether the recognition events are of Mtb-infected cells or not.

CD8⁺ T cell recognition of Mtb-infected cells

Mature, cytolytic (CTL) CD8⁺ T cells migrate from the lymph node to the lungs to kill Mtb-infected macrophages, and this killing is evident both *in vitro* and *in vivo* in mice [60]. After Mtb infection of mice, antigen-specific CD8⁺ T cells can be detected in the lungs as early as 3 weeks post infection [114, 116]. Presumably, to execute their functions, CD8⁺ T cells must recognize those infected macrophages. Many studies have investigated recognition indirectly when examining the cytolytic effects of CD8⁺ T cells. These studies assess recognition based on the CD8⁺ T cells' 1) cytolytic ability via target cell killing or CD107 degranulation [190], 2) activation using cell surface markers such as CD69 [113, 191] and CD25 [192], or 3) IFN γ production [192]. Backing up these studies is the finding that Mtb infection does not inhibit the presentation of soluble OVA proteins to SIINFEKL-specific RF33.70 hybridoma T cells [193]. Ex vivo polyclonal CD8⁺ T cells from infected mice can reduce bacterial burden and make IFN γ when co-cultured with Erdman-infected BMDMs [76, 78, 80], though DCs may be better at presenting to CD8⁺ T cells than macrophages [194]. Several studies have also investigated recognition in BCG-infected cells. CD8⁺ T cells specific to the MPT64

antigen produce IFN γ when co-cultured with BCG-infected DCs [195]. Antigen-specific CD8 $^{+}$ hybridoma T cells recognize BCG-infected cells by producing IL-2 [196]. Finally, BCG infection of macrophages does not inhibit the presentation of OVA protein to OVA-specific hybridoma CD8 $^{+}$ T cells [197, 198]. Thus, in murine studies, from BCG to Mtb infections, CD8 $^{+}$ T cells seem to be able to produce cytokines and reduce bacterial burden, though it is unknown whether these actions are TCR-dependent or not.

Human CD8 $^{+}$ T cells can recognize Mtb-infected cells, when assessed for the above-mentioned markers. Several Mtb antigens recognized by CD8 $^{+}$ T cells from patients have been identified, indirectly suggesting that CD8 $^{+}$ T cells may recognize Mtb-infected cells [199]. Human CD8 $^{+}$ T cells isolated from PPD $^{+}$ donors' PBMCs proliferate when incubated with Mtb antigens in the absence of APCs, and antibody depletion of MHC class I molecules reduces the amount of proliferation [192]. Monocyte derived macrophages infected with virulent and avirulent Mtb can be recognized, based on CTL activity or IFN γ ELISpot, by CD8 $^{+}$ T cells from TB patients, either polyclonal or clonally expanded by Mtb-peptide [200-204]. Cho et al have identified 3 MHC class I restricted CD8 $^{+}$ T cells from TB patients that produce IFN γ and lyse Mtb-infected macrophages [203]. CD8 $^{+}$ T cells from PBMC of human patients also can release granulysin and reduce intracellular bacterial burden upon co-culture with infected cells [205]. In studying MDDCs, multiple CD8 $^{+}$ T cell clones have been identified from PPD $^{+}$ donors when co-cultured with Mtb-infected MDDCs [206]. Grotzke et al demonstrate that Mtb-

infected phagosomes are competent for presentation to two CD8⁺ T cell clones from donors, further suggesting antigen presentation to CD8⁺ T cells [207]. Using fluorescent bacteria, Lewinsohn et al show that human, CFP-10-specific CD8⁺ T cells recognize a small subset of MDDCs that are heavily infected [208]. Epithelial cells in the human airways can harbor Mtb, and these cells are efficiently recognized by CD8⁺ T cells based on T cell IFN γ production [209].

There is some evidence against CD8⁺ T cell recognition of Mtb-infected cells. CD8⁺ T cells from BCG immunized mice do not produce IFN γ or IL-2 when co-cultured with BCG-infected DCs [210], nor do they reduce bacterial burden in vitro [211]. Schaible et al have demonstrated that the MHC class I presentation pathway does not sample Mtb inside the infected cell, instead, uninfected, bystander APCs take up apoptotic vesicles containing Mtb antigens or live Mtb and present them to CD8⁺ T cells [212].

From the above studies, only two murine studies, which show some CTL activity and weak recognition by the CD8⁺ T cells, use MHC restriction to show that these processes are TCR-dependent [113, 195]. An additional study has shown conjugate formation between CD8⁺ T cells isolated from PPD⁺ healthy donors and Mtb-infected macrophages [213]. However, this study only examines the conjugate formation after one hour of co-culturing. Given that CD8⁺ T cells require sustained interaction with their targets to perform cytolytic actions, determining the dynamics of contact-dependent recognition in Mtb-infected cells requires a more continuous observation of conjugate formation [214]. Some of the human studies used

monoclonal antibody blocking to restrict the responses to only CD8⁺ T cells [203, 204, 215, 216]. Overall, the CD8⁺ T cell recognition literature shows that there is recognition of Mtb-infected cells. However, murine studies deal mostly with polyclonal CD8⁺ T cells and do not focus on cognate recognition while human studies investigate a vast repertoire of antigens produced during Mtb infection. Very few studies focus on the recognition by CD8⁺ T cells elicited by immunodominant antigens.

Immunodominant antigens

During Mtb infection, immunodominant antigens are the primary targets of T cell responses [217]. Immunodominance arises from the observation that despite host APCs generating numerous, often thousands, of peptides from a pathogen, the majority of the T cells only respond to a handful of those peptides [218]. This signifies that a select few antigens cause the expansion of the majority of the T cell response. When a biological product of a pathogen, whether it is a piece of the cell wall or a secreted protein, enters the MHC class I or II presentation pathway, that product first gets processed, either in the phagosome or, more commonly, the proteasome [152]. The proteasome randomly cleaves the antigen into peptides of varying lengths, and some will then be loaded onto MHC class I or II molecules for presentation. A few of those peptides might be recognized more often or earlier than others during an infection, leading to a large expansion of T cells during priming made specific for those peptides. The sequences of the peptides

recognized by the T cells are immunodominant epitopes, and the antigens where the peptides are derived from are immunodominant antigens.

Several immunodominant antigens have been identified in the murine TB model, including Ag85a, Ag85b, CFP-10, ESAT-6 and TB10.4 [219]. T cell responses to these antigens are also frequently detectable in Mtb-infected people, and these highly prevalent responses represent the basis for TB immunodiagnostic tests [220]. Many of these immunodominant antigens serve as components in vaccines, with the logical expectation that T cells specific to these immunodominant antigens will contain the infection before Mtb can establish a niche and evade host immunity [217]. Yet, vaccines based on some of these immunodominant antigens have largely been ineffective. MV85A, a vaccine based on the immunodominant antigen Ag85A and the only vaccine after BCG to go beyond a phase I safety trial, showed no increased efficacy as a booster for BCG-vaccinated infants [221]. Studies examining preclinical data and memory T cell function have shed some light as to why vaccination may not have been efficacious [145, 183, 222]. However, there remains a gap of knowledge in understanding the effectiveness of immunodominant-antigen-elicited T cell responses.

Setting the stage

This chapter introduces the topics of TB pathogenesis, immune responses to Mtb with specific regards to CD4⁺ and CD8⁺ T cells, immune evasion by Mtb, T cell recognition of Mtb infected cells, and immunodominant antigens. The main

themes that underlie these sections are 1) Mtb has adapted to survive despite host immunity, and 2) there are Mtb antigens that generates large T cell responses, but we do not completely understand whether these responses correlate with protection. The work presented in this thesis focuses on T cell responses generated against two immunodominant antigens, TB10.4 and Ag85b. In Chapter II, the recognition of infected cells by TB10.4-specific CD8⁺ and Ag85b-specific CD4⁺ T cells will be addressed. In Chapter III, an investigation into the mechanisms contributing to inefficient antigen recognition will be presented. In Chapter IV, a discussion of the presented data will highlight areas of improvement and raise questions for future investigation. The data and interpretation presented in this thesis will hopefully enhance our understanding of how immunodominant-antigen-elicited T cells respond during TB and better inform future vaccine designs.

**CHAPTER II. *MYCOBACTERIUM TUBERCULOSIS*-SPECIFIC CD4 AND CD8
T CELLS DIFFER IN THEIR CAPACITY TO RECOGNIZE MTB-INFECTED
CELLS**

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Abstract

Containment of *Mycobacterium tuberculosis* (Mtb) infection requires T cell recognition of infected macrophages. Mtb has evolved to tolerate, evade, and subvert host immunity. Despite a vigorous and sustained CD8⁺ T cell response during Mtb infection, CD8⁺ T cells make limited contribution to protection. Here, we ask whether the ability of Mtb-specific T cells to restrict Mtb growth is related to their capacity to recognize Mtb-infected macrophages.

We derived CD8⁺ T cell lines that recognized the Mtb immunodominant epitope TB10.4₄₋₁₁ and compared them to CD4⁺ T cell lines that recognized Ag85b₂₄₀₋₂₅₄ or ESAT6₃₋₁₇. While the CD4⁺ T cells recognized Mtb-infected macrophages and inhibited Mtb growth in vitro, the TB10.4-specific CD8⁺ T cells neither recognized Mtb-infected macrophages nor restricted Mtb growth. Importantly, polyclonal CD8⁺ T cells specific for Mtb antigens other than TB10.4 recognized Mtb-infected macrophages in a MHC-restricted manner. As TB10.4 elicits a dominant CD8⁺ T cell response that poorly recognizes Mtb-infected macrophages, we propose that TB10.4 acts as a decoy antigen.

Introduction

Mycobacterium tuberculosis (Mtb), the cause of human tuberculosis (TB), subverts and evades host immunity [122]. Mtb subverts vesicular trafficking, prevents phagolysosome fusion, and replicates in an intracellular niche within macrophages, allowing it to evade detection by humoral immunity [130]. Mtb also delays the initiation and recruitment of T cell immunity to the lung, promoting the establishment of a persistent infection [60]. Despite these challenges, T cell immunity does occur and plays an essential role in controlling the infection in both mice and humans [60, 70, 223]. With 10 million new TB cases annually, an effective vaccine would offer a cost-effective way to prevent TB and attenuate this persistent global pandemic. Given the importance of T cells during host defense, strategies for TB vaccines largely aim at generating memory T cells rather than neutralizing antibodies. Most subunit vaccines incorporate immunodominant Mtb antigens, which elicit large T cell responses [217]. The expectation is that antigen-specific T cells elicited by these immunodominant-antigen-based vaccines will contain the infection before Mtb can adapt to the host immune system.

T cell recognition of Mtb-infected macrophages is fundamental to containment of TB infection [170]. However, even though many presume that Mtb-infected cells present immunodominant antigens, the data validating this assumption is surprisingly inconsistent. For example, Ag85b₂₄₁₋₂₅₆ elicits a CD4⁺ T cell response early after infection, but Mtb reduces Ag85b production within 3 weeks after in vivo infection, subsequently diminishing Ag85b-specific CD4⁺ T cell

response [144]. Furthermore, Mtb has other mechanisms to evade T cell recognition, including dysregulating MHC class II expression and inhibiting antigen presentation by stimulating antigen export by the infected antigen presenting cells (APCs) [122, 144, 151, 185]. Whether the immunodominant antigens recognized by CD8⁺ T cells are presented by Mtb-infected macrophages remains unknown. Here, we investigated cognate T cell recognition of Mtb-infected macrophages by CD8⁺ T cells specific to the immunodominant antigen TB10.4.

TB10.4 (EsxH) is an ESAT-6-like protein secreted by the ESX-3 type VII secretion system, important in iron and zinc acquisition, and essential for Mtb growth in vitro and in vivo [224, 225]. Following Mtb infection, TB10.4 is a target of CD4⁺ and CD8⁺ T cell responses in humans and mice [226-230]. In Mtb-infected mice, TB10.4 elicits immunodominant responses in both BALB/c and C57BL/6 mice, and 30-40% of lung CD8⁺ T cells are specific to single epitopes (Figure 2.1) [226, 227]. Whether these TB10.4-specific CD8⁺ T cells can mediate protection is unclear. Adoptive transfer of TB10.4-specific CD8⁺ T cells into Mtb-infected, immunocompromised mice reduces the bacterial burden and promotes survival [227]. However, despite eliciting large numbers of TB10.4-specific CD8⁺ T cells, a vaccine incorporating the H-2 K^b-restricted epitope, TB10.4₄₋₁₁, failed to protect mice from Mtb infection [231]. We hypothesize that the inability of TB10.4-specific CD8⁺ T cells to mediate protection is due to inefficient recognition of Mtb-infected macrophages.

We used primary CD4⁺ and CD8⁺ T cells lines to investigate the recognition of Mtb-infected macrophages by T cells specific to Ag85b, ESAT-6, or TB10.4. Ag85b- and ESAT-6-specific CD4⁺ T cells recognized Mtb-infected macrophages, but under the same conditions, TB10-specific CD8⁺ T cells did not recognize infected macrophages or inhibit bacterial growth. This was true even upon examination of numerous conditions and permutations including length of infection, duration of T cell and macrophage co-culture, and multiplicity of infection. Interestingly, polyclonal CD8⁺ T cells specific for Mtb antigens other than TB10.4 recognized Mtb-infected macrophages in a MHC class I-restricted manner. Thus, while TB10.4-specific CD8⁺ T cells do not recognize Mtb-infected macrophages, there exist other CD8⁺ T cells that recognize subdominant antigens presented by Mtb-infected cells. Based on these data, we propose that TB10.4 is a decoy antigen: it elicits a massive and persistent CD8⁺ T cell response, which cannot recognize Mtb-infected macrophages. Such a decoy antigen may distract the CD8 response from focusing on subdominant antigens presented by infected cells, leading to evasion from host immunity.

Moreover, it appears that this response overshadows a subdominant CD8⁺ T cell response that can recognize Mtb-infected macrophages. The ability of Mtb to subvert the CD8⁺ T cell response may explain why CD8⁺ T cells make a disproportionately small contribution to host defense compared to CD4⁺ T cells. The selection of Mtb antigens for vaccines has focused on antigens that generate immunodominant responses. We propose that establishing whether vaccine-

elicited, Mtb-specific T cells recognize Mtb-infected macrophages could be a useful criterion for preclinical vaccine development.

Results

TB10.4-specific CD8⁺ T cells dominate the pulmonary CD8⁺ T cell response during Mtb infection

We first examined if the Mtb antigen TB10.4 elicited a large CD8⁺ T cell response. 6 weeks post aerosol infection of C57BL/6 mice, 35% of total lung CD8⁺ T cells were TB10.4₄₋₁₁-tetramer positive (Figure 2.1). This confirms findings from previously published data [227, 231].

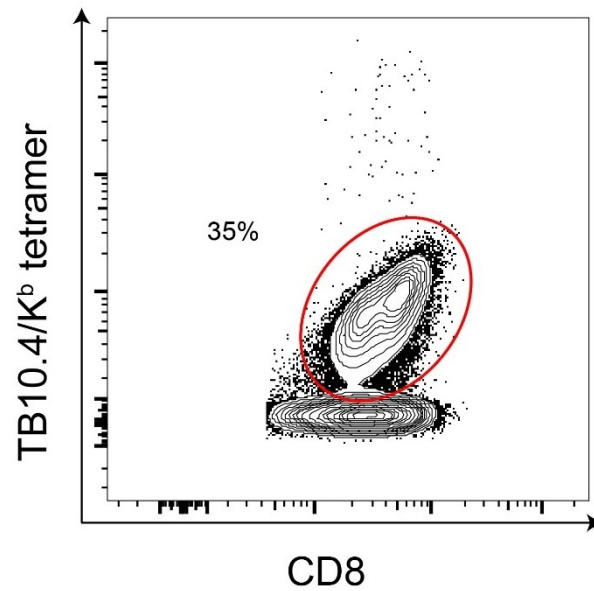


Figure 2.1. TB10.4₄₋₁₁-tetramer positive CD8⁺ dominates the pulmonary CD8⁺ T cell response during Mtb infection in C57BL/6 mice. Representative flow plot showing the percent of TB10.4₄₋₁₁-tetramer positive CD8⁺ T cells among lung cells isolated from mice infected with Erdman via the aerosol route 6 weeks post-infection.

TB10.4-specific CD8⁺ and Ag85b-specific CD4⁺ T cell lines sensitively recognize their cognate antigens

To study T cell recognition of Mtb-infected macrophages, we established antigen-specific T cell lines, which unlike T cell hybridomas, facilitate the study of T cell function as well as recognition. The TB10.4₄₋₁₁-specific CD8⁺ T cell line, referred hereafter as TB10Rg3, has a distinct TCR cloned originally from TB10.4₄₋₁₁-tetramer⁺ CD8⁺ T cells isolated from infected mice and expressed in retrogenic mice [227]. The Ag85b₂₄₀₋₂₅₄-specific CD4⁺ T cell line, referred hereafter as P25 cells, was derived from P25 TCR transgenic mice [232]. To confirm their antigen-specificity, we co-cultured the P25 or TB10Rg3 T cells with thioglycolate-elicited peritoneal macrophages (TGPMs) pulsed with or without their cognate peptides and then measured their expression of CD69 and Nur77. While both CD69 and Nur77 are T cell activation markers, increases in Nur77 expression indicate TCR-mediated activation more specifically [163, 233]. After co-culture with TGPMs pulsed with Ag85b₂₄₁₋₂₅₆ peptide, Nur77 expression by P25 cells peaked after 2 hours (Figure 2.2a, b), while CD69 expression continued to increase (Figure 2.2c, d). TB10Rg3 T cells exhibited similar Nur77 and CD69 expression patterns after their co-culture with TGPMs pulsed with the TB10.4₄₋₁₁ peptide (IMYNYPAM) but not with a control peptide (IMANAPAM) (Figure 2.2e-h). Since the increase in Nur77 expression was transient, we next tested whether CD69 and IFN γ could be useful markers of antigen recognition for longer experiments. During 72 hours of co-culture with peptide-pulsed TGPMs, P25 and TB10Rg3 T cells continued to

express CD69 and secreted IFN γ in a peptide dose-dependent manner (Figure 2.2i-l). These experiments show that P25 and TB10Rg3 T cells can recognize their cognate antigens presented by TGPMs, both in short-term and long-term co-culture assays.

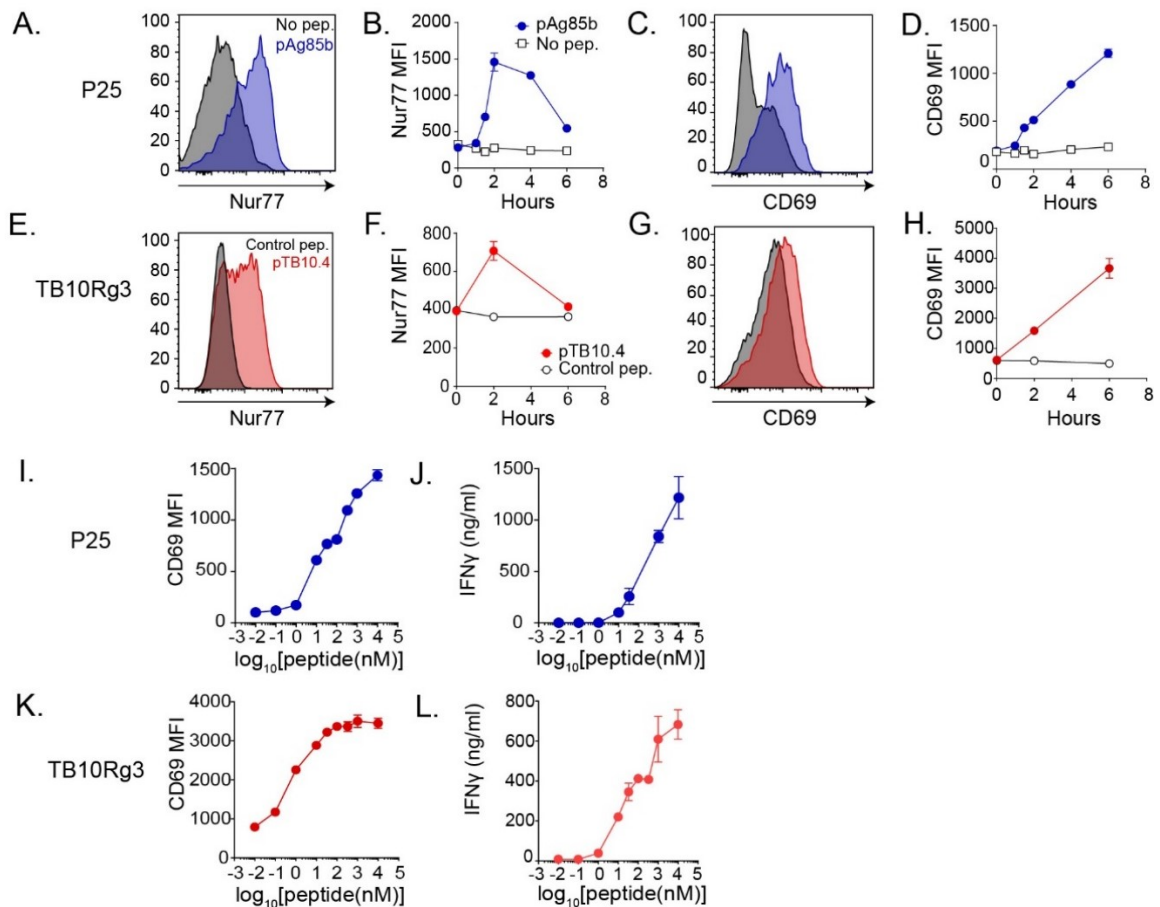


Figure 2.2. TB10.4-specific CD8⁺ (TB10Rg3) and Ag85b-specific CD4⁺ (P25) T cells both recognize their cognate peptides. (a) Representative histogram of Nur77 expression in P25 T cells after 2 hours of co-culture with macrophages and (b) time course of Nur77 MFI in P25 T cells. (c) Representative histogram of CD69 in P25 T cells after 2 hours of co-culture with macrophages and (d) time course of CD69 MFI in P25 T cells. (e) Representative histogram of Nur77 in TB10Rg3 T cells after 2 hours of co-culture and (f) time course of Nur77 MFI in TB10Rg3 T cells. (g) Representative histogram of CD69 in TB10Rg3 T cells at 2 hours of co-

culture with macrophages and (h) time course of CD69 MFI in TB10Rg3 T cells. (i) CD69 MFI and (j) IFN γ production by P25 T cells after 72 hours of co-culture. (k) CD69 MFI and (l) IFN γ production by TB10Rg3 cells after 72 hours of co-culture. MFI, mean fluorescence intensity; m ϕ , macrophage.

Ag85b-specific CD4⁺ T cells, but not TB10.4-specific CD8⁺ T cells, restrict intracellular bacterial replication

Given that a primary function of T cells during Mtb infection is to restrict bacterial growth, we determined whether these T cell lines could limit intracellular mycobacterial growth *in vitro*. We infected TGPMs with H37Rv, a virulent Mtb strain that expresses both TB10.4 and Ag85b *in vitro* [229, 234]. To assess whether any bacterial growth inhibition observed was dependent on cognate recognition, we infected both MHC-matched (i.e., H-2^b) and mismatched (i.e., H-2^k) macrophages. T cells were added on day 1 post-infection, and the number of colony forming units (CFU) was assayed 96 hours later. In the absence of T cells, Mtb grew significantly ($p < 0.01$) (Figure 2). P25 T cells significantly inhibited intracellular bacterial growth in H37Rv-infected TGPMs ($p < 0.0001$). Addition of Ag85b peptide to the infected macrophages did not further enhance the ability of P25 T cells to inhibit bacterial growth, suggesting that their activation was maximal. As expected, P25 T cells only inhibited bacterial growth in MHC-matched macrophages, indicating that growth inhibition mediated by T cells required cognate recognition under these conditions.

In contrast, TB10Rg3 T cells did not inhibit bacterial growth (Figure 2.3). We considered whether the inability of TB10Rg3 to inhibit bacterial growth was due to a lack of recognition of the infected macrophages or a defect in the T cells' effector functions. When Mtb-infected TGPMs were pulsed with the TB10.4₄₋₁₁ peptide for one hour prior to adding the T cells, TB10Rg3 T cells significantly reduced bacterial

growth ($p < 0.0001$) (Figure 2.3). We wondered whether a TCR with stronger affinity might be more effective at inhibiting bacterial growth. We derived another TB10.4-₁₁-specific CD8⁺ T cell line, referred hereafter as TB10Rg4 T cells, that has a higher affinity than TB10Rg3 based on previous publication [227]. Co-culture of Mtb-infected macrophages with TB10Rg4 T cells also did not lead to a significant reduction in bacterial burden, and, similar to TB10Rg3 T cells, the TB10Rg4 T cells did significantly inhibit bacterial growth when the infected macrophages were pretreated with the TB10.4 peptide (Figure 2.3).

Thus, under the same conditions where P25 T cells significantly suppressed intracellular Mtb growth in a MHC-restricted manner, TB10Rg3 and TB10Rg4 T cells failed to inhibit bacterial growth. Since TB10Rg3 and TB10Rg4 T cells did inhibit bacterial growth when their cognate peptide was added to Mtb-infected macrophages, we conclude that both T cells could express the effector function required to restrict intracellular bacterial growth; however, they simply did not recognize Mtb-infected macrophages.

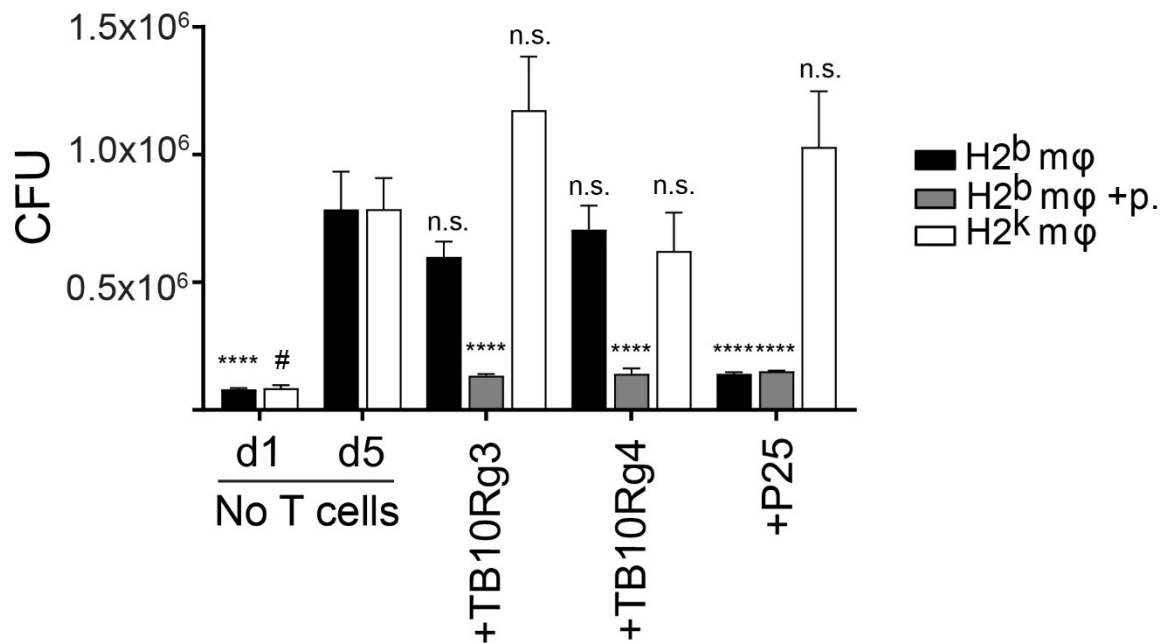


Figure 2.3. Ag85b-specific CD4⁺ T cells, but not TB10.4-specific CD8⁺ T cells, inhibit bacterial growth *in vitro*. Matched (H-2^b) or mismatched (H-2^k) macrophages were infected with H37Rv for 2 hours, and then, one day post-infection, T cells were added. CFU were determined 4 days later. Separately, TB10.4 or Ag85b peptide was added to Mtb-infected (H-2^b) macrophages, and then T cells were added. Results are representative of at least three experiments. Statistical testing was by one-way ANOVA, using the Dunnett posttest compared to d5. *, #, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.0001. φ, macrophages.

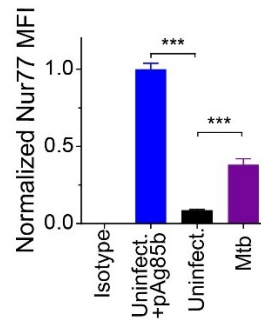
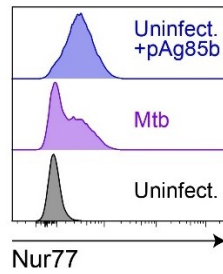
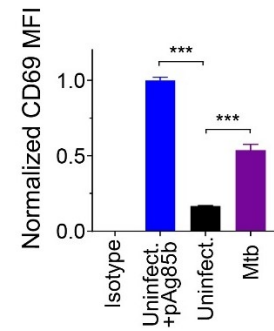
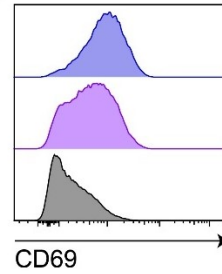
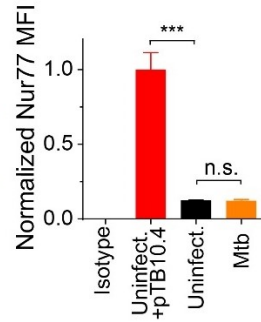
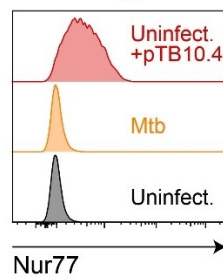
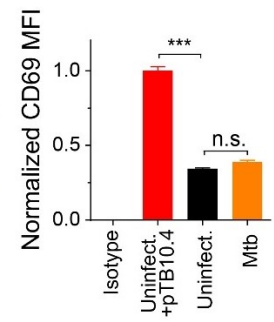
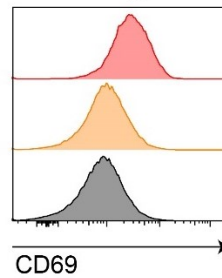
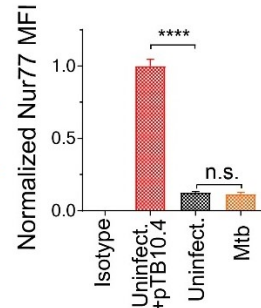
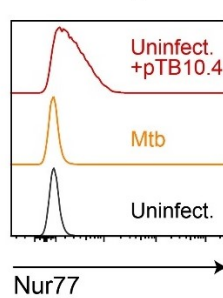
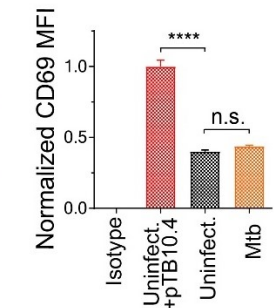
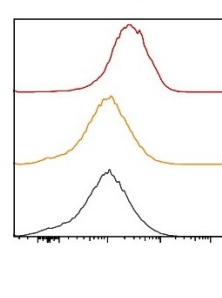
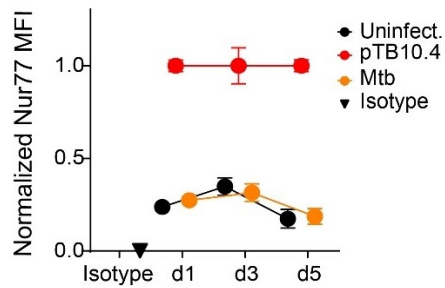
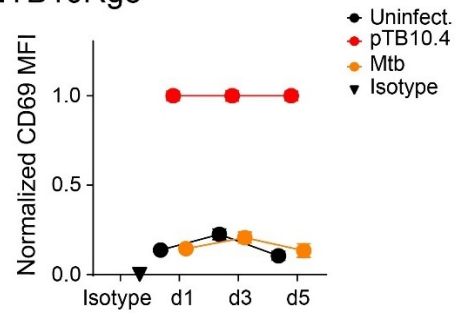
Ag85b-specific CD4⁺ T cells, but not TB10.4-specific CD8⁺ T cells, recognize Mtb-infected macrophages.

To further investigate TB10Rg3 and P25 T cells recognition of Mtb-infected cells, we next investigated the kinetics of Mtb antigen presentation. After Mtb infection, TGPMs were cultured for various lengths of time before adding the T cells. To assay antigen presentation, we added the T cells for two hours and then measured Nur77 and CD69 (see Figure 2.2. for kinetics). When added immediately after infection (i.e., day 0), P25 T cells recognized Mtb-infected macrophages based on the induction of Nur77 and CD69 (Figure 2.4a, b). Under these conditions, there was no increase in Nur77 or CD69 expression by TB10Rg3 T cells (Figure 2.4c, d). To assess whether increased TCR affinity of TB10.4 might lead to better recognition, we examined TB10Rg4 T cells. Similar to TB10Rg3, the TB10Rg4 T cells did not show increased Nur77 or CD69 expression (Figure 2.4e, f). We next chose later time points, which might allow Mtb to adapt to the intracellular environment and potentially let the TB10.4 antigen accumulate. TB10Rg3 T cells were added to infected macrophages on days 1, 3, or 5 post-infection. Again, we did not observe any increase in Nur77 or CD69 expression (Figure 2.4g, h). As a control for T cell health and function, we co-cultured TB10.4-₁₁-peptide-pulsed-, uninfected-macrophages with either TB10Rg3 or TB10Rg4 T cells and observed significant increases in their Nur77 and CD69 expression (Figure 2.4).

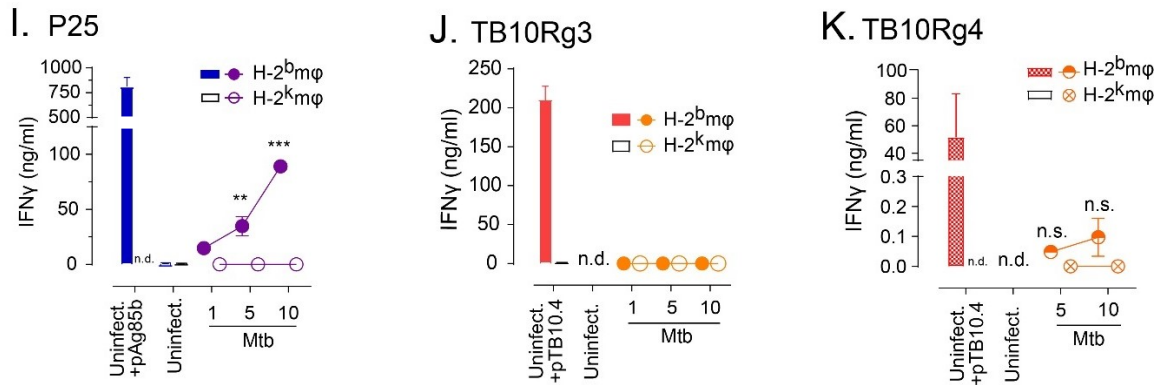
Despite assessing recognition on multiple days, we considered whether the short assay period (i.e. 2 hours) might not detect recognition of Mtb-infected macrophages by TB10Rg3 T cells, especially if presentation of TB10.4 is inefficient or asynchronous. Therefore, we used IFN γ production as a cumulative indicator of T cell activation during a 72-hour co-culture experiment. Since cytokine-driven activation (e.g., IL-12, IL-18) can stimulate IFN γ production by T cells independently of TCR signaling, we used MHC-matched (H-2^b) or mismatched (H-2^k) TGPM to assess cognate recognition [55, 161-163]. Over the first 3 days post infection (i.e., d0-d3), as the infectious dose (MOI, multiplicity of infection) increased, more IFN γ was measured when the P25 T cells were co-cultured with MHC-matched, Mtb-infected TGPMs compared to when they were co-cultured with MHC-mismatched, Mtb-infected TGPMs (Figure 2.4i). In contrast, TB10Rg3 as well as TB10Rg4 T cells did not produce IFN γ when co-cultured with Mtb-infected TGPMs (Figure 2.4j, k). Interestingly, in 2 out of 3 experiments, TB10Rg4 did show a slight increase in IFN γ production as the MOI increased (Figure 2.4k). However, while this increase was MHC restricted, it was not a significant increase when analyzed by two-way ANOVA with Sidak posttest. This reflects that increased affinity leads to a better T cell response, but it is still much smaller compared to that of the P25 T cells. We also investigated whether adding the T cells at a later time (i.e., d3) might lead to better recognition. Over the latter 3 days of infection (i.e., d3 to d6), P25 T cells produced significant IFN γ while both TB10Rg3 and TB10Rg4 T cells did not (Figure 2.4l-n). Of note, TB10Rg4 did show a trend in

increased IFN γ production, but the trend was not consistent or specific in 2 experiments. Together, these data show that TB10.4-specific CD8 $^{+}$ T cells do not produce IFN γ when co-cultured either during early or late infection.

As before, TB10Rg3 and TB10Rg4 T cells produced IFN γ when co-cultured with uninfected macrophages pulsed with the TB10.4₄₋₁₁ peptide (Figure 2.4j, k). These data show that, regardless of the time point of T cell addition or the length of co-culture, P25 T cells, but not TB10Rg3 or TB10Rg4 T cells, recognized Mtb-infected macrophages, based on their increased Nur77 and CD69 expression as well as their IFN γ production.

A. P25**B. P25****C. TB10Rg3****D. TB10Rg3****E. TB10Rg4****F. TB10Rg4****G. TB10Rg3****H. TB10Rg3**

Day0 to Day3 post infection:



Day3 to Day6 post infection:

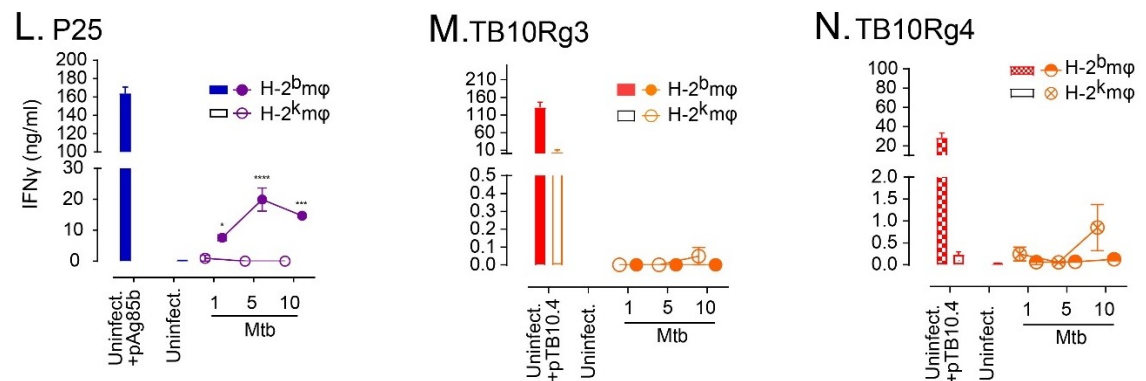


Figure 2.4. Ag85b-specific CD4⁺ T cells, but not TB10.4-specific CD8⁺ T cells, recognize Mtb-infected macrophages. (a-d) T cells were co-cultured with peptide-pulsed, Mtb-infected, or uninfected macrophages for 2 hours. (a) Representative histogram of Nur77 expression in P25 T cells and the normalized Nur77 MFI. (b) Representative histogram of CD69 expression in P25 T cells and the normalized CD69 MFI. (c) Representative histogram of Nur77 in TB10Rg3 T cells and the normalized Nur77 MFI. (d) Representative histogram of CD69

expression in TB10Rg3 T cells and the normalized CD69 MFI. (e) Representative histogram of Nur77 in TB10Rg4 T cells and the normalized Nur77 MFI. (f) Representative histogram of CD69 expression in TB10Rg4 T cells and the normalized CD69 MFI. (g-h) TB10Rg3 T cells were co-cultured with uninfected, peptide-pulsed, or Mtb-infected macrophages for 2 hours on d1, d3, and d5 post infection. Normalized expression of (g) Nur77 or (h) CD69 by TB10Rg3 T cells. P25 (i), TB10Rg3 (j), or TB10Rg4 (k) T cells were co-cultured with uninfected, peptide-pulsed, or Mtb-infected macrophages, and IFN γ production was measured after 72 hours from d0 to d3 post infection. P25 (l), TB10Rg3 (m), or TB10Rg4 (n) T cells were co-cultured with uninfected, peptide-pulsed, or Mtb-infected macrophages, and IFN γ production was measured after 72 hours from d3 to d6 post infection. Figures are representative of at least 5 (a-f, TB10Rg3), 3, (a-f, TB10Rg4), 2 (a-f, P25, and l-n), or 3 (g-k) experiments. Statistical analysis was done by one-way ANOVA with Dunnett posttest (a-d) or two-way ANOVA with Sidak posttest (g-h). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$. ϕ , macrophages.

TB10Rg4 CD8⁺ T cells recognize Mtb-infected dendritic cells.

Dendritic cells are critical in priming CD8 T cells during Mtb infection [61]. DCs are known to be better antigen presenters than macrophages, even during Mtb infection [80, 194]. Interestingly, by 4 weeks post infection, CD11b^{+/hi}CD11c^{+/hi} myeloid DCs represent a high percentage of infected cells in the lungs of infected mice, but, by 10 weeks post infection, CD11b^{+/lo}CD11c^{-/lo} recruited monocytes/macrophages are the dominant infected macrophages [235]. We next investigated whether Mtb-infected dendritic cells may lead to recognition by the TB10Rg3 and TB10Rg4 T cells. After 72 hours of co-culture, TB10Rg3 T cells did not consistently produce significant amount of IFN γ as significant IFN γ production was measurable in only 2 out of 4 experiments. However, TB10Rg4 T cells, which has a higher affinity than TB10Rg3 T cells, consistently produced IFN γ , as did P25 T cells, in a MHC-restricted manner (Figure 2.5a, b). This shows that BMDCs can present TB10.4 to TB10.4-specific CD8 T cells, and that affinity may be an important contributing factor for recognition of the immunodominant TB10.4 antigen.

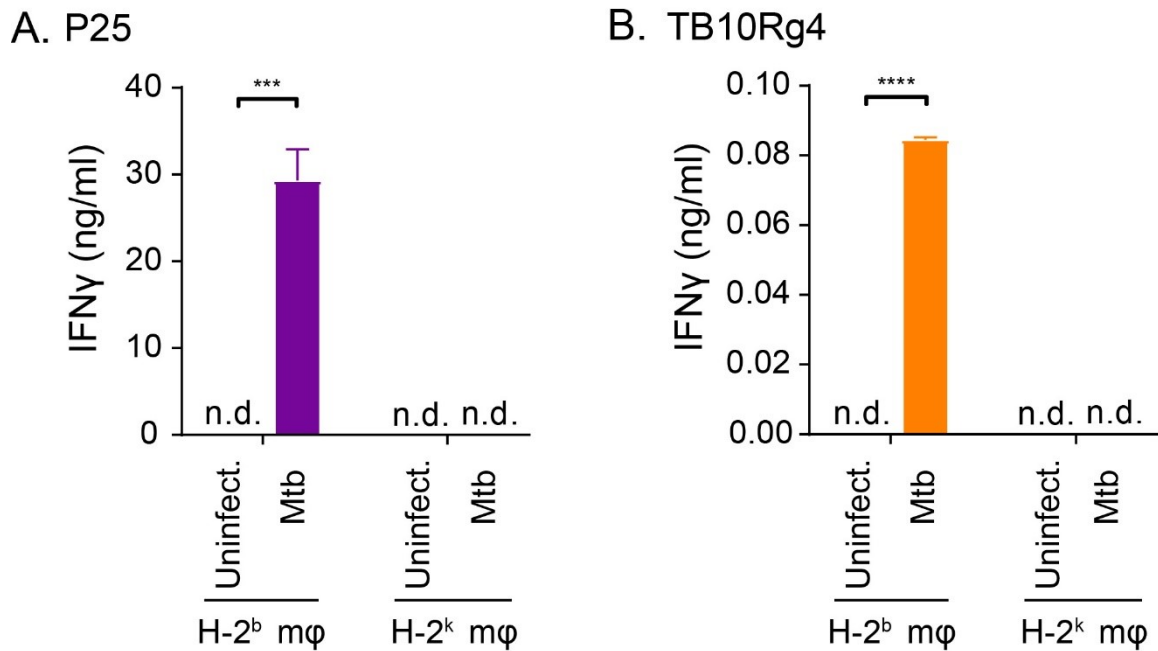


Figure 2.5. Ag85b-specific CD4⁺ T cells and TB10.4-specific CD8⁺ T cells recognize Mtb-infected bone marrow derived dendritic cells (BMDCs). P25 (a) or TB10Rg4 (b) T cells were co-cultured with uninfected or Mtb-infected BMDCs, and IFN γ production was measured after 72 hours. Figures are representative of 3 experiments. Statistical analysis was done by two-way ANOVA with Sidak posttest. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$. ϕ , macrophages.

TB10.4-specific CD8⁺ T cells do not recognize lung cells from Mtb-infected mice.

During *in vivo* infection, Mtb infects a variety of myeloid cells, and this diversity changes over the course of the infection [236-238]. We considered that lung myeloid cells from Mtb-infected mice are more physiologically relevant than TGPMs. Thus, we isolated MHC class II⁺ lung cells from Erdman-infected, RAG-1^{-/-} mice 4 weeks post infection and tested their ability to present Mtb antigens to TB10Rg3 T cells. We used RAG-1^{-/-} mice because of the possibility that CD8⁺ T cells in the lungs of Mtb-infected, wild type mice may recognize and eliminate any lung cells presenting the TB10.4 antigen. Since Mtb downregulates Ag85b expression by 3 weeks post infection [144, 145], we used an ESAT-6-specific CD4⁺ T cell line we derived from C7 transgenic mice, which we refer to as C7 T cells [183, 239]. The immunodominant antigen ESAT-6 retains high levels of expression throughout infection and elicits a dominant CD4⁺ T cell response in C57Bl/6 mice [145]. Due to the difficulty in obtaining large numbers of MHC class II⁺ cells from uninfected, RAG-1^{-/-} mice, we used TGPMs from age-matched, RAG-1^{-/-} mice as a source of uninfected, inflammatory macrophages. Cognate recognition is a requirement for T cell proliferation [179, 180], thus we examined the proliferative abilities of these T cells after co-culture with the lung myeloid cells. We stained C7, TB10Rg3, TB10Rg4 T cells with 5uM of the proliferation dye eFluor450 (eBioscience) before co-culturing them with the lung myeloid cells. After 72 hours, we measured their proliferation. C7 T cells proliferated extensively when co-

cultured with the infected lung myeloid cells but not when co-cultured with uninfected TGPMs (Figure 2.6a, b). In contrast, TB10Rg3 T cells did not proliferate when co-cultured with the lung myeloid cells (Figure 2.6c, d). To assess whether TB10Rg3 T cells could proliferate if TB10.4 was present, we pulsed the lung APCs with the TB10.4₄₋₁₁ peptide for 1 hour before adding the TB10Rg3 T cells. As predicted, TB10Rg3 T cells proliferated after 72 hours of co-culture with peptide-pulsed, lung myeloid cells (Figure 2.6c, d). Despite having a higher affinity, TB10Rg4 T cells also did not proliferate after the co-culture (Figure 2.6e, f)

We considered the possibility that Mtb in lung myeloid cells may not grow well in vitro, leading to altered antigen abundance that could affect T cell recognition. To address this possibility, we measured the bacterial burden in the lung myeloid cells. There was a 3-fold increase in the bacterial numbers between the beginning (d1) and the end (d4) of the experiment, indicating that the bacteria remained viable (Figure 2.6g). Together, these data indicate that, under the conditions in which C7 T cells recognized lung myeloid cells from Mtb-infected mice, TB10Rg3 and TB10Rg4 T cells did not recognize these lung myeloid cells.

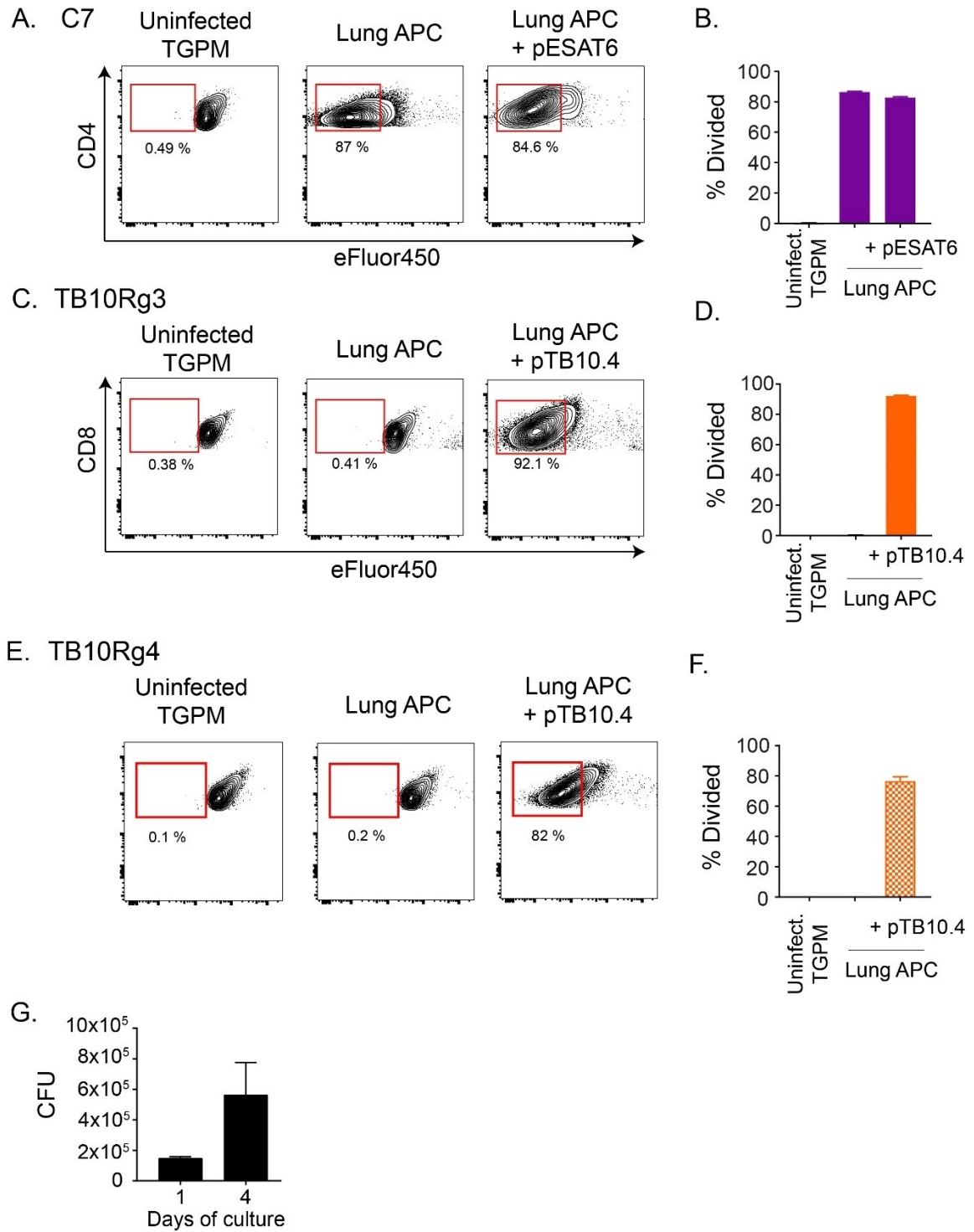


Figure 2.6. TB10.4-specific CD8⁺ T cells do not recognize lung APCs from infected mice. (a-d) T cell proliferation after coculture with lung APC from infected

mice, with or without cognate peptide, or uninfected TGPM, based on eFluor450 fluorescence dilution after 72 hours. Representative flow plot (a) and quantification (b) of C7 T cell proliferation. Representative flow plot (c) and quantification (d) of TB10Rg3 T cell proliferation. Representative flow plot (e) and quantification (f) of TB10Rg4 T cell proliferation. (g) Bacterial burden in the lung APCs during *in vitro* culture over the course of the experiment in the absence of T cells. Representative of 4 (TB10Rg3 or TB10Rg4) or 2 (C7) similar experiments.

Polyclonal, TB10.4₁₁-tetramer negative CD8⁺ T cells from the lungs of Mtb-infected mice recognize infected macrophages.

Along with the previous finding that TB10.4₁₁-specific CD8⁺ T cells make up ~40% of total lung CD8⁺ T cells during infection (2.1 Figure) [227], our finding that TB10Rg3 and TB10Rg4 T cells do not recognize Mtb-infected macrophages suggests that TB10.4 may be a decoy antigen. This raises the question whether the inability to recognize Mtb-infected macrophages is a general feature of the CD8⁺ T cell response to Mtb, or if this is a unique feature of TB10.4-specific CD8⁺ T cells. Therefore, we determined whether polyclonal CD8⁺ T cells from the lungs of infected mice could recognize Mtb-infected macrophages. We carried out aerosol infection of C57BL/6 mice with the virulent Mtb strain Erdman, and, 6-8 weeks post infection, we purified polyclonal CD4⁺ or CD8⁺ T cells from their lungs and co-cultured them with Mtb-infected macrophages. After 72 hours of co-culture, polyclonal CD4⁺ T cells produced high amounts of IFN γ in a MHC-restricted manner (Figure 2.7a). Interestingly, polyclonal CD8⁺ T cells also produced IFN γ in a MHC-restricted manner when co-cultured with Mtb-infected macrophages (Figure 2.7b). These results indicate that other antigen-specific CD8⁺ T cells recognizing Mtb-infected macrophages do exist, and infected TGPMs can present Mtb antigens to CD8⁺ T cells. However, based on the high abundance of TB10.4-specific CD8⁺ T cells post infection (Figure 2.1), the non-TB10.4-specific, Mtb-specific CD8⁺ T cells may be dwarfed by the dominant TB10.4-specific CD8⁺ T cells.

To better assess whether the IFN γ production by polyclonal CD8 $^{+}$ T cells arose predominantly from non-TB10.4-specific CD8 $^{+}$ T cells, we used the TB10.4 $_{4-11}$ -tetramer to separate TB10.4-specific and non-TB10.4-specific, polyclonal CD8 $^{+}$ T cells from the lungs of infected mice. After 72-hour co-culture with Mtb-infected macrophages, TB10.4 $_{4-11}$ -tetramer negative CD8 $^{+}$ (non-TB10.4-specific CD8 $^{+}$) T cells produced significantly higher IFN γ compared to that of uninfected control ($p < 0.005$), and the production was MHC class I restricted (Figure 2.7c). In contrast, TB10.4 $_{4-11}$ -specific CD8 $^{+}$ T cells produced IFN γ in a non-MHC-restricted manner during co-culture with both uninfected and Mtb-infected macrophages (Figure 2.7d). We cannot exclude the possibility that the tetramer isolation might have inadvertently activated the TB10.4 $_{4-11}$ -specific CD8 $^{+}$ T cells. Nevertheless, these data show that polyclonal, TB10.4 $_{4-11}$ -tetramer negative CD8 $^{+}$ T cells recognized Mtb-infected macrophages, supporting the notion of a subdominant T cell response that may be effective at detecting Mtb.

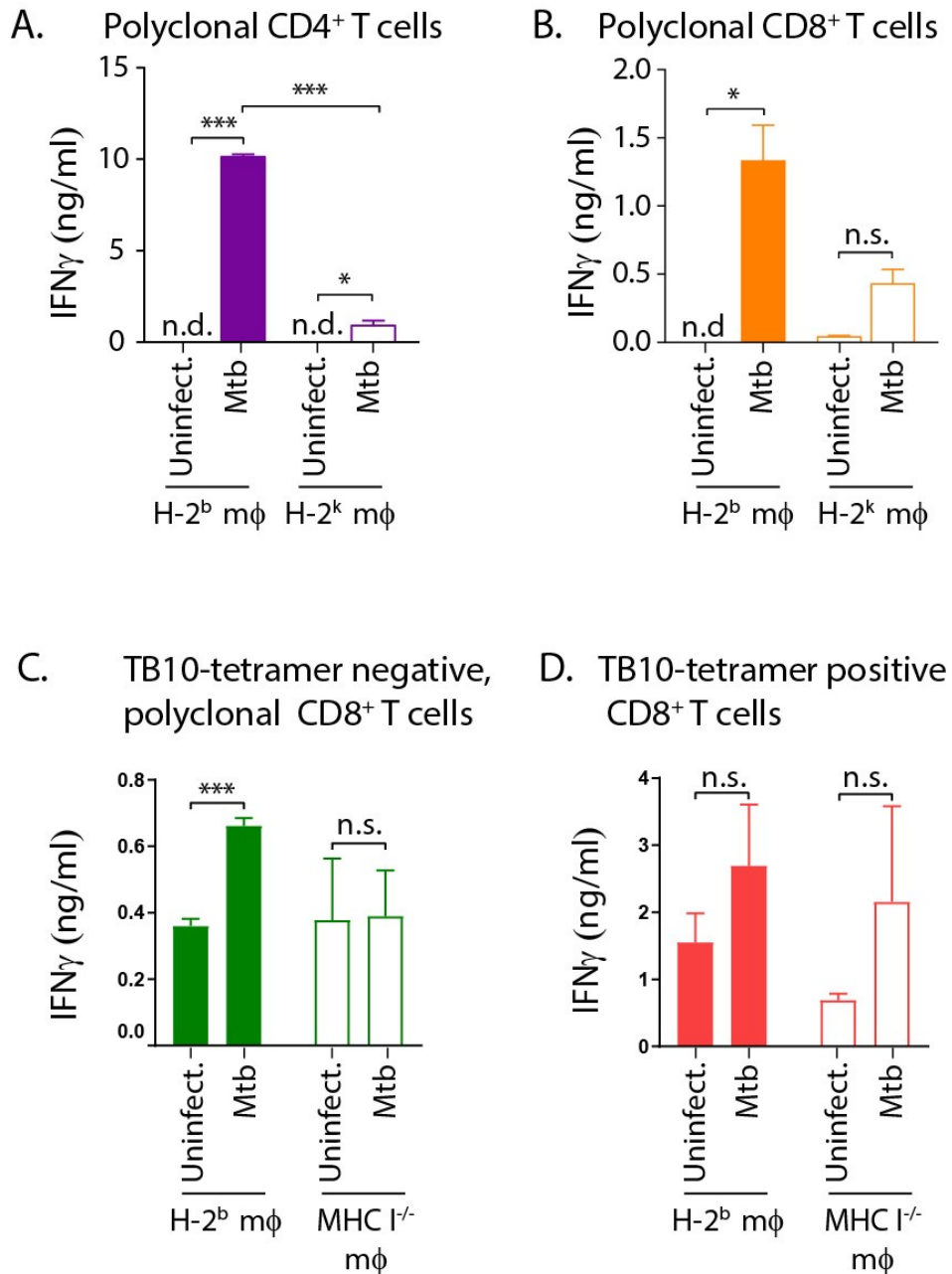


Figure 2.7. Polyclonal CD8⁺ T cells from the lungs of Mtb-infected mice recognize infected macrophages. IFN γ production by polyclonal CD4⁺ (a) or CD8⁺ (b) T cells after co-culture with either MHC-matched (H-2^b) or MHC-mismatched (H-2^k), Mtb-infected macrophages. IFN γ production by TB10.4-11-

tetramer-depleted (c) or tetramer-enriched (d) polyclonal CD8⁺ T cells after co-culture with either MHC-matched (H-2^b) or MHC-mismatched (H-2^k), Mtb-infected macrophages. Data is representative of at least 2 experiments. Statistical testing by a two-tailed, unpaired Student's T test. *, p<0.05; **, p<0.01; and ***, p<0.005. ϕ , macrophages.

Discussion

A complexity in defining T cell recognition is distinguishing cognate from non-cognate recognition. T cell IFN γ production, a common readout for recognition, can be stimulated by IL-12 and IL-18, two cytokines secreted by Mtb-infected cells [55, 161-163]. Even cognate recognition does not always signify recognition of infected cells. Uninfected macrophages and dendritic cells (DCs) can acquire exosomes, soluble proteins, apoptotic vesicles or necrotic debris containing non-viable bacilli or its antigens and present these to T cells [151, 240-242]. This detour pathway allows T cells to be activated by uninfected DCs [212, 240]. Thus, T cell recognition of infected macrophages, which is central to our fundamental paradigm of TB pathogenesis, remains poorly defined.

Our study advances the understanding of T cell recognition of Mtb-infected cells. By focusing on TCR-mediated recognition, our data show that T cells specific to immunodominant antigens vary in their ability to recognize Mtb-infected macrophages. Despite being a persistent and dominant population of CD8 $^{+}$ T cells in the lungs of Mtb-infected mice, TB10.4₄₋₁₁-specific CD8 $^{+}$ T cells do not recognize Mtb-infected macrophages. While we primarily used TGPMs, which have been used to model human macrophages [135, 243], we also showed that TB10.4-specific CD8 $^{+}$ T cells also failed to recognize lung APCs from infected mice. Importantly, concurrent with our analysis of CD8 $^{+}$ T cells, we systematically assessed recognition of Mtb-infected macrophages by Ag85b-specific (i.e., P25) and ESAT-6-specific (i.e., C7) CD4 $^{+}$ T cells. Both recognized Mtb-infected

macrophages and inhibited bacterial growth (here and [183]). Thus, under conditions that activated Mtb-specific CD4⁺ T cells, no activation of TB10.4-specific CD8⁺ T cells occurred. This finding has many implications, among which the most important is that not all Mtb-specific T cells recognize Mtb-infected macrophages.

Ag85b is an immunodominant antigen with an epitope recognized by CD4⁺ T cells in C57BL/6 mice. In vivo data shows that Ag85b-specific CD4⁺ T cells can recognize Mtb-infected cells early during infection; however, recognition decreases after infection is established [89, 144, 184, 185, 244]. The inability of Ag85b-specific CD4⁺ T cells to efficiently recognize Mtb-infected bone-marrow derived macrophages (BMDMs) or bone-marrow derived dendritic cells (BMDCs) stems from a combination of reduced Ag85b expression by Mtb and because infected cells actively export Ag85b into the extracellular milieu [144, 151]. In our experiments, we found that P25 T cells recognized Mtb-infected macrophages and inhibited bacterial growth in a MHC-restricted manner. A difference between the studies is the duration of macrophage and T cell co-culture. Grace et al examined 16-24 hours and found a lack of recognition, whereas our assays focused on 72-96 hours and detected recognition. Moreover, it is unknown whether Mtb-infected cells still exported antigens after the initial 24 hours of infection. Furthermore, the exported Ag85b could be taken up by infected cells during longer co-cultures, leading to their recognition by T cells. A recent study by Lai et al have found that Mtb-infected DCs can lead to the proliferation of P25 T cells [184], consistent with our findings that P25 T cells can recognize Mtb-infected macrophages. Finally, it

is possible that cognate recognition of uninfected cells that present Ag85b can activate CD4⁺ T cells in a TCR-mediated manner, inducing IFN γ and indirectly controlling Mtb replication in macrophages. Nonetheless, under conditions that activate Mtb-specific CD4⁺ T cells, we could not observe activation of TB10.4-specific CD8⁺ T cells.

These results led us to re-examine the evidence that CD8⁺ T cells recognize infected cells. In our evaluation of the literature, among the best evidence is: (1) direct ex vivo recognition of Mtb-infected macrophages and DC by CD4⁺ and CD8⁺ T cells [80, 176, 194, 245]; (2) murine T cells' cytolytic activity (CTL) of MTb-infected cells [76, 78, 113]; (3) human CD8⁺ T cells that recognize Mtb-infected DC [208, 216, 246]. However, these data have limitations. The murine studies rarely demonstrated cognate recognition, and the frequencies were lower than expected. The human studies were only done using DC and not macrophages and used a high MOI, raising concerns about death of infected cells and presentation of nonviable antigen. Nevertheless, these studies are consistent with the idea that CD8⁺ T cells recognize infected cells, but the frequency that recognize infected macrophages might be lower than we previously expected. Such a finding might explain why CD8⁺ T cells make a disproportionately small contribution to host defense, even though Mtb infection elicits a robust CD8 T cell response.

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We would like to thank Dr. Kenneth Rock (University of Massachusetts Medical School, MA) for the generous gift of C57BL/6 $K^{b-/-}D^{b-/-}$ (MHC $I^{-/-}$) mice. We also thank the Animal Medicine team at University of Massachusetts Medical School (Worcester, MA) for their technical assistance.

CHAPTER III. INVESTIGATING MECHANISMS OF NON-RECOGNITION BY TB10.4-SPECIFIC CD8⁺ T CELLS

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Attributions:

S.C. derived original TB10.4-specific CD8⁺ and P25 T cells, and J.D.Y. maintained TB10.4-specific CD8⁺ and P25 T cell lines for all experiments. D.M. and Y.J.L. performed the experiments in Figure 3.3, and F.R. performed the experiments in Figure 3.5a and b. J.D.Y. performed all other experiments and performed statistical analysis for all experiments. G.B. and S.F. made the TB10.4-overexpressing Mtb strains and performed qPCR to quantify overexpression measurement in Figure 3.4. S.S.W. provided the Listeria strains. B.K. provided SIINFEKL-bound magnetic microbeads used in Figure 3.2. S.C., and S.B. provided critical feedback for interpretation of the data. J.D.Y. and S.B. designed all experiments.

Abstract

T cells specific to immunodominant antigens are generated during Mtb infection, yet whether they recognize Mtb-infected macrophages is debatable. We have previously shown that TB10.4-CD8⁺ T cells do not efficiently recognize Mtb-infected macrophages, whereas the Ag85b-specific CD4⁺ T cells do. We also found that there are polyclonal CD8⁺ T cells that recognize Mtb-infected macrophages, yet we do not know which antigens are presented. These data suggest that there may be a unique mechanism that prevents the presentation of TB10.4 during infection.

Here, we investigate the lack of recognition by the TB10.4-specific CD8⁺ T cells. First, we examined whether Mtb may interfere with antigen presentation. We found that infection with Mtb did not inhibit expression of MHC I or MHC II molecules, nor did it interfere with presentation of OVA-bound magnetic beads. Moreover, we also examined whether the TGPMs could process and present TB10.4 whole protein during an infection. *Listeria monocytogenes* expressing TB10.4 whole protein was successfully recognized by TB10.4-specific CD8⁺ T cells after infection in TGPMs. Over-expression of TB10.4 in Mtb also did not result in better recognition by TB10.4-specific CD8⁺ T cells. Finally, we investigated whether Mtb may specifically inhibit the presentation of TB10.4. TB10.4-specific CD8⁺ T cells recognized macrophages pulsed with irradiated Mtb, indicating that macrophages can efficiently cross-present the TB10.4 protein and raising the possibility that viable bacilli might suppress cross-presentation.

Introduction

Mycobacterium tuberculosis has evolved many mechanisms of evading the host immune system. Other than preventing phagolysosomal fusion, Mtb also interferes with innate microbicidal activities of the macrophages [131] [132] and preferentially leads to the necrosis of the infected cells [135] [136]. Both human and mouse studies have shown that Mtb infection prevents the upregulation of IFN γ -induced MHC class II expression [148, 149], and the Mtb 19-kDa lipoprotein is responsible for this interference [122]. Mtb can also selectively turn down its expression of certain antigens so that antigen-specific T cells cannot recognize the infected cells late during infection [144]. Mtb also evades adaptive immunity by delaying T cell priming and recruitment, in both mice and human studies [60] [141, 142]. These data present a complicated host-pathogen interaction that favors the survival of Mtb within its host.

We have previously shown that Mtb antigen-specific CD8⁺ and CD4⁺ T cells differed in their capacity to recognize Mtb-infected cells. In a Mtb-infected, C57BL/6 mouse, TB10.4-specific CD8⁺ T cells dominate the CD8⁺ T cell response in the lung (Figure 2.1). Ag85b-specific CD4⁺ T cells also dominate the CD4⁺ T cell response in the lung during infection of C57BL/6 mice [247]. Ag85b-specific CD4⁺ T cells recognized Mtb-infected macrophages and reduced bacterial burden (Figures 2.3-2.5). However, TB10.4-specific CD8⁺ T cells did not recognize Mtb-infected macrophages (Figures 2.3-2.4), though higher affinity TB10.4-specific CD8⁺ T cells did recognize Mtb-infected DCs (Figure 2.5). The in vitro macrophage

study results were confirmed using ex vivo lung APCs, which also did not trigger recognition by the TB10.4-specific CD8⁺ T cells (Figure 2.6). Yet, there are other antigens presented by the infected macrophages as polyclonal, non-TB10.4-specific CD8⁺ T cells recognized those macrophages (Figure 2.7). These results suggest a unique environment in the infected macrophage that prevents successful antigen presentation of TB10.4.

We took several different approaches to investigate the lack of antigen recognition of TB10.4 during infection. We found that the infected macrophages retained their antigen presentation ability during infection (i.e. no decrease in MHC I or II expression and being able to present other antigens). Next, we found that TB10.4-specific CD8⁺ T cells recognized macrophages infected with recombinant *Listeria monocytogenes* expressing TB10.4, but only if the bacilli could escape into the cytosol. However, overexpressing TB10.4 in Mtb did not confer recognition. Importantly, macrophages pulsed with irradiated bacteria, as well as activated, Mtb-infected macrophages, efficiently cross-presented TB10.4 to CD8⁺ T cells, suggesting that live Mtb actively inhibited presentation.

Together, these data suggest that Mtb does not interfere with the antigen presentation ability of the macrophage on a whole cell scale. Live infection may impair the presentation of TB10.4 specifically. The impairment of this presentation may help Mtb evade T cell immunity as 35% of the CD8⁺ T cells, which are TB10.4-specific, cannot recognize the Mtb-infected macrophages.

Results

Mtb infection does not significantly impair MHC class I and II expression of macrophages.

We investigated whether Mtb may inhibit MHC class I expression by infected TGPMs. Mtb and TLR2 agonists inhibit IFN γ -induced MHC class II expression by bone marrow derived macrophages, and the mycobacterial PPE38 protein can inhibit MHC class I expression in RAW264.7 macrophages and TGPMs infected with *Mycobacterium smegmatis* [149, 248]. Therefore, we asked whether Mtb impaired MHC class I expression in our in vitro infection system, especially since the TGPMs were not pre-activated with IFN γ prior to infection. We measured MHC class I and II expression by macrophages on each of the five days following infection. At baseline, uninfected TGPMs expressed high MHC class I, and Mtb infection did not alter MHC class I expression compared to the baseline (Figure 3.1a, c; solid lines). Although IFN γ pretreatment of macrophages led to an increase in MHC class I expression in uninfected TGPMs, infected TGPMs did not achieve the same peak levels (Figure 3.1a, c; dotted lines). As expected, the regulation of MHC class II was more sensitive to IFN γ . Uninfected TGPMs expressed low baseline levels of MHC class II (Figure 3.1b, d; solid lines). IFN γ pretreatment resulted in a >100-fold increase in MHC class II median fluorescence intensity (MFI) in the uninfected TGPMs, which peaked on day 3 with a >2000-fold increase over the baseline (Figure 3.1b, d; dotted lines). Mtb-infection alone did not significantly affect MHC class II expression, and consistent with previous studies,

Mtb significantly impaired the induction of MHC class II by IFN γ pretreatment (Figure 3.1b, d). These data show that in our in vitro infection model, in which the TGPMs were unstimulated, Mtb infection did not inhibit class I and II MHC expression. Importantly, the differences in MHC class I or class II expression by Mtb-infected macrophages cannot explain why P25 T cells, but not TB10Rg3 or TB10Rg4 T cells, recognized infected macrophages.

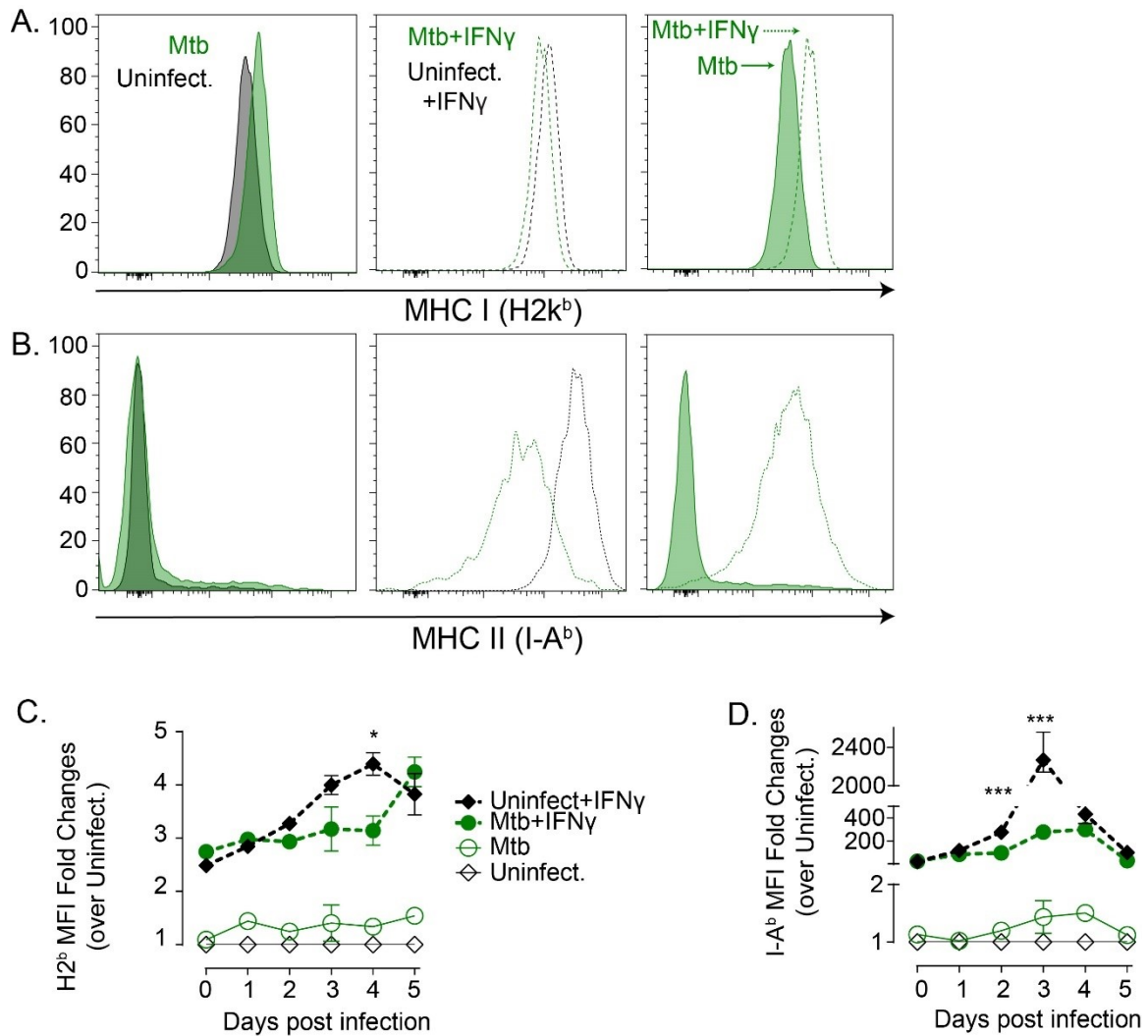


Figure 3.1. Mtb infection does not significantly impair MHC class I and II expression of macrophages. (a-b) MHC class I and II expression by Mtb-infected-macrophages. Representative histograms and fold-change of MHC class I (a, c) or class II (b, d) expression on infected cells. Data is representative of 3 experiments. Statistical testing by one-way ANOVA with Dunnett posttest. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.005$.

Mtb infection does not interfere with global presentation of antigens.

Mtb can interfere with a series of host immune signaling pathways in the macrophage in order to survive. In 1996, Mazzaccaro et al found that feeding soluble OVA protein to Mtb-infected BMDMs led to production of IL-2 by SIINFEKL-specific, MHC I-restricted CD8⁺ T cell hybridoma, RF33.70 [193]. However, more recent studies have suggested that live Mtb infection may impede the presentation of antigens [210-212]. Our data previously showed that polyclonal CD8⁺ T cells from the lungs of infected mice recognized Mtb-infected macrophages, suggesting that live Mtb infection does not impede antigen presentation of all Mtb antigens. Nonetheless, we thought to test whether Mtb infection may interfere with antigen presentation within the entire infected cell.

TGPMs were either uninfected, uninfected treated with γ -irradiated Mtb (i.e. dead bacteria), or infected with live Mtb. All samples were then treated with either the SIINFEKL peptide or a titration of OVA-bound magnetic beads (a generous gift from Dr. Kenneth Rock, University of Massachusetts, Worcester, MA) and then co-cultured with SIINFEKL-specific RF33.70 CD8⁺ T cells, which is a MHC class I restricted [249]. After 24 hours of co-culture, we found that the RF33.70 T cells produced IL-2 when co-cultured with any of the three different macrophage populations, regardless of the titration of the OVA-bound beads (Figure 3.2). At the highest concentration of OVA-bound beads (i.e. 40 μ g/ml), we detected a trend of infected macrophages eliciting more T-cell-produced IL-2 compared to uninfected macrophages and γ -irradiated-Mtb-treated macrophages (Figure 3.2). This

increasing trend, while not statistically significant by two-way ANOVA with Tukey posttest, was seen in 3 independent experiments. There was also a decreasing trend where γ -irradiated-Mtb-treated macrophages elicited less T-cell-produced IL-2 compared to uninfected macrophages (Figure 3.2). However, this decreasing trend was seen in only 1 out of 3 independent experiments.

This data shows that OVA-bound beads can be phagocytosed and processed correctly for MHC class I presentation by macrophages treated with either dead Mtb or infected with live Mtb. Moreover, live Mtb infection did not inhibit the cross presentation of phagocytosed antigens. The limitation of this experiment is that we could not tell whether the phagocytosed antigens resided in the same phagosomes as the Mtb-containing phagosome. Therefore, this data can only tell us that the global, or the whole cell, ability of the infected macrophage to present antigen is not interfered, but it does not tell us whether the Mtb-containing phagosome is capable of participating in the MHC class I presentation pathway. Nonetheless, taking into account of the fact that polyclonal CD8⁺ T cells recognized Mtb-infected macrophages, this data supports the conclusion that Mtb-infected macrophages can present antigens.

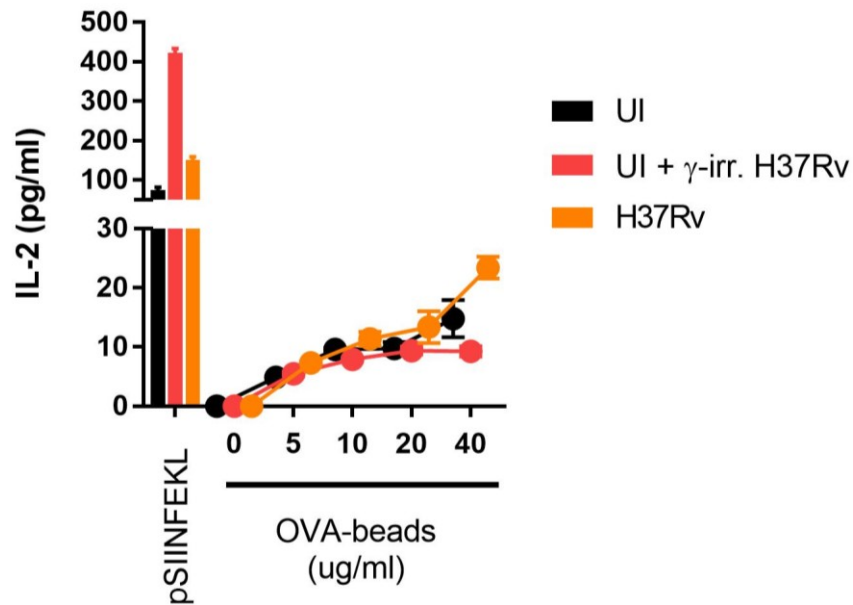


Figure 3.2. Mtb infection does not interfere with global presentation of antigens. TGPMs were either uninfected, uninfected treated with γ -irradiated Mtb (i.e. dead bacteria), or infected with live Mtb. All samples were then treated with either the SIINFEKL peptide or a titration of OVA-bound magnetic beads before co-culturing with RF33.70 T cells. The amount of IL-2 secretion was assayed via ELISA.

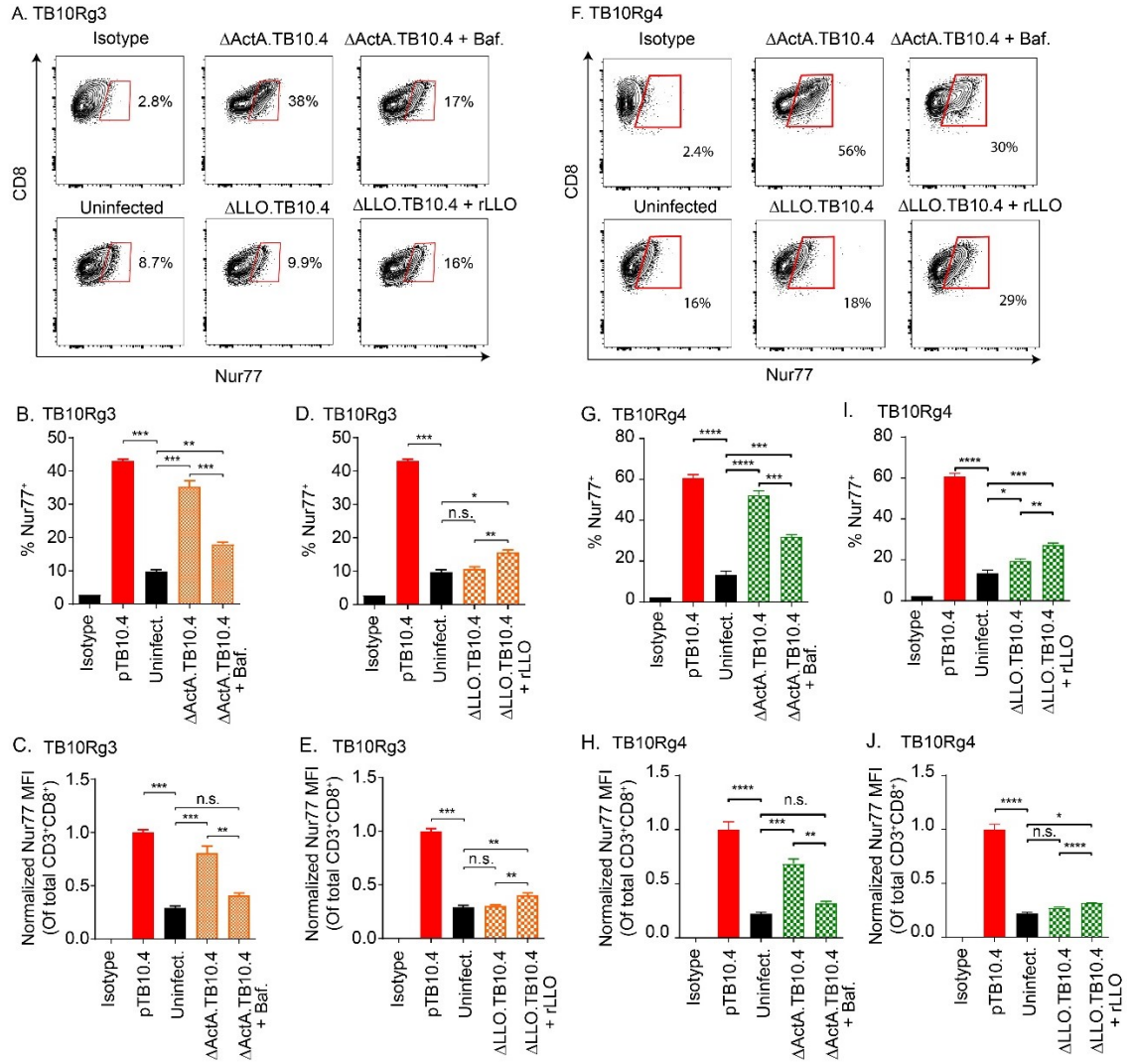
TB10.4-specific CD8⁺ and P25 CD4⁺ T cells recognize macrophages infected with TB10.4- or Ag85b-expressing *Listeria*.

We next investigated whether the location of the antigen might affect the presentation of TB10.4 since the MHC class I antigen presentation pathway primarily samples the cytosol, whereas Mtb is a classic phagosomal pathogen. TB10.4-specific CD8⁺ T cells are primed and expanded during Mtb infection, so the TB10.4 antigens must be cross-presented; however, whether murine, Mtb-infected macrophages are competent to cross-present mycobacterial antigens is unknown. We investigated these possibilities using Δ LLO or Δ ActA mutant strains of *Listeria monocytogenes* engineered to express the full length TB10.4 protein, hereafter referred to as Δ LLO.TB10.4 or Δ ActA.TB10.4, respectively. Both are attenuated strains: the Δ LLO.TB10.4 mutant cannot escape from the vacuole, while the Δ ActA.TB10.4 mutant can escape from the vacuole but not from the cell. Hence, the TB10.4 protein made by the Δ LLO.TB10.4 strain will remain trapped in the phagosome, but the TB10.4 protein made by the Δ ActA.TB10.4 strain will gain access to the cytosol.

TB10Rg3 T cells recognized Δ ActA.TB10.4-infected TGPMs based on an increased frequency of Nur77-expressing cells ($p < 0.005$) and the Nur77 MFI ($p < 0.005$) (Figure 3.3a-c). Bafilomycin, which inhibits vacuolar acidification and impairs the entry of the Δ ActA.TB10.4 strain into the cytosol, diminished the frequency of Nur77-expressing cells ($p < 0.005$) and Nur77 MFI ($p < 0.01$) (Figure 3.3a, top, b, c). In contrast, TB10Rg3 T cells co-cultured with Δ LLO.TB10.4-

infected TGPMs showed no increase in the frequency of Nur77-expressing cells or the Nur77 MFI (Figure 3.3a bottom, d, e). If recombinant listeriolysin (rLLO), the protein missing from the Δ LLO.TB10.4 strain, was added to the infected macrophages, an increase in the frequency of Nur77-expressing TB10Rg3 T cells ($p < 0.01$) and the Nur77 MFI ($p < 0.01$) became apparent. TB10Rg4 T cells responded similarly to the TB10Rg3's, though their responses are consistently higher (Figure 3.3f-j). Of note, TB10Rg4 increased significantly in the frequency of Nur77-expressing T cells ($p < 0.05$) even during co-culture with Δ LLO.TB10.4-infected TGPMs (Figure 3.3i). However, this significant increase was not seen when measuring the MFI of the TB10Rg4 T cell population (Figure 3.3j). Nonetheless, both correlates of recognition increased significantly when rLLO was added to the co-culture prior to adding the T cells (Figure 3.3i, j).

We also determined whether P25 T cells recognized Ag85b-expressing *Listeria monocytogenes* using the recombinant *Listeria* strains Δ ActA.Ag85b and Δ LLO.Ag85b. Based on the propensity of MHC class II to present extracellular and vacuolar antigens, P25 cells recognized TGPMs infected with either Δ ActA.Ag85b or Δ LLO.Ag85b, based on an increase in the frequency of Nur77-expressing T cells and Nur77 MFI ($p < 0.005$) (Figure 3.3k-m). These results show that 1) TGPMs can efficiently process the full length TB10.4 protein and present the TB10.4₄₋₁₁ epitope via MHC class I; 2) this process is more efficient when the bacteria are in the cytosol; and 3) TB10Rg3 T cells can efficiently recognize TB10.4₄₋₁₁ presented during a live infection.



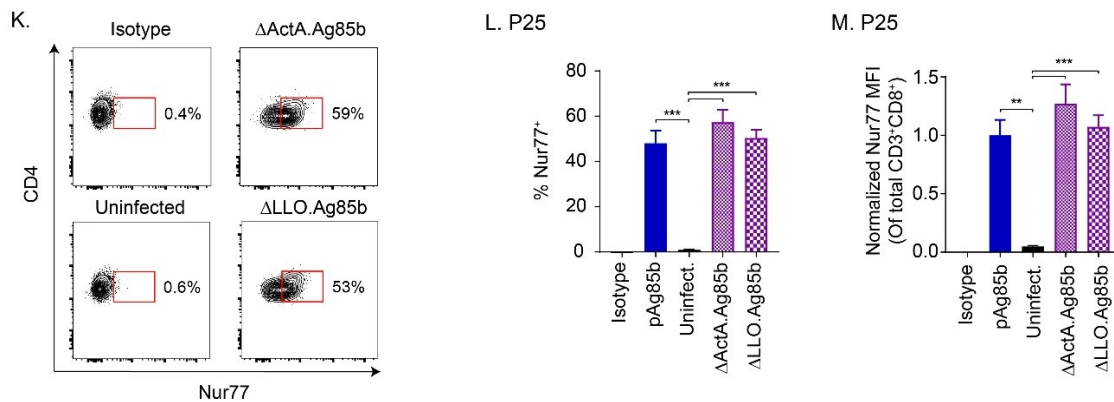


Figure 3.3. TB10Rg3, TB10Rg4 and P25 T cells can recognize macrophages infected with *Listeria monocytogenes* expressing TB10.4 and Ag85b proteins, respectively. (a) Representative flow plots showing Nur77 induction by TB10Rg3 T cells after co-culture with macrophages infected with Δ ActA.TB10 (top row) or Δ LLLO.TB10 (bottom row) *Listeria*. (b-e) Analysis of the frequency of Nur77-expressing TB10Rg3 T cells (b, d) or normalized MFI (c, e) after co-culture with Δ ActA.TB10 (b, c) or Δ LLLO.TB10 (d, e) infected macrophages. (f) Representative flow plots showing Nur77 induction by TB10Rg4 T cells after co-culture with macrophages infected with Δ ActA.TB10 (top row) or Δ LLLO.TB10 (bottom row) *Listeria*. (g-j) Analysis of the frequency of Nur77-expressing TB10Rg4 T cells (g, i) or normalized MFI (h, j) after co-culture with Δ ActA.TB10 (g, h) or Δ LLLO.TB10 (i, j) infected macrophages. (k) Representative flow plots showing Nur77 induction by P25 T cells after co-culture with macrophages infected with Δ ActA.TB10 (top row) or Δ LLLO.TB10 (bottom row) *Listeria*. Analysis of the frequency of Nur77-expressing P25 T cells (l) or normalized MFI (m) after co-culture with Δ ActA.TB10-

or Δ LLO.TB10-infected macrophages. Representative of at least two experiments.

Statistical testing by one-way ANOVA with Dunnett posttest. *, $p < 0.05$; **, $p < 0.01$;

, $p < 0.005$; *, $p < 0.0001$.

TB10.4-specific CD8⁺ T cells do not recognize Mtb overexpressing TB10.4.

Antigen abundance can affect T cell recognition, so we next tested whether increasing the level of TB10.4 protein expression might enhance TB10Rg3 T cell recognition of Mtb-infected macrophages. Since Mtb secretes esxH (TB10.4) together with esxG as a heterodimer [250], we developed a recombinant strain of H37Rv (esxGH-OE.Mtb), which overexpresses both esxG and esxH under the control of a tet^{ON} promoter. After tetracycline induction for 24 hours, the esxG and esxH mRNA expression increased multiple folds (Figure 3.4a). Prior to in vitro infection, we treated esxGH-OE.Mtb with or without tetracycline. The next day, TGPMs were infected with uninduced or tetracycline-induced esxGH-OE.Mtb. P25 T cells produced similar amounts of IFN γ when co-cultured with macrophages infected with either uninduced or induced esxGH-OE.Mtb, which was expected since Ag85b expression should not be altered (Figure 3.4b). Despite increasing the production of TB10.4 by Mtb, TB10Rg3 T cells still did not recognize Mtb-infected macrophages (Figure 3.4c). Moreover, we also assessed the ability of TB10Rg4 T cells to recognize TGPMs infected with esxGH-OE.Mtb. Out of 4 experiments, 2 experiments showed no specific IFN γ production while the other 2 experiments showed specific increase in IFN γ production even when co-cultured with TGPMs infected with uninduced esxGH-OE.Mtb (data not shown). Due to this inconsistency, it was challenging to make a formal conclusion for TB10Rg4 T cells. Nonetheless, the trend that TB10Rg4 T cells may produce IFN γ when co-cultured with Mtb-infected TGPMs hint at the importance of the increased affinity for

TB10.4. Although we cannot be certain that the induction of EsxGH leads to an increased amount of antigen delivered to the antigen processing pathway, this result suggests that antigen abundance is not limiting TB10.4-specific CD8⁺ T cell recognition of Mtb-infected macrophages.

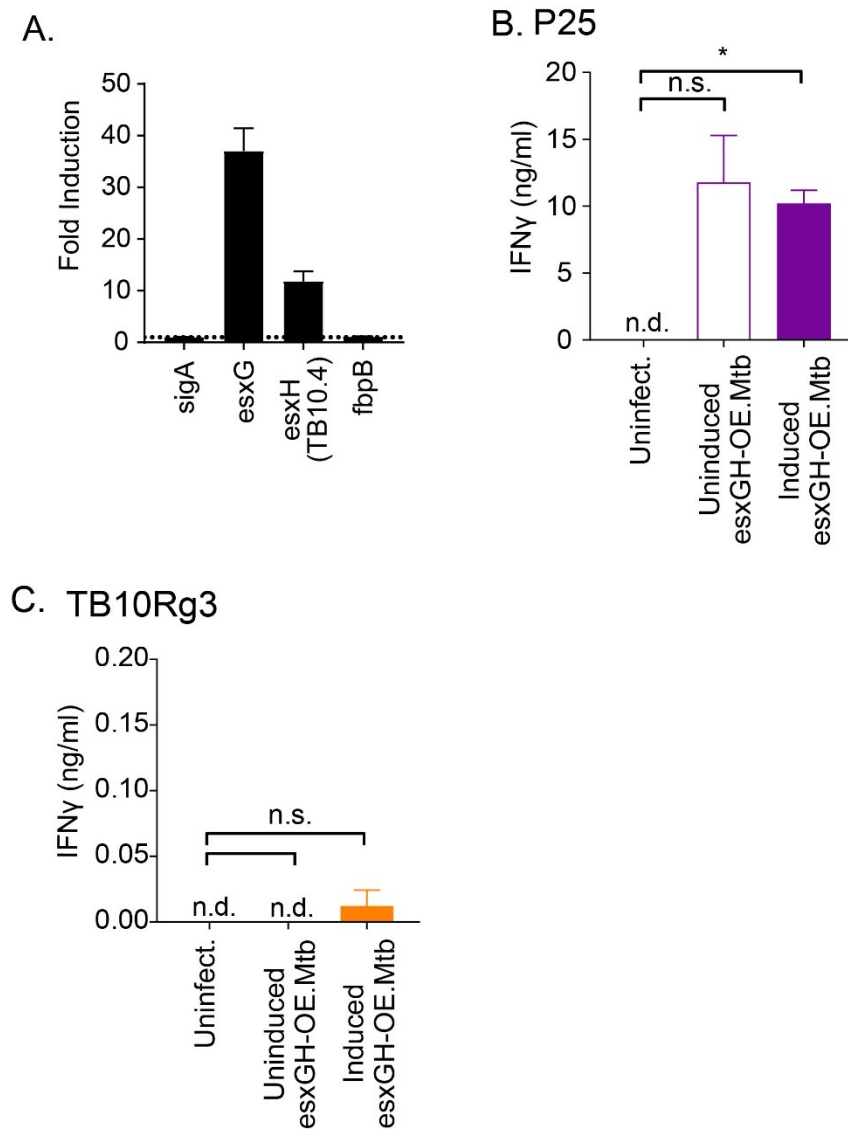


Figure 3.4. TB10.4-specific CD8⁺ T cells do not recognize Mtb overexpressing TB10.4. (a-c) EsxG (TB10.4) and its partner EsxH were overexpressed together in H37Rv to determine whether increasing TB10.4 abundance would lead to recognition of infected macrophages (esxGH-OE.Mtb). (a) Tetracycline treatment of esxGH-OE.Mtb in broth culture induces esxG and esxH, but not fbpB and sigA, mRNA as measured by qPCR. Fold-induction was normalized to baseline (i.e.,

uninduced). IFN γ production by (b) P25 or (c) TB10Rg3 T cells after co-culture with macrophages infected with uninduced or induced esxGH-OE.Mtb.

Macrophages cross-present antigens from non-viable Mtb to TB10.4-specific CD8⁺ T cells.

Next, we hypothesized that Mtb may interfere with MHC class I presentation of mycobacterial antigens, specifically TB10.4. Therefore, we tested the ability of the P25 and TB10Rg3 T cell lines to recognize TGPMs cultured with γ -irradiated, nonviable Mtb. Activation of pattern recognition receptors such as TLR2 and TLR4 by large amounts of dead bacteria might induce large amounts of IL-12 and IL-18, resulting in cytokine-driven T cell activation. Taking this concern into consideration, we used MHC-mismatched TGPMs as a control. We pulsed macrophages with a dose titration of γ -irradiated Mtb, then added TB10Rg3 or P25 T cells, and measured IFN γ secretion by the T cells after 72 hours. Both P25 and TB10Rg3 T cells produced high amounts of IFN γ when cultured with MHC-matched (i.e., H-2^b) but not with MHC-mismatched (i.e., H-2^k), TGPMs, and this response was dose dependent (Figure 3.5a, b). The ability of macrophages to process and present TB10.4 after phagocytosing γ -irradiated Mtb but not viable bacteria raises the possibility that live Mtb actively inhibit MHC class I presentation of TB10.4.

Given that cross presentation of non-viable Mtb can lead to efficient recognition by TB10.4-specific CD8⁺ T cells, we next investigated whether pre-activating macrophages might lead to better recognition. Pre-activation of macrophages with IFN γ has been shown to lead to enhanced antimicrobial effects [251] [252] [44, 253, 254]. Thus, TGPMs were pre-treated with 25ng/ml of IFN γ overnight, washed, and infected with Mtb. When T cells were co-cultured from d0

to d3 post infection, no consistent increase in IFN γ was observed for TB10Rg3 and TB10Rg4 T cells (data not shown). We also examined later infection time point, from d3 to d6 post infection. After 72 hours of co-culture, there was minimal amount of background IFN γ , indicating that the washing step did not leave residual IFN γ in the co-culture (Figure 3.5c-e). P25 T cells showed increased IFN γ production when the TGPMs were pretreated with IFN γ ($p < 0.05$) (Figure 3.5c). TB10Rg3 T cells showed large increase in IFN γ production when the TGPMs were pretreated with IFN γ , however, this increase was not significant when analyzed via two-way ANOVA with Sidak posttest (Figure 3.5d). TB10Rg4 T cells showed significant, MHC class I-restricted increase when co-cultured with TGPMs pretreated with IFN γ ($p < 0.05$) (Figure 3.5e).

We examined an additional molecule for pre-activating macrophages by pretreating TGPMs with IL-32. IL-32 is a human cytokine important in protecting the human host against Mtb [255]. Interestingly, IL-32 can also protect mice against Mtb infection [256]. In Mtb-infected THP-1 macrophages, IL-32 has been shown to lead to increased apoptosis of the infected cells [257]. We pre-treated TGPMs with IL-32 for 1 hour prior to infection. After co-culturing Mtb-infected, IL-32-pretreated TGPMs with T cells, we found that TB10Rg3 did not have consistent increase in IFN γ production (Figure 3.5f). However, TB10Rg4 T cells exhibited significant, MHC class I-restricted IFN γ production, and that production was dependent on the dose of IL-32 used (Figure 3.5g).

These results show that the non-viable Mtb (i.e. γ -irradiated Mtb) can be presented by TGPMs, and that presentation leads to MHC-specific recognition by the TB10Rg3 T cells. Moreover, pre-activating macrophages with either IFN γ or IL-32 led to MHC-specific recognition as well, though TB10Rg4 responded better than TB10Rg3 T cells. These data support the notion that live Mtb may actively inhibit the presentation of TB10.4.

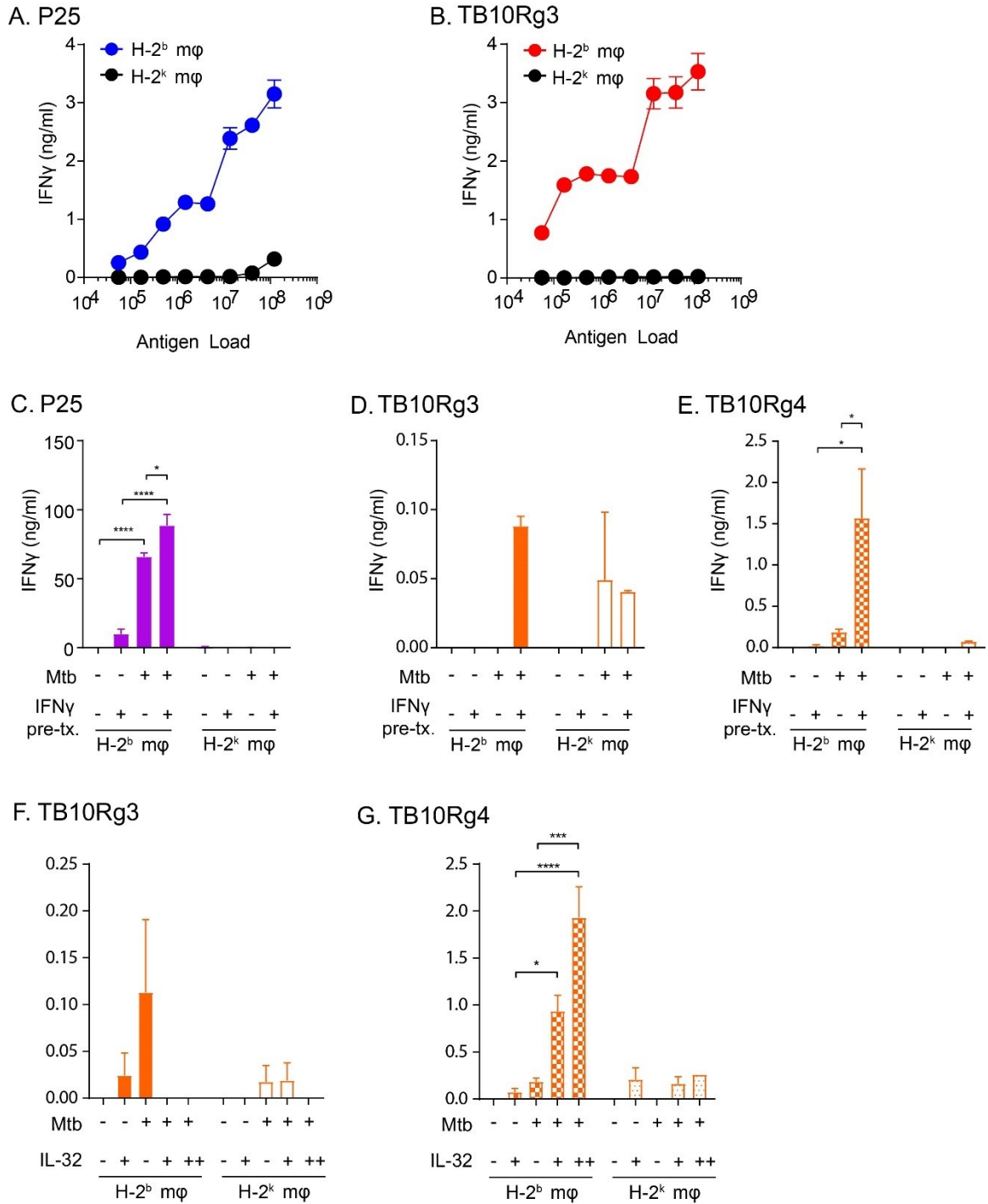


Figure 3.5. Macrophages cross-present antigens from non-viable Mtb to TB10.4-specific CD8⁺ T cells. (a) P25 and (b) TB10Rg3 production of IFN γ after

co-culture with macrophages pulsed with titrated amounts of γ -irradiated (non-viable) H37Rv. (c-e) TGPMS were pretreated with 25ng/ml IFN γ overnight prior to infection. After 72 hours of co-culture, the IFN γ produced by (c) P25, (d) TB10Rg3, and (e) TB10Rg4 T cells were measured. (f-g) TGPMS were pretreated with either 100ng/ml (+) or 200ng/ml (++) IL-32 for 1 hour prior to infection. After 72 hours of co-culture, the IFN γ produced by (f) TB10Rg3 and (g) TB10Rg4 T cells were measured. Data is representative of 3 (a-b) or 2 (c-g) experiments. Statistical testing by one-way ANOVA with Dunnett posttest (a-b) or two-way ANOVA with Sidak posttest (c-g). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.0001$. ϕ , macrophages.

Discussion

We investigated several mechanisms that might explain why TB10.4-specific CD8⁺ T cells do not recognize infected macrophages. We confirmed that Mtb-infected macrophages only had decreased MHC I and MHC II expression when the macrophages were pre-activated with IFN γ . In our in vitro model, where the macrophages were not activated with IFN γ , we found there was no decrease in either MHC I or II expression, indicating that Mtb did not interfere with their expression levels and that the lack of recognition could not be explained by this. Pre-activating macrophages with either IFN γ or IL-32 led to increased MHC-specific recognition by the TB10.4-specific CD8⁺ T cells, with the TB10Rg4 T cells having a better overall response. We also found that Mtb infection did not hinder the overall presentation of antigens during the OVA-coated beads studies, and this supports our finding that other antigens can be presented as evidenced by the recognition from the polyclonal CD8⁺ T cells.

Another possibility is the access of the TB10.4 antigen to the MHC class I processing pathway. Mtb can disrupt the phagosomal membrane and translocate into the cytosol [258], though this action often occurs later in infection and leads to necrosis of the macrophage [259]. We saw no evidence of recognition even at late time points such as days 4-5 post infection (Fig 3). The importance of antigen location became apparent during the *Listeria* infection experiments, where infected macrophages presented TB10.4₄₋₁₁ only when the bacteria could enter the cytosol (i.e., Δ ActA.TB10 but not Δ LLO.TB10). The *Listeria* experiments also provided an

additional insight. Lindenstrom et al report that vaccination with TB10.4 (EsxH), which has a leucine at position 12 (i.e., IMYNYPAML), inefficiently generates TB10.4-specific CD8⁺ T cells [260]. However, vaccination with TB10.3 (EsxR), a related antigen that also contains the same epitope followed by a methionine (i.e., IMYNYPAMM), elicits TB10.4-specific CD8⁺ T cells. This led them to conclude there is a processing defect that prevents the generation of the TB10.4₄₋₁₁ epitope from the TB10.4 protein. However, they also find that TB10.4-specific CD8⁺ T cells elicited by TB10.3 vaccination recognize splenocytes pulsed with the rTB10.4 proteins, showing that the full length TB10.4 protein can be processed and presented. These data indicate that the lack of vaccine-elicited TB10.4-specific CD8⁺ T cells is due to a problem with priming after vaccination instead of an inability to process the IMYNYPAM epitope. Moreover, our data using TB10.4 expressed by *Listeria* show that TGPMs can process the full length TB10.4 protein and present the TB10.4₄₋₁₁ epitope. Therefore, we conclude that the processing of TB10.4 is not hindered by its amino acid sequence, and antigen location may be an important factor in antigen presentation. While the *Listeria* experiments show the potential importance of antigen location and raise the possibility that sequestration of the TB10.4 antigen in the phagosome renders it inaccessible to the MHC class I presentation pathway, another important variable is antigen abundance.

We have previously argued that there is limited amount of TB10.4 antigen presentation in the lungs of infected mice, leading to extreme bias in the TCR

repertoire of the TB10.4-specific CD8⁺ T cell response and defects in the memory-recall response in vivo [227, 231]. We considered whether low TB10.4 abundance could explain why Mtb-infected macrophages do not present TB10.4. To test this possibility, we overexpressed EsxG and EsxH (TB10.4) together but did not see greater T cell recognition of Mtb-infected macrophages, suggesting that abundance might not be the issue.

Unexpectedly, macrophages pulsed with γ -irradiated Mtb were recognized by TB10.4-specific CD8⁺ T cells, raising the possibility that live Mtb actively inhibits MHC class I presentation of TB10.4. This is particularly interesting since the presentation of CFP-10, another ESAT-6-like protein, by human DCs to CD8⁺ T cells requires viable Mtb; DCs given heat-killed bacteria do not present CFP10 to T cells [216]. While these data suggest that presentation requires active secretion of CFP10 [261, 262], the heat-killing process could have destroyed CFP10, or there might not have been sufficient amounts of CFP10 available in the non-viable bacteria. On the other hand, Russell et al demonstrate that infection of mice with recombinant ovalbumin (rOVA)-expressing BCG reduces CD8⁺ T cell priming compared to when the mice are infected with rOVA-expressing *Listeria monocytogenes*; but increasing the amount of OVA being expressed corrects the defect [143]. This finding also suggests that live mycobacterial infection may hinder antigen presentation of antigens that are presumed to be in the same phagosome as the bacterium. Our data supports this idea. In combination with our data showing polyclonal CD8⁺ T cells recognize Mtb-infected macrophages, these data

suggest that it is possible that certain antigens are presented by live Mtb while others are actively prevented from being sampled by MHC class I. Together, the investigation into the mechanisms behind the lack of recognition have shown that there is an active inhibitory process by the Mtb that prevents efficient presentation of the TB10.4 protein. Future studies may focus on how exactly Mtb manipulates the host cell or modifies its own behavior to prevent presentation of TB10.4.

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CHAPTER IV. DISCUSSION

In 2015, TB overtook HIV to become the leading cause of death by an infectious disease in the world [1]. While global public health efforts have drastically reduced the mortality rate, the rate at which multi-drug resistant strains of TB are emerging is alarming. Therefore, research efforts focusing on new therapeutics and vaccine targets are of great significance. It is our goal that, by examining how the host interacts with the pathogen, we may gain a better understanding of the pathogenesis and identify processes that may be exploited against Mtb. To that end, we began our study with a fundamental question: Do antigen specific CD8⁺ and CD4⁺ T cells recognize Mtb-infected macrophages?

In Chapter II, we examined this question by assessing the ability of TB10.4-specific CD8⁺ T cells or antigen-specific CD4⁺ T cells to recognize Mtb-infected cells. We found that, while antigen-specific CD4⁺ T cells recognized Mtb-infected macrophages, TB10.4-specific CD8⁺ T cells did not. This was a particularly interesting result as other Mtb-specific CD8⁺ T cells have been found to produce IFN γ , upregulate activation markers and reduce bacterial burden (See Chapter I. Introduction). However, only two of those studies showed that recognition MHC-restricted. This prompted us to investigate the second question: Why is there a lack of efficient recognition by TB10.4-specific CD8⁺ T cells?

In Chapter III, we took multiple approaches to probe the mechanisms that could lead to poor recognition. Live Mtb infection did not hinder antigen presentation at a whole cell level. Nor did it inhibit MHC class I and II expression

in un-activated TGPMs. Interestingly, TB10.4-specific CD8⁺ T cells recognized TGPMs infected with TB10.4-expressing *Listeria monocytogenes* when the bacteria are allowed into the cytosol. The most important finding was that macrophages could present TB10.4 from non-viable Mtb to TB10.4-specific CD8⁺ T cells. This supports the idea that live Mtb may actively prevent the presentation of TB10.4 by MHC class I.

Together, our data from these two chapters highlight the importance of assessing recognition as part of understanding the host-pathogen interaction. Our systematic approach has uncovered a difference in the recognition of Mtb-infected macrophages by TB10.4-specific CD8⁺ T cells and antigen-specific CD4⁺ T cells, raising several intriguing questions for further investigation. In the following discussion, I will summarize the major findings and areas for improvement from each chapter, raise new questions for investigation, and outline the approaches that might enable us to better understand how Mtb evades T cell immunity.

***Mycobacterium tuberculosis*-specific CD4⁺ and CD8⁺ T cells differ in their capacity to recognize Mtb-infected cells**

Summary of results

We investigated the ability of TB10.4-specific CD8⁺ and Ag85b-specific CD4⁺ T cells to recognize Mtb-infected cells. TB10.4 (EsxH) is a secreted protein important in iron and zinc acquisition, and Mtb requires it in order to survive both in vitro and in vivo [224, 225]. Our interest in TB10.4 comes from the finding that TB10.4₄₋₁₁-specific CD8⁺ T cells make up ~35-40% of total lung CD8⁺ T cells during murine infection (Figure 2.1) [226, 227]. Yet, despite eliciting large numbers of TB10.4-specific CD8⁺ T cells, a vaccine incorporating the H-2 K^b-restricted epitope, TB10.4₄₋₁₁, failed to protect mice from Mtb infection [231]. We hypothesized that the inability of TB10.4-specific CD8⁺ T cells to mediate protection may be due to inefficient recognition of Mtb-infected macrophages. We also investigated recognition by Ag85b-specific CD4 T cells. In vivo data shows that Ag85b-specific CD4⁺ T cells can recognize Mtb-infected cells early during infection; however, recognition decreases after infection is established [89, 144, 184, 185, 244]. Whether these cells recognize Mtb-infected macrophages in vitro remains controversial.

In our in vitro infection model, we found that TB10.4-specific CD8⁺ T cells did not recognize or reduce bacterial burden in Mtb-infected macrophages. Increasing the MOI, examining at different time points, or the length of the co-culture did not lead to better recognition by the TB10Rg3 T cells. TB10Rg4 T cells,

which have a higher affinity to TB10.4₄₋₁₁ than TB10Rg3 T cells, showed some ability to recognize Mtb-infected macrophages based on IFN γ production. However, those results were not always significant or reproducible. In co-cultures with Mtb-infected BMDCs, TB10Rg4 T cells consistently produced IFN γ in a MHC-restricted manner, whereas similar results from TB10Rg3 T cells were not reproducible. The finding that DCs present TB10.4 is not surprising as DCs are the primary cells found in the LN that prime the T cells [61]. The lack of recognition of Mtb-infected macrophages is still important since macrophages remain a dominant population of infected cells during established infection [235]. Under the same conditions where TB10Rg3 and TB10Rg4 T cells did not recognize Mtb-infected macrophages, P25 T cells recognized and reduced bacterial burden. Extending these experiments to ex vivo cells, we discovered that TB10Rg3 and TB10Rg4 did not proliferate when co-cultured with lung APCs from infected mice. Since Ag85b expression is decreased after 3 weeks post infection, we used an ESAT-6-specific CD4⁺ T cell line [144]. ESAT-6 is another immunodominant antigen that can help form pores in the phagosomes to allow Mtb to escape into the cytosol [263]. Co-culture of the ex vivo APCs with ESAT-6-specific CD4⁺ T cells showed that those CD4⁺ T cells could proliferate, which requires cognate recognition [179, 264]. We also wondered whether the lack of recognition by TB10.4-specific CD8⁺ T cells may indicate a global defect in which all CD8⁺ T cells generated during Mtb infection may have limited recognition ability, and this was further explored in the Chapter III. Interestingly, polyclonal CD8⁺ T cells from the lungs of infected mice

recognized Mtb-infected macrophages in a MHC-restricted manner, and it was the non-TB10.4-tetramer CD8⁺ T cells that recognized those macrophages. This important finding also illustrates that there is no global defect in antigen presentation or processing. Nonetheless, we could not exclude the possibility that there may still be decreased antigen presentation.

Future directions

Our findings show that TB10.4-specific CD8⁺ T cells do not recognize Mtb-infected macrophages while Ag85b-specific CD4 and ESAT-6-specific CD4⁺ T cells do. Before discussing the future plans, there are a few limitations to our study that could be addressed in the future.

Our findings that the TB10.4-specific CD8⁺ T cells have limited recognition of Mtb-infected macrophages is set in a specifically defined context. First, while we exhaustively investigated various infection and experimental parameters to detect recognition, our in vitro infection model cannot reflect the physiologic environment during chronic infection. Infected TGPMs can stay healthy for about one week after infection, after which the TGPMs will start to die due to increased bacterial burden (observations from the lab). There is evidence that suggests that CD8⁺ T cells are more effective during later stages of infection [107, 108]. Both CD4⁺ and CD8⁺ T cells can become exhausted during chronic infection [265], and it is possible that the effectiveness of CD8⁺ T cells is better appreciated during a setting when CD4⁺ T cells are functionally exhausted [145]. On the other hand, the infected

macrophages and the Mtb may change as well [266] Nonetheless, it is still unknown whether TB10.4-specific CD8⁺ T cells recognize Mtb-infected cells in late infection in vivo. TB10.4-specific CD8⁺ T cells persist after 3 months in infected C57BL/6 [231] or 9 months in infected BALB/c mice [226]. Whether these persistent TB10.4-specific CD8⁺ T cells may recognize during late infection is not easy to dissect in an in vitro model. While we attempted this by looking into co-cultures of T cells and Mtb-infected macrophages from d3 to d6, this may not represent a real late infection. Future directions should examine whether the TB10.4-specific CD8⁺ T cells in the lungs of infected mice in late infection might recognize Mtb-infected macrophages. To examine the antigen recognition during late infection or in an in vivo setting, we can use a similar approach as taken by Bold et al [144]. Briefly, we can adoptively transfer TB10.4-specific CD8⁺ T cells expressing the congenic marker CD45.1 into Mtb-infected mice and then isolate these cells during late infection and assess their production of IFN γ ex vivo without stimulation. IFN γ production can be an indicator that these T cells may be recognizing TB10.4 in the lung environment.

Our study of antigen-specific CD8⁺ and CD4⁺ T cell recognition takes place in only one host genotype, C57BL/6. The C57BL/6 and BALB/c mice are known to be more resistant to Mtb compared to other mouse strains [267], and the TB10.4-specific CD8⁺ T cell response is dominant in both C57BL/6 and BALB/c mice [226, 227]. Smith et al expanded on this concept by examining 10 different inbred mouse strains and identified new susceptible and resistant mouse genotypes [268]. It will

be interesting to see the frequency of TB10.4-specific CD8⁺ T cells in the lungs of these newly defined resistant and susceptible mice. This will be met with the challenge of having to map which epitopes the T cells respond to in the different host genotypes. One could also expand that search to cover other major Mtb antigens. The end result will illustrate whether the generation of TB10.4 or the lack therefore may or may not correlate with susceptibility to Mtb.

For ex vivo CD4⁺ and CD8⁺ T cell studies, we are limited by our current set of reagents to analyze TB10.4-specific responses. The isolation of TB10.4-specific CD8⁺ T cells using tetramers may inadvertently have activated those T cells, making it difficult to assess whether they can recognize Mtb-infected macrophages during co-cultures due to high background activation. To circumvent this, we can try to use the Nur77-GFP mouse developed by Moran et al [233]. Specifically, Nur77 is a transcription factor upregulated after TCR activation, and the Nur77-GFP mouse will have the GFP expression after TCR activation. We can infect the Nur77-GFP mice, which are on a C57BL/6 background, and, at different times after infection, we can isolate the lung CD8⁺ T cells and co-culture them with Mtb-infected macrophages. Then, we can use tetramer staining on the GFP⁺ and GFP⁻ population to confirm whether the activated T cells are TB10.4-specific or not. This would enhance our finding that polyclonal, non-TB10.4-tetramer-positive T cells recognize Mtb-infected macrophages.

We considered the possibility of using BMDMs in our study to expand the applicability of our findings. However, preliminary studies showed that the BMDMs

triggered high background and non-specific recognition (i.e. high signal from the MHC mismatched BMDMs). This prompted us to use the ex vivo APCs as a way to illustrate the recognition capabilities in the presence of a heterogeneous population of lung APCs. While the lung APCs isolated using MHC II microbeads do contain dendritic cells and macrophages, we may need to isolate and enrich specific populations such as alveolar macrophages for a more specific investigation. Moreover, our lab has found that aerosol infection of mice does not lead to a high percentage of lung APCs being actually infected. While this finding is consistent with natural infection where the initial infection burden is not very high, it raises the question of how many infected cells are present in the co-culture. A more effective approach may be to do a high dose aerosol, or IV, or intratracheal infection with a fluorescent-protein-expressing-Mtb, such as YFP-Mtb. We can subsequently isolate the lung cells, sort only the infected cells based on fluorescence, and then plate those cells and see if TB10-specific CD8⁺ T cells might recognize them better. This approach does also investigate the question of antigen abundance, which is explained more in detail in the next section.

While the above approaches help to improve the study, there are exciting future directions that we should pursue as well. Despite showing that TB10.4-specific CD8⁺ T cells do not recognize Mtb-infected cells, there is overwhelming evidence that these CD8⁺ T cells persist during infection. Presumably, there must be a population of cells that are recognized that lead to the persistence of those cells. We return to the idea that there is a detour pathway in antigen presentation.

Through the works of Winau et al and Schaible et al, we now know that DCs can pick up apoptotic vesicles containing live Mtb or pieces of bacteria and prime naïve T cells in the LN [212, 240, 269]. While they describe this scenario in the context of priming, it is possible that such a process may occur in the infected lung. One can imagine the scenario where an uninfected bystander macrophage phagocytoses a dying, Mtb-infected macrophage, and, through efferocytosis, eliminate the bacterium inside. However, during that process, peptides can be generated from the dead bacterium and its proteins. These can then be presented to the surrounding T cells. This activation will lead to MHC-restricted or TCR-dependent recognition; however, the end result is the recognition of an uninfected cell rather than an infected cell. The persistence of the T cell response without focusing on the infected cells could lead to inflammation without necessarily promoting protecting. To test whether the detour pathway may lead to better recognition, we can first infect MHC mismatched macrophages, induce apoptosis via chemical agents or UV light, and then add uninfected, MHC matched macrophages. If co-culture with T cells lead to TB10.4-specific CD8⁺ T cell recognition, then we can conclude that cross presentation of apoptotic vesicles can lead to activation. This also supports the notion that TB10.4 in the presence of dead bacteria can be presented. In this experiment, we must be careful to ensure that the uninfected bystander macrophages do not become infected by Mtb that have escaped from dying cells.

Earlier, we alluded to the fact that our T cells represent 2 TCRs that recognize TB10.4₄₋₁₁. The generation of these TCRs comes from isolating TB10.4-specific CD8⁺ T cells from infected, $\alpha 2\text{var}$ mice [227]. To broaden the scope of our study, we can generate more TB10.4-specific CD8⁺ T cells and try to investigate whether other TCRs may also have difficulty in recognizing Mtb-infected macrophages. Indeed, others in the lab have generated several new TB10.4-specific T cell lines with TCRs isolated from infection of C57BL/6 mice. Data from those experiments have so far been negative, in the sense that TB10.4-specific CD8⁺ T cells do not recognize Mtb-infected macrophages (Rujapak Sutiwisesak).

Another future direction is to focus on the T cells that recognize Mtb-infected macrophages and, consequently, the antigens being presented. The polyclonal CD8⁺ T cells from infected mice recognized Mtb-infected macrophages, but their IFN γ production response was relatively small compared to that of the CD4⁺ T cells. While this could be due to an inherent capacity of CD4⁺ T cells to produce more IFN γ than CD8⁺ T cells [76, 270], it also suggests that the population of CD8⁺ T cells recognizing Mtb-infected macrophages is small. We can quantify the frequency of the CD8⁺ T cells recognizing Mtb-infected macrophages. Efforts are currently underway in the lab using polyclonal CD8⁺ T cells from infected mice to co-culture with Mtb-infected macrophages and calculating the frequency of IFN γ produced spots in an ELISPOT assay (Yash Patankar). Correlating the frequency of CD8⁺ T cell responding to Mtb-infected macrophages in a MHC-restricted

manner will, predictably, further highlight the small frequency of responding CD8⁺ T cells, which dwarf in comparison to TB10.4-specific CD8⁺ T cells.

Next, we can try to identify the antigen specificity of the responding CD8⁺ T cells. We can isolate CD8⁺ T cells from the lungs of Nur77-GFP infected mice, use GFP to sort out the activated polyclonal CD8⁺ T cells after co-culture with Mtb-infected macrophages. We can attempt to culture them in vitro with anti-CD3 and CD28 antibodies and supportive cytokines such as IL-2 and IL-7. Finally, we will screen a peptide library consisting Mtb antigen epitopes using an IFN γ ELISA or ELISPOT as readout. This screening method, though tedious, has been used before in identifying epitopes recognized by T cells from human and murine studies [114, 228]. The result will illustrate the epitopes recognized by the polyclonal CD8⁺ T cells and indicate a pool of peptides that may be presented during infection. Identifying which epitopes are presented and recognized during infection will allow for new vaccine targets.

In our studies, we found that Ag85b-specific and ESAT-6-specific CD4⁺ T cells recognized Mtb-infected macrophages and ex vivo APCs. As highlighted in Chapter I and Chapter II Discussion, there are studies that support Ag85b-specific CD4⁺ T cells recognizing Mtb-infected macrophages and studies that challenge that idea. An important finding that supports antigen recognition is that the reduction of bacterial burden requires cognate recognition via cell-to-cell contact [170]. Given that Ag85b-specific CD4⁺ T cells reduced bacterial burden in a MHC-

restricted manner, we believe that the Ag85b-specific CD4⁺ T cells do recognize Mtb-infected macrophages.

Nonetheless, we cannot exclude the possibility that the P25 T cells in our in vitro infection are recognizing bystander macrophages that have picked up Ag85b or vesicles through the detour pathway. Uninfected APCs can acquire Mtb antigens through the engulfment of apoptotic cells, necrotic debris, exosomes or uptake of exported antigens, and T cells may recognize these uninfected APCs but miss the infected cells [212, 240]. To fully investigate this, we can take advantage of live cell imaging. Unlike other experimental setups, live cell time lapse imaging allows direct observation of interactions between T cells and Mtb-infected cells. Macrophages infected with fluorescent bacteria can be visualized under the microscope, and the length of time when the T cells are interacting with those macrophages can be recorded. We can draw a baseline interaction time based on the T cells interaction with uninfected macrophages and compare that to the times with the infected macrophages. We have already set up preliminary experiments and are currently analyzing this data. Direct observation and quantification will allow a much better assessment of whether the detour pathway may be causing P25 T cell recognition, or whether the recognition is indeed of Mtb-infected cells. Moreover, we can extend this method to also assess ex vivo CD4⁺ and CD8⁺ T cells to also ascertain that the recognition that we have observed comes from recognition of infected cells as well.

In this chapter, we investigated the recognition ability of CD8⁺ and CD4⁺ T cells specific to immunodominant antigens. Our findings show that immunodominant antigens may generate CD8⁺ T cells that do not recognize Mtb-infected macrophages, implicating the antigen as a potential decoy antigen. Rather than discounting the TB10.4-specific CD8⁺ T cells as unimportant, we can approach this from an alternative angle. TB10.4-specific CD8⁺ T cells exist in great numbers in both human and murine models. The challenge is that they do not recognize the infected targets. If we can figure out how to enable these T cells to recognize Mtb-infected macrophages, then we already have a large T cell response that can eliminate the bacteria. To get us closer to that reality, we must first understand the mechanism by which the TB10.4-specific CD8⁺ T cells do not recognize Mtb-infected macrophages.

Investigating mechanisms of non-recognition by TB10.4-specific CD8⁺ T cells

Summary of results

In this chapter, we aimed to investigate the mechanisms that may explain why there was a lack of recognition of Mtb-infected macrophages by the TB10.4-specific CD8⁺ T cells. As mentioned in Chapter I and Chapter III, there are many mechanisms by which Mtb can escape or evade T cell immunity. We approached this investigation by focusing on the APC first, then the T cells, and, finally, the bacterium itself. This approach allowed us to eliminate quality control issues and arrive at the conclusion that there is an active process that inhibits the presentation of TB10.4-specifically.

In investigating the TGPMs, we found that Mtb did not interfere with MHC I or II expression in the infected macrophages. Mtb did inhibit MHC I and II expression during later days of infection if the TGPMs were pretreated with IFN γ , and this is consistent with published studies [148, 149]. We also found that Mtb did not interfere with the global presentation of antigens when the OVA-coated beads were added to the Mtb-infected macrophages. Indeed, SIINFEKL-specific hybridoma, RF33.70, T cells made IL-2 when the OVA-coated beads were co-cultured with MTb-infected macrophages.

We next investigated whether TB10.4 CD8⁺ T cells could recognize TB10.4 expressing-*Listeria monocytogenes*. We found that, if the *Listeria* were allowed to escape into the cytosol, then the TB10.4-specific CD8⁺ T cells recognized them.

This experiment confirmed that the TB10.4-specific CD8⁺ T cells could recognize the TB10.4₄₋₁₁ epitope generated during infection. Prior to this experiment, our positive control that both TB10Rg3 and TB10Rg4 T cells recognized TB10.4₄₋₁₁ came from peptide pulsed studies. Although TB10Rg4 recognized Mtb-infected BMDCs, we had no positive data on the recognition of macrophages. Second, this study also confirmed that the macrophages were capable of processing the full length TB10.4 protein into the minimally recognized epitope for presentation. As indicated during the previous discussion, studies have argued that the processing of the TB10.4 epitope may be inefficient and leads to suboptimal activation [260]. However, our findings show that the macrophages can process the whole protein. Third, this experiment showed that the location of the bacteria might be important given that the recognition of the TB10.4 expressing *Listeria* was only achieved when the bacteria could escape from the phagosome into the cytosol. Several studies have shown that Mtb can form pores on the phagosomal membranes and may translocate into the cytosol after infection [258, 263, 271, 272]. However, that translocation often indicates imminent cell necrosis, which disrupts antigen presentation [258, 259, 273, 274].

Knowing that the TB10.4-specific CD8⁺ T cells could recognize TB10.4 during an infection, we next investigated whether there was a process by the Mtb bacterium that is inhibiting presentation. As mentioned above, we have previously argued that there is limited amount of TB10.4 antigen presentation in the lungs of infected mice, leading to extreme bias in the TCR repertoire of the TB10.4-specific

CD8⁺ T cell response and defects in the memory-recall response in vivo [227, 231]. In the context of learning that Ag85b can be downregulated during infection, we wondered whether antigen abundance of TB10.4 might be an issue. Using a *esxGH*-over-expression strain of *Mtb* (*esxGH-OE.Mtb*), we found that there was no increased recognition by the TB10.4-specific CD8⁺ T cells, suggesting that antigen abundance may not be the issue that prevents efficient recognition.

Finally, we investigated whether there was an active process of inhibition of TB10.4 presentation. It is important to keep in mind that we have shown that *Mtb* does not interfere with the global antigen presentation of the infected cell (Figure 3.2), and *Mtb*-infected macrophages present antigens other than TB10.4 during infection (Figure 2.7). We found that TB10.4-specific CD8⁺ T cells recognized macrophages pulsed with γ -irradiated *Mtb*. Using IFN γ and IL-32 to pre-activate the macrophages, we found TB10.4-specific CD8⁺ T cells, especially TB10Rg4, could efficiently recognize these pre-activated, *Mtb*-infected macrophages. These results show that pulsing with non-viable *Mtb* or pre-activation of macrophages led to better presentation of TB10.4. The non-viable *Mtb* experiment also indicates an active process by live *Mtb* that results in the prevention of TB10.4 presentation. However, it remains unknown exactly how that process works.

Future directions

There are a few improvements that we can make to further our investigation. First, the OVA-coated beads experiment only assessed the antigen presentation

of the entire cell during Mtb infection. It was unlikely that the OVA-coated beads were in the same phagosome as the Mtb bacterium. Thus, this study only addressed whether Mtb infection may limit the antigen presentation ability of the whole cell, not just the phagosome that the Mtb is in. To address the question of whether Mtb-containing phagosome may be inhibited in their presentation of antigens, we need an alternative approach. Since Mtb-infected macrophages presented antigens to polyclonal CD8 T cells, we can assume that the Mtb containing phagosome is not inhibited from antigen presentation. Interestingly, Grotzke et al show that Mtb containing phagosomes from human DCs have MHC class I molecules and TAP, leading to the conclusion that those phagosomes are capable of antigen presentation [207]. Less is known about whether Mtb phagosomes in murine models also can present antigens. One way to address this question is to assess the presentation of a MHC class I antigen localized to the same phagosome as Mtb during Mtb infection. Using a Tet-inducible, SIINFEKL-expressing Mtb strain, we have preliminary data showing inefficient recognition of the SIINFEKL peptide by the RF33.70 T cells. However, more work needs to be done to validate that the optimal induction of SIINFEKL is achieved (data not shown).

The limitation with the *Listeria* studies is that 1) the TB10.4 is expressed via a plasmid, meaning that the protein most likely is not secreted by the bacterium, and 2) the expression plasmid does not express EsxG, which partners with TB10.4 (EsxH) to form a heterodimer. It is likely that the TB10.4 protein is not functional

during *Listeria* infection. Studies have shown that TB10.4 may be involved in impairing the ESCRT pathway [189, 275] and in the inhibition of presentation to Ag85b-specific CD4⁺ T cells. However, we found that Mtb-infected macrophages presented to P25 T cells and polyclonal CD4⁺ T cells, and thus, we do not fully support the idea that TB10.4 may be detrimental to antigen presentation to CD4⁺ T cells. Differences between the studies include 1) our long-term co-culture vs. their short-term co-culture, and 2) our use of TGPMs and ex vivo APCs vs. their use of BMDMs. Furthermore, they find that CD4⁺ T cells mediated better bacterial reduction when co-cultured with BMDMs infected with a mutant strain of Mtb lacking TB10.4 (Δ esxH.Mtb) [189]. However, while the WT Mtb grew intracellularly in the samples without T cells, the mutant strain did not grow at all. This raises the question about the health of the mutant strain and suggests that the macrophages were already restricting bacterial growth. The enhanced bacterial burden control from the T cells may have been partially helped by the enhanced killing by innate macrophage antimicrobial mechanisms. Our studies with the OVA-coated beads, polyclonal CD8⁺ and CD4⁺ T cells from infected mice, along with Ag85b-specific and ESAT-6-specific T cell recognition all indicate that TB10.4 is not interfering with antigen presentation. However, in order to rule this out definitively, it will be important to obtain the Δ esxH.Mtb and see if we get even better recognition from P25 T cells when TB10.4 is not present.

The *Listeria* studies also show that the location of the antigen and bacterium may be important. The interest in allowing Mtb to access the cytosol as a way to

increase T cell response was piqued by the finding that a BCG vaccine that expresses listeriolysin has increased efficacy [276]. We have attempted to elicit phagosomal escape using several methods. We have tried to add recombinant listeriolysin to the Mtb-infected macrophage culture prior to adding T cells (data not shown). However, this did not yield promising results for two potential reasons: 1) listeriolysin requires a pH of below 5 to work, whereas Mtb phagosomes usually are in the pH > 5.5 [125, 126]; 2) addition of listeriolysin may not deliver the listeriolysin to the Mtb containing phagosome. We also tried using perfringolysin, the toxin secreted by *Clostridium perfringens* bacterium that forms pores in lipid membranes [277]. Interestingly, a perfringolysin-expressing-BCG strain (AFRO-1) that over-expresses Ag85A, Ag85B and TB10.4 also showed increased efficacy in protecting mice against TB [278]. However, in our hands, AFRO-1 infection did not lead to increased recognition by the TB10.4-specific CD8 T cells (data not shown). We found a lot of cell death during the co-culture after infection, and it is possible that the pore forming abilities of perfringolysin may have lysed not only the phagosomes but also the plasma membranes of the infected macrophages. Moreover, the TB10.4 may not have been expressed, which the authors admitted that they could not detect TB10.4 via Western blotting [278].

While we investigated the role of antigen abundance during Mtb infection, we have not ruled out definitively that enough TB10.4 was produced during infection. To assess this, we need to probe for antigen abundance during Mtb infection. We have tried to quantify the amount of TB10.4 produced during infection

by doing Western blotting on cell lysates from Mtb-infected macrophages. However, we have not been able to consistently detect the protein (data not shown). This may be partially due to the commercial polyclonal antibody that has not been validated. Our next approach is to use mass spectrometry to quantify the amount of protein present. Furthermore, we should also assess the amount of TB10.4 present during in vivo infection. We can use a similar approach as Bold et al have done by examining the mRNA expression levels of TB10.4 at different time points after aerosol infection of mice [144]. We can correlate the mRNA expression levels with the tetramer positive frequency of CD8⁺ T cells to see if the sustained tetramer positive population corresponds to a sustained level of TB10.4 production.

We can also improve upon experiments concerning macrophages cross presenting antigens from non-viable bacteria and pre-activated, Mtb-infected macrophages. We used IFN γ and IL-32 to pre-activate the macrophages. While the results hold promise, we need to ensure that the effects we are seeing are due to enhanced killing versus the generation of non-viable bacteria. We can do parallel CFU assays to assess whether IFN γ and IL-32 pretreated macrophages do restrict bacterial burden better after infection. To fully appreciate the effects of killing, and not just restricting bacterial growth, we can use a longer infection course. After infection, we will rest the macrophages from d0 to d3 to allow intracellular bacterial to grow. Then, on d3, we add IFN γ , IL-32, or antibiotics such as isoniazid (INH) and assess whether the addition may lead to decrease in

bacterial burden. If it does, then on d5, we can add T cells for 24-72 hours to see if there is any recognition. The important parameter is to make sure to let the Mtb grow first and then reduce the bacterial burden, leading to an accumulation of dead bacteria that may be presented to the T cells.

The above approaches will enhance existing findings. However, two questions remain: 1) what active process is preventing the presentation of TB10.4? 2) what is sustaining the high frequency of TB10.4-specific CD8 T cells through chronic infection?

To probe the process that actively inhibits antigen presentation of TB10.4, we can use a screening approach. We infect two groups of RAG-1^{-/-} or TCR α ^{-/-} mice, one as is and the other one with adoptively transferred TB10.4-CD8⁺ T cells. We infect these mice with a transposon insertion library of Mtb mutants [225, 279, 280]. After 4 weeks, we can plate the resulting lung homogenates, recover the bacterial DNA and sequence them. If there are mutants in the library that can be recognized by TB10.4-specific CD8⁺ T cells, then we should detect their absence in the mice that received T cells. We can potentially identify new genes that play a role in inhibition of antigen presentation. Of course, this approach may return gene candidates that we already know. However, the data will still be useful as we can define new mechanisms by which known genes are inhibiting the presentation of one specific antigen.

Alternatively, we can examine cases where TB10.4-specific CD8⁺ T cells are not present. The clinical isolate 667 has a natural polymorphism that changes

an alanine to a threonine in the TB10.4₄₋₁₁ epitope [281], and efforts in our lab have identified that infection with the clinical isolate 667 does not generate TB10.4-specific CD8⁺ T cells during murine infection (Rujapak Sutiwisesak). It will be important to assess the virulence of the bacterium, and, if there is a decreased virulence, whether that virulence is due to the absence of TB10.4-specific CD8⁺ T cells. We can also attempt to identify other Mtb antigens that may arise as immunodominant in the absence of a TB10.4 response, and we can measure the frequency of polyclonal CD8⁺ T cells that recognize Mtb-infected cells to see if there is an enrichment of that population. This study is critical in confirming the theory that TB10.4 is a decoy antigen.

The sustained frequency of TB10.4-specific CD8⁺ T cells during murine infection is of great interest because it suggests there must be ongoing recognition. To answer this question, we need to accomplish 2 objectives. First, we need to identify the cells that are presenting TB10.4 during infection. Second, we need to ascertain whether these TB10.4-presenting cells contain live Mtb or apoptotic vesicles from Mtb-infected cells. To address the first question, we will use TCR tetramers. Whereas MHC-peptide tetramers are useful for identifying and quantifying the frequency of epitope specific T cell populations, TCR tetramers identify the frequency of antigen presenting cells that have epitopes that bind to TCRs [282]. Previous work has shown that engineering Mtb antigen-specific CD4⁺ TCR tetramer is possible and can be used to detect APCs in human peripheral blood that present Mtb antigens [283]. We can engineer TCR tetramers that are

specific to the TB10.4₄₋₁₁ epitope and use them to screen lung cells isolated from Mtb-infected mice. The mice will be infected with a live-dead strain of Mtb, which expresses mCherry constitutively and expresses GFP when induced with tetracycline [46]. Those that bind to the TCR tetramers can then be stained for various myeloid markers to identify whether they are DCs, alveolar macrophages, recruited monocytes, or another subset of APCs. Furthermore, to answer the second question about whether the APCs contain live or dead Mtb, we can use flow cytometry to assess whether the TCR tetramer positive APCs also contain live Mtb, which should still express GFP. If the TCR tetramer positive APCs contain dead Mtb or no detectable Mtb, then we can conclude that the sustained CD8⁺ T cell population comes from the presentation of non-viable Mtb via the above-mentioned detour pathway. If this detour pathway is indeed what drives the maintenance of the TB10.4-specific CD8⁺ T cells, then we can confirm that TB10.4 is a decoy antigen.

Concluding remarks

The TB10.4₄₋₁₁ epitope has been extensively used to characterize CD8⁺ T cell responses in the mouse model of TB, and TB10.4-specific CD8⁺ T cell responses have also been characterized in people with tuberculosis [227, 229, 231, 260, 284-286]. The finding that TB10.4-specific CD8⁺ T cells do not recognize infected macrophages was unexpected, particularly since TB10.4-specific CD8⁺ T cells persist in the lungs of infected mice and become more dominant with time [226, 227]. The data presented in this thesis help to define a set of questions that warrant further investigation. Briefly, the main questions are:

1. Do TB10.4-specific CD8⁺ T cells exist in other diverse host genotypes? Do they correlate with susceptibility to TB? Do they recognize Mtb-infected cells?
2. What are the antigens that infected cells present to the small group of polyclonal CD8⁺ T cells that recognize infected macrophages?
3. What is the expression level of TB10.4 and does it change throughout in vivo infection?
4. What sustains the high frequency of TB10.4-specific CD8⁺ T cells during murine infection? Is it the detour pathway?
5. What bacterial genes may contribute to the inhibition of TB10.4 presentation in infected cells?

The various approaches that we can take to answer these questions are outlined in the above discussion sections. Answering these questions will not only confirm whether TB10.4 is a decoy antigen or not but also inform future vaccine designs.

In retrospect, our findings may partially explain why eliciting TB10.4-specific CD8⁺ T cells by vaccination fails to protect mice against Mtb infection [231, 260]. While vaccination with immunodominant antigens recognized by CD4⁺ T cells (e.g., Ag85b, ESAT-6) induce moderate protection [287, 288], we must consider the possibility that these antigens may not be the best stimulators of protective immunity. Ag85b-specific CD4⁺ T cells have variable efficacy, in large part due to its reduced expression by the bacterium as early as 3 weeks after infection [144, 145]. However, by their nature, the recruitment of memory T cell responses specific for immunodominant antigens is only incrementally faster than the primary T cell response [183, 231]. Thus, a crucial question for vaccine development is whether other Mtb antigens resemble TB10.4, in that they elicit T cell responses that fail to recognize infected macrophages. We did detect polyclonal CD8⁺ T cells that recognized Mtb-infected macrophages, corroborating a previous study showing that polyclonal CD8⁺ T cells from infected mice can lyse Mtb-infected cells [113]. These data indicate that there are antigens presented by Mtb-infected cells, even if those antigens may be subdominant compared to TB10.4. Thus, future vaccine developments will benefit by identifying antigen targets based on their ability of being presented rather than only their immunogenicity.

Priming of TB10.4-specific CD8⁺ T cells occurs early after Mtb infection in the lung draining lymph node (LN) [138, 231]. Yet it is unknown whether priming of naïve T cells occurs via Mtb-infected DCs, or by uninfected DCs that acquire antigen through uptake of apoptotic blebs containing Mtb proteins [212, 240], or by

the transfer of antigen from cell to cell [242]. Being primed by an uninfected cell can have detrimental consequences if the infected cell presents a different repertoire of Mtb antigens. Considering our findings, we propose that TB10.4 is a decoy antigen: TB10.4-specific CD8⁺ T cells expand in the LN during priming, accumulate in the lungs, but ineffectively recognize Mtb-infected macrophages. This raises the hypothesis that not all immune responses elicited by Mtb provide benefits to the host. Interestingly, Mtb genes encoding epitopes recognized by T cells are more highly conserved than other DNA elements, implying that T cell recognition of these Mtb epitopes may provide a survival advantage to the bacterium [12, 13]. For example, T cell dependent inflammation may benefit Mtb by promoting transmission. Even though TB10.4 is more variable than most other antigens, our results support these genetic data [12, 13]. Thus, Mtb focuses the CD8⁺ T cell response on the decoy antigen TB10.4 and distracts the immune response from other antigens that might be targets of protective immunity, successfully evading T cell immunity and enabling it to establish itself as persistent infection.

CHAPTER V. METHODS AND MATERIALS

Ethics Statement

Studies involving animals were conducted following relevant guidelines and regulations, and the studies were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (Animal Welfare A3306-01), using the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Office of Laboratory Animal Welfare.

Mice

C57BL/6J, RAG-1^{-/-} (B6.129S7-Rag1^{tm1Mom}), B10 (C57BL/10J), B10.BR (B10.BR-H2^{k2} H2-T18^a/SgSnJJrep), P25 (C57BL/6-Tg(H-2K^b-Tcr α ,Tcr β)P25Ktk/J) [64, 232], mice were obtained from Jackson Laboratories (Bar Harbor, ME). C57BL/6J and B10 mice were used for isolating MHC-matched TGPMs while B10.BR mice were used for isolating MHC-mismatched TGPMs. C57BL/6 K^b^{-/-}D^b^{-/-} (MHC I^{-/-}) mice were a generous gift from Dr. Kenneth Rock (University of Massachusetts Medical School, MA). C7 TCR transgenic mice were a generous gift from Dr. Eric Pamer (Memorial Sloan Kettering Cancer Center, NY)[239].

Thioglycolate-elicited peritoneal macrophages

Thioglycolate-elicited peritoneal macrophages were obtained 4-5 days after intra-peritoneal injection of mice with 3% thioglycolate solution, as described [55]. 1×10^5 macrophages were plated per well. Macrophages were maintained in culture with RPMI 1640 media (Invitrogen Life Technologies, ThermoFisher, Waltham, MA) supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine (all from Invitrogen Life Technologies) and 10% heat-inactivated fetal bovine serum (HyClone, GE Healthcare Life Sciences, Pittsburgh, PA), referred hereafter as supplemented complete media.

Bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells were prepared as previously described [289]. Briefly, bone marrow cells isolated from C57BL/6 were cultured in vitro with complete RPMI1640 and 10ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ) for 8 days before being harvested, counted and plated for experiments. For infection, BMDCs were infected at MOI 10 for 2 hours, and the wells were washed extensively after infection to get rid of extracellular bacteria.

Generation of CD8⁺ and CD4⁺ T cell lines

Retrogenic mice expressing TB10Rg3 TCR specific for the TB10.44-11 epitope were generated as previously described [227]. The TB10Rg3 CD8⁺ T cells

were isolated from these mice, stimulated *in vitro* with irradiated splenocytes pulsed with the peptide TB10.4₄₋₁₁ in complete media containing IL-2. P25 or C7 CD4⁺ T cells were isolated from transgenic P25 or C7 mice, respectively [232, 239]. The P25 and C7 cells were stimulated *in vitro* with irradiated splenocytes pulsed with the Ag85b₂₄₁₋₂₅₆ peptide or the ESAT-6₁₋₁₅, respectively, in complete media containing IL-2 and anti-IL-4. After the initial stimulation, these T cells were split every two days for 3-4 divisions and rested for two to three weeks. After the initial stimulation, the cells were cultured in complete media containing IL-2 and IL-7.

Peptides

The following synthetic peptide epitopes were used as antigens: TB10.4₄₋₁₁ (IMYNYPAM); Ag85b₂₄₁₋₂₅₆ (QDAYNAAGGHNAVFN); and ESAT-6₁₋₁₅ (MTEQQWNFAGIEAAA). We also generated a negative control peptide predicted to not bind to H-2 K^b: IMANAPAM. The peptides were obtained from New England Peptides (Gardner, MA).

As positive controls assessing the function of macrophages to present antigen, uninfected macrophages and, in certain experiments, infected macrophages were pulsed with the peptides of interest. We pulsed macrophages by incubating 10uM of the peptides of interest with the macrophages in supplemented complete RPMI 1640 media for 1 hour. After incubation, the cells were washed 3 to 5 times with fresh supplemented complete RPMI 1640 media.

The cells were then resuspended in supplemented complete RPMI 1640 media for experiments.

In vitro Mtb infection

H37Rv was grown as previously described [55]. Bacteria was grown to an OD₆₀₀ of 0.6 – 1.0, washed in RPMI, opsonized with TB coat (RPMI 1640, 1% heat-inactivated FBS, 2% human serum, 0.05% Tween-80), washed again and filtered through a 5-micron filter to remove bacterial clumps. The bacteria were counted using a Petroff-Hausser chamber. Infection was performed as previously described [55]. The final multiplicity of infection (MOI), based on plating CFU, was 0.2-0.8 for all experiments. For CFU-based, bacterial growth inhibition assays, T cells were added at a ratio of 5 T cells to each macrophage. Four replicate wells were used for each condition. Cell cultures were lysed by adding 1/10th volume of with 10% Triton X-100 in PBS (final concentration of 1%), and CFUs were determined by plating in serial dilutions of the lysates on Middlebrook 7H10 plates. CFUs were enumerated after culture for 21 days at 37°C and 5% CO₂.

In vivo aerosol Mtb infection and lung cell isolation

Aerosol infection of mice was done with the Erdman strain of Mtb using a Glas-Col aerosol-generation device. A bacterial aliquot was thawed, sonicated for 1 minute and then diluted in 0.9% NaCl-0.02% Tween-80 to 5 ml. The number of Mtb deposited in the lungs was determined for each experiment, by plating

undiluted lung homogenate from a subset of the infected mice within 24 hours of infection. The inoculum varied between 37-120 CFU. For the ex vivo APC experiments, lung cells were isolated from Erdman-infected, RAG-1^{-/-} mice, 4-weeks post-infection, and the APCs were enriched by positive selection using anti-MHC class II⁺ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and the Miltenyi AutoMACS. On average, the isolated cells were 89% CD11c⁺ or CD11c⁺CD11b⁺. The APCs were counted on a hemocytometer and plated at 1x10⁵ per well in supplemented complete RPMI 1640 media.

For the ex vivo CD4⁺ and CD8⁺ T cell experiments, single cell suspensions were isolated from the lungs of infected C57BL/6 mice, 6 to 8 weeks post-infection, as described [183]. Polyclonal CD4⁺ and CD8⁺ T cells were enriched by positive selection using Mouse CD4⁺ and Mouse CD8⁺ T cell isolation kits, respectively (Miltenyi Biotec). After enrichment, average purity for polyclonal CD4⁺ and CD8⁺ T cells were 93% and 95%, respectively. For experiments investigating TB10.4₄₋₁₁-tetramer positive cells and polyclonal, non-TB10.4-specific, CD8⁺ T cells, the following isolation was done. Single cell suspensions from the lungs of infected mice were incubated with APC-conjugated, TB10.4₄₋₁₁-loaded, H-2^b tetramers from the National Institute of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Tetramer positive CD8⁺ T cells were then selected via the AutoMACS separator by anti-APC microbeads (Miltenyi Biotec). Average purity of TB10.4₄₋₁₁-tetramer positive, CD8⁺ T cells was 85%, with 1.4% contaminating CD4⁺ T cells. The tetramer negative population was subsequently

washed and then enriched for polyclonal CD8⁺ T cells via Mouse CD8⁺ T cell isolation kit (Miltenyi Biotec). Average purity of polyclonal, non-TB10.4₄₋₁₁-tetramer positive, CD8⁺ T cells was 75% with 0.8% contaminating CD4⁺ T cells and 13% contaminating TB10.4₄₋₁₁-tetramer positive CD8⁺ T cells. The T cells were counted using a hemocytometer and resuspended in supplemented complete RPMI 1640 media before being used in experiments.

Listeria infections

The recombinant *Listeria* strains have been previously described [231]. For in vitro infections, they were grown to an OD₆₀₀ of 0.6-1.0 in BHI media (Sigma Aldrich) with 10 µg/ml chloramphenicol (Sigma Aldrich) at 30°C. Macrophages were infected with the *Listeria* strains using a MOI 50, for 45 minutes. Extracellular bacteria were eliminated by adding 60 µg/ml gentamicin (Sigma Aldrich) for 20 minutes. Bacterial burden was assessed by lysing the infected macrophages with 1% TritonX-100 in PBS and plating serial dilutions of the lysate on BHI agarose supplemented with 10µg/ml chloramphenicol (Sigma Aldrich, St. Louis, MO). Recombinant listeriolysin (Prospec, East Brunswick, NJ) was added in some experiments at 2 µg/ml for 30 minutes, and any excess was washed away. Bafilomycin (InvivoGen, San Diego, CA) was added in some experiments at 5 µM for 30 minutes, before being washed away.

Generation of TB10.4-overexpressing Mtb strains

pJR1103 was cleaved with EcoRI-HF and Sall-HF [290]. mCherry preceded by the groEL2 promoter from H37Rv was inserted by HiFi Assembly. The resulting plasmid was cleaved with NdeI and NotI-HF. The esxGH gene from H37Rv, along with 12 upstream nucleotides, was inserted by HiFi Assembly following the plasmid-borne tetracycline-inducible promoter. All enzymes used above were purchased from New England Biolabs. The resulting plasmid (pGB6) was electroporated into Mtb H37Rv and integrated at the L5 site. RNA was purified from induced and uninduced cultures using TRIzol (ThermoFisher) and chloroform extraction, followed by purification on Zymo columns. cDNA was produced with Superscript IV (ThermoFisher), and quantitative PCR was performed using the iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA) on an Applied Biosystems Viia 7 thermocycler.

Irradiated H37Rv

The following reagent was obtained through BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv, Gamma-Irradiated Whole Cells, NR-14819. Briefly, H37Rv was grown to late-log phase, and γ -irradiation was carried out using 2.4 megaRads dose via ^{137}Cs source. The irradiated H37Rv was gently sonicated using a cup-horn sonicator at a low power to disperse bacterial clumps while limiting bacterial lysis. The number of bacteria was approximated by measuring the turbidity at OD₆₀₀ and correlating it with live H37Rv (OD₆₀₀ = 1 is

equivalent to 3.0×10^8 CFU/ml). To pulse TGPMs, diluted, sonicated, γ -irradiated H37Rv were added to adherent macrophages for one hour before repeatedly washing the cultures to remove residual extracellular bacteria. Subsequently, TB10Rg3 or P25 T cells were added at a ratio of 1 T cell to 1 macrophages. After 72 hours, the amount of IFN γ in the supernatants was measured using Mouse IFN γ ELISA MAX kits (Biolegend, San Diego, CA).

Flow Cytometry Analysis

The following cell surface antigens were detected by flow cytometry using the following antibodies: mouse CD4 (clone GK1.5), CD8 (clone 53-6.7), CD3 ϵ (clone 145-2C11), CD69 (clone H1.2F3), I-A/I-E (clone M5/114.15.2), and H-2K^b (clone AF6-88.5) (all from Biolegend). BV421- and APC-conjugated, TB10.4₄₋₁₁-loaded, H-2K^b tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). Zombie Violet Fixable viability dye (Biolegend) or the Live/Dead Fixable Far Red Dead Cell stain (ThermoFisher) were used for distinguishing live from dead cells. To stain for the Nur77 transcription factor, the Nur77 monoclonal antibody (clone 12.14) was used in combination with the Foxp3 Transcription Factor Staining Buffer Set (both from ThermoFisher) by the manufacturer's protocol. Live/dead viability staining and surface staining were done for 20 minutes at 4°C, and intracellular staining was done for 30 minutes at room temperature. Samples were then fixed with 1% paraformaldehyde/PBS for 1 hour before being analyzed by a MACSQuant flow

cytometer (Miltenyi Biotec). FlowJo Software (Tree Star, Portland, OR) was used to analyze the collected data. Single lymphocytes were gated by forward scatter versus height and side scatter for size and granularity, and dead cells were excluded.

Normalization and statistical analysis

To compare the cellular expression of Nur77 and CD69 expression levels between time points, the MFI values were normalized as follows: experimental values were divided by the difference between the isotype control MFI (minimum response) and the peptide control MFI (maximum response).

Each Figure represents a minimum of 2 similar experiments, with 2 to 4 biological replicates in each experiment. Data are represented as mean \pm standard error of the mean (SEM). For comparing two groups, a two-tailed, unpaired student's t-test was used. For more than two groups, the data were analyzed using a one-way ANOVA. A p value < 0.05 was considered to be statistically significant. Analysis was performed using GraphPad Prism, Ver. 7 (GraphPad Software, La Jolla, CA).

CHAPTER VI: REFERENCES

1. Staff. Global Tuberculosis Report 2017. World Health Organization, 2017 9789241565394.
2. Frith J. History of Tuberculosis. Part 1-phthisis, consumption and the white plague. *Journal of Military and Veterans' Health*. 2014;22(2).
3. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. *PLoS Med*. 2016;13(10):e1002152. Epub 2016/10/26. doi: 10.1371/journal.pmed.1002152. PubMed PMID: 27780211; PubMed Central PMCID: PMC5079585.
4. Colditz GA. Efficacy of BCG Vaccine in the Prevention of Tuberculosis. *Jama*. 1994;271(9). doi: 10.1001/jama.1994.03510330076038.
5. Fine PEM. Variation in protection by BCG: implications of and for heterologous immunity. *The Lancet*. 1995;346(8986):1339-45. doi: 10.1016/s0140-6736(95)92348-9.
6. Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PE, et al. Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. *Clin Infect Dis*. 2014;58(4):470-80. Epub 2013/12/18. doi: 10.1093/cid/cit790. PubMed PMID: 24336911.
7. Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, et al. Effect of BCG vaccination against *Mycobacterium tuberculosis* infection in children: systematic review and meta-analysis. *BMJ*. 2014;349:g4643. Epub 2014/08/07. doi: 10.1136/bmj.g4643. PubMed PMID: 25097193; PubMed Central PMCID: PMC4122754.
8. Ma Z, Lienhardt C, McIlleron H, Nunn AJ, Wang X. Global tuberculosis drug development pipeline: the need and the reality. *The Lancet*. 2010;375(9731):2100-9. doi: 10.1016/s0140-6736(10)60359-9.
9. Gelmanova IY. Barriers to successful tuberculosis treatment in Tomsk, Russian Federation: non-adherence, default and the acquisition of multidrug resistance. *Bulletin of the World Health Organization*. 2007;85(09):703-11. doi: 10.2471/blt.06.038331.
10. Ottenhoff TH, Kaufmann SH. Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog*. 2012;8(5):e1002607. Epub 2012/05/17. doi: 10.1371/journal.ppat.1002607. PubMed PMID: 22589713; PubMed Central PMCID: PMC3349743.
11. Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, et al. Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nature Genetics*. 2013;45:1176. doi: 10.1038/ng.2744.
12. Comas I, Chakravarti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nat Genet*. 2010;42(6):498-503. Epub 2010/05/25. doi:

10.1038/ng.590. PubMed PMID: 20495566; PubMed Central PMCID: PMC2883744.

13. Coscolla M, Copin R, Sutherland J, Gehre F, de Jong B, Owolabi O, et al. M. tuberculosis T Cell Epitope Analysis Reveals Paucity of Antigenic Variation and Identifies Rare Variable TB Antigens. *Cell Host Microbe*. 2015;18(5):538-48. Epub 2015/11/27. doi: 10.1016/j.chom.2015.10.008. PubMed PMID: 26607161; PubMed Central PMCID: PMC2883744.
14. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, et al. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet*. 2013;45(7):784-90. Epub 2013/06/12. doi: 10.1038/ng.2656. PubMed PMID: 23749189; PubMed Central PMCID: PMC3777616.
15. Lambert PA. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. *Journal of Applied Microbiology*. 2002;92(s1):46S-54S. doi: 10.1046/j.1365-2672.92.5s1.7.x.
16. Slama N, Jamet S, Frigui W, Pawlik A, Bottai D, Laval F, et al. The changes in mycolic acid structures caused by hadC mutation have a dramatic effect on the virulence of Mycobacterium tuberculosis. *Mol Microbiol*. 2016;99(4):794-807. Epub 2015/11/06. doi: 10.1111/mmi.13266. PubMed PMID: 26538472.
17. James BW, Williams A, Marsh PD. The physiology and pathogenicity of Mycobacterium tuberculosis grown under controlled conditions in a defined medium. *J Appl Microbiol*. 2000;88(4):669-77. PubMed PMID: 10792526.
18. von Groll A, Martin A, Portaels F, da Silva PE, Palomino JC. Growth kinetics of Mycobacterium tuberculosis measured by quantitative resazurin reduction assay: a tool for fitness studies. *Braz J Microbiol*. 2010;41(2):300-3. doi: 10.1590/S1517-83822010000200006. PubMed PMID: 24031495; PubMed Central PMCID: PMC2883744.
19. Munoz-Elias EJ, Timm J, Botha T, Chan WT, Gomez JE, McKinney JD. Replication dynamics of Mycobacterium tuberculosis in chronically infected mice. *Infect Immun*. 2005;73(1):546-51. Epub 2004/12/25. doi: 10.1128/IAI.73.1.546-551.2005. PubMed PMID: 15618194; PubMed Central PMCID: PMC538940.
20. Guirado E, Schlesinger LS, Kaplan G. Macrophages in tuberculosis: friend or foe. *Semin Immunopathol*. 2013;35(5):563-83. Epub 2013/07/19. doi: 10.1007/s00281-013-0388-2. PubMed PMID: 23864058; PubMed Central PMCID: PMC3763202.
21. Armstrong JA. Response of Cultured Macrophages to Mycobacterium Tuberculosis, with Observations on Fusion of Lysosomes with Phagosomes. *Journal of Experimental Medicine*. 1971;134(3):713-40. doi: 10.1084/jem.134.3.713.
22. Ernst JD. The immunological life cycle of tuberculosis. *Nat Rev Immunol*. 2012;12(8):581-91. Epub 2012/07/14. doi: 10.1038/nri3259. PubMed PMID: 22790178.

23. Nunes-Alves C, Booty MG, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM. In search of a new paradigm for protective immunity to TB. *Nat Rev Microbiol.* 2014;12(4):289-99. Epub 2014/03/05. doi: 10.1038/nrmicro3230. PubMed PMID: 24590243; PubMed Central PMCID: PMC4085047.
24. Golden MP, Vikram HR. Extrapulmonary tuberculosis: an overview. *Am Fam Physician.* 2005;72(9):1761-8. PubMed PMID: 16300038.
25. Frankel A, Penrose C, Emer J. Cutaneous tuberculosis: a practical case report and review for the dermatologist. *J Clin Aesthet Dermatol.* 2009;2(10):19-27. PubMed PMID: 20725570; PubMed Central PMCID: PMC2923933.
26. Thomas S, Suhas S, Pai KM, Raghu AR. Lupus vulgaris--report of a case with facial involvement. *Br Dent J.* 2005;198(3):135-7. Epub 2005/02/12. doi: 10.1038/sj.bdj.4812038. PubMed PMID: 15706374.
27. Oni T, Berkowitz N, Kubjane M, Goliath R, Levitt NS, Wilkinson RJ. Trilateral overlap of tuberculosis, diabetes and HIV-1 in a high-burden African setting: implications for TB control. *Eur Respir J.* 2017;50(1). Epub 2017/07/22. doi: 10.1183/13993003.00004-2017. PubMed PMID: 28729474; PubMed Central PMCID: PMC5538899.
28. Sekar R, Mythreyee M. Tuberculosis, HIV/AIDS and Diabetes – Is it Time to Think Together? *Journal of Medical Microbiology & Diagnosis.* 2012;01(01). doi: 10.4172/2161-0703.1000e103.
29. van Crevel R, Ottenhoff THM, van der Meer JWM. Innate Immunity to *Mycobacterium tuberculosis*. *Clinical Microbiology Reviews.* 2002;15(2):294-309. doi: 10.1128/cmr.15.2.294-309.2002.
30. Ma N, Zalwango S, Malone LL, Nsereko M, Wampande EM, Thiel BA, et al. Clinical and epidemiological characteristics of individuals resistant to *M. tuberculosis* infection in a longitudinal TB household contact study in Kampala, Uganda. *BMC Infect Dis.* 2014;14:352. doi: 10.1186/1471-2334-14-352. PubMed PMID: 24970328; PubMed Central PMCID: PMC4091673.
31. Al-Orainey IO. Diagnosis of latent tuberculosis: Can we do better? *Annals of Thoracic Medicine.* 2009;4(1):5-9. doi: 10.4103/1817-1737.44778. PubMed PMID: PMC2700471.
32. Lerner TR, Borel S, Gutierrez MG. The innate immune response in human tuberculosis. *Cell Microbiol.* 2015;17(9):1277-85. Epub 2015/07/03. doi: 10.1111/cmi.12480. PubMed PMID: 26135005; PubMed Central PMCID: PMC4832344.
33. Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat Immunol.* 2015;16(1):27-35. Epub 2014/12/19. doi: 10.1038/ni.3045. PubMed PMID: 25521682; PubMed Central PMCID: PMC4318521.
34. Middleton AM, Chadwick MV, Nicholson AG, Dewar A, Groger RK, Brown EJ, et al. Interaction of *Mycobacterium tuberculosis* with human respiratory mucosa. *Tuberculosis.* 2002;82(2-3):69-78. doi: 10.1054/tube.2002.0324.
35. Nair VR, Franco LH, Zacharia VM, Khan HS, Stamm CE, You W, et al. Microfold Cells Actively Translocate *Mycobacterium tuberculosis* to Initiate

- Infection. Cell Rep. 2016;16(5):1253-8. Epub 2016/07/28. doi: 10.1016/j.celrep.2016.06.080. PubMed PMID: 27452467; PubMed Central PMCID: PMC4972672.
36. Ernst JD. Macrophage receptors for Mycobacterium tuberculosis. Infect Immun. 1998;66(4):1277-81. Epub 1998/04/07. PubMed PMID: 9529042; PubMed Central PMCID: PMC4972672.
 37. Cambier CJ, Falkow S, Ramakrishnan L. Host evasion and exploitation schemes of Mycobacterium tuberculosis. Cell. 2014;159(7):1497-509. doi: 10.1016/j.cell.2014.11.024. PubMed PMID: 25525872.
 38. Domingo-Gonzalez R, Prince O, Cooper A, Khader SA. Cytokines and Chemokines in Mycobacterium tuberculosis Infection. Microbiol Spectr. 2016;4(5). Epub 2016/10/21. doi: 10.1128/microbiolspec.TBTB2-0018-2016. PubMed PMID: 27763255; PubMed Central PMCID: PMC4972672.
 39. Henderson RA, Watkins SC, Flynn JL. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. J Immunol. 1997;159(2):635-43. PubMed PMID: 9218578.
 40. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwiertman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med. 2001;345(15):1098-104. doi: 10.1056/NEJMoa011110. PubMed PMID: 11596589.
 41. Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol. 2001;19:93-129. Epub 2001/03/13. doi: 10.1146/annurev.immunol.19.1.93. PubMed PMID: 11244032.
 42. Fabri M, Stenger S, Shin DM, Yuk JM, Liu PT, Realegeno S, et al. Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages. Sci Transl Med. 2011;3(104):104ra2. Epub 2011/10/15. doi: 10.1126/scitranslmed.3003045. PubMed PMID: 21998409; PubMed Central PMCID: PMC3269210.
 43. Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev. 2015;264(1):182-203. doi: 10.1111/imr.12266. PubMed PMID: 25703560; PubMed Central PMCID: PMC4368383.
 44. Herbst S, Schaible UE, Schneider BE. Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis. PLoS One. 2011;6(5):e19105. Epub 2011/05/12. doi: 10.1371/journal.pone.0019105. PubMed PMID: 21559306; PubMed Central PMCID: PMC3085516.
 45. Martin CJ, Peters KN, Behar SM. Macrophages clean up: efferocytosis and microbial control. Curr Opin Microbiol. 2014;17:17-23. Epub 2014/03/04. doi: 10.1016/j.mib.2013.10.007. PubMed PMID: 24581688; PubMed Central PMCID: PMC3942671.
 46. Martin CJ, Booty MG, Rosebrock TR, Nunes-Alves C, Desjardins DM, Keren I, et al. Efferocytosis is an innate antibacterial mechanism. Cell Host Microbe. 2012;12(3):289-300. Epub 2012/09/18. doi:

- 10.1016/j.chom.2012.06.010. PubMed PMID: 22980326; PubMed Central PMCID: PMC3517204.
47. Kramnik I, Dietrich WF, Demant P, Bloom BR. Genetic control of resistance to experimental infection with virulent *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2000;97(15):8560-5. doi: 10.1073/pnas.150227197. PubMed PMID: 10890913; PubMed Central PMCID: PMC26987.
 48. Pan H, Yan BS, Rojas M, Shebzukhov YV, Zhou H, Kobzik L, et al. *lpr1* gene mediates innate immunity to tuberculosis. *Nature*. 2005;434(7034):767-72. doi: 10.1038/nature03419. PubMed PMID: 15815631; PubMed Central PMCID: PMC1388092.
 49. Lowe DM, Redford PS, Wilkinson RJ, O'Garra A, Martineau AR. Neutrophils in tuberculosis: friend or foe? *Trends Immunol*. 2012;33(1):14-25. Epub 2011/11/19. doi: 10.1016/j.it.2011.10.003. PubMed PMID: 22094048.
 50. Eum SY, Kong JH, Hong MS, Lee YJ, Kim JH, Hwang SH, et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest*. 2010;137(1):122-8. Epub 2009/09/15. doi: 10.1378/chest.09-0903. PubMed PMID: 19749004; PubMed Central PMCID: PMC2803122.
 51. Pedrosa J, Saunders BM, Appelberg R, Orme IM, Silva MT, Cooper AM. Neutrophils play a protective nonphagocytic role in systemic *Mycobacterium tuberculosis* infection of mice. *Infect Immun*. 2000;68(2):577-83. PubMed PMID: 10639420; PubMed Central PMCID: PMC297179.
 52. Eruslanov EB, Lyadova IV, Kondratieva TK, Majorov KB, Scheglov IV, Orlova MO, et al. Neutrophil responses to *Mycobacterium tuberculosis* infection in genetically susceptible and resistant mice. *Infect Immun*. 2005;73(3):1744-53. Epub 2005/02/26. doi: 10.1128/IAI.73.3.1744-1753.2005. PubMed PMID: 15731075; PubMed Central PMCID: PMC1064912.
 53. Nandi B, Behar SM. Regulation of neutrophils by interferon-gamma limits lung inflammation during tuberculosis infection. *J Exp Med*. 2011;208(11):2251-62. Epub 2011/10/05. doi: 10.1084/jem.20110919. PubMed PMID: 21967766; PubMed Central PMCID: PMC3201199.
 54. Vankayalapati R, Garg A, Porgador A, Griffith DE, Klucar P, Safi H, et al. Role of NK Cell-Activating Receptors and Their Ligands in the Lysis of Mononuclear Phagocytes Infected with an Intracellular Bacterium. *The Journal of Immunology*. 2005;175(7):4611-7. doi: 10.4049/jimmunol.175.7.4611.
 55. Rothchild AC, Jayaraman P, Nunes-Alves C, Behar SM. iNKT cell production of GM-CSF controls *Mycobacterium tuberculosis*. *PLoS Pathog*. 2014;10(1):e1003805. Epub 2014/01/07. doi: 10.1371/journal.ppat.1003805. PubMed PMID: 24391492; PubMed Central PMCID: PMC3879349.
 56. Sia JK, Georgieva M, Rengarajan J. Innate Immune Defenses in Human Tuberculosis: An Overview of the Interactions between *Mycobacterium tuberculosis* and Innate Immune Cells. *J Immunol Res*. 2015;2015:747543. Epub 2015/08/11. doi: 10.1155/2015/747543. PubMed PMID: 26258152; PubMed Central PMCID: PMC4516846.

57. Petersen HJ, Smith AM. The role of the innate immune system in granulomatous disorders. *Front Immunol.* 2013;4:120. Epub 2013/06/08. doi: 10.3389/fimmu.2013.00120. PubMed PMID: 23745122; PubMed Central PMCID: PMC3662972.
58. Davis JM, Clay H, Lewis JL, Ghorri N, Herbomel P, Ramakrishnan L. Real-Time Visualization of Mycobacterium-Macrophage Interactions Leading to Initiation of Granuloma Formation in Zebrafish Embryos. *Immunity.* 2002;17(6):693-702. doi: 10.1016/s1074-7613(02)00475-2.
59. Cooper AM. T cells in mycobacterial infection and disease. *Curr Opin Immunol.* 2009;21(4):378-84. Epub 2009/08/04. doi: 10.1016/j.coi.2009.06.004. PubMed PMID: 19646851; PubMed Central PMCID: PMC2746551.
60. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. *Semin Immunol.* 2014;26(6):559-77. Epub 2014/10/15. doi: 10.1016/j.smim.2014.09.003. PubMed PMID: 25311810; PubMed Central PMCID: PMC4250436.
61. Tian T, Woodworth J, Skold M, Behar SM. In Vivo Depletion of CD11c+ Cells Delays the CD4+ T Cell Response to Mycobacterium tuberculosis and Exacerbates the Outcome of Infection. *The Journal of Immunology.* 2005;175(5):3268-72. doi: 10.4049/jimmunol.175.5.3268.
62. Reiley WW, Calayag MD, Wittmer ST, Huntington JL, Pearl JE, Fountain JJ, et al. ESAT-6-specific CD4 T cell responses to aerosol Mycobacterium tuberculosis infection are initiated in the mediastinal lymph nodes. *Proc Natl Acad Sci U S A.* 2008;105(31):10961-6. doi: 10.1073/pnas.0801496105. PubMed PMID: 18667699; PubMed Central PMCID: PMC2504808.
63. Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunol.* 2011;4(3):288-93. Epub 2011/04/01. doi: 10.1038/mi.2011.10. PubMed PMID: 21451503; PubMed Central PMCID: PMC3206635.
64. Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura T, Takatsu K, et al. Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J Exp Med.* 2008;205(1):105-15. Epub 2007/12/26. doi: 10.1084/jem.20071367. PubMed PMID: 18158321; PubMed Central PMCID: PMC2234384.
65. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium tuberculosis Is Influenced by Host Factors and Precedes the Initiation of T-Cell Immunity. *Infection and Immunity.* 2002;70(8):4501-9. doi: 10.1128/iai.70.8.4501-4509.2002.
66. Griffiths KL, Ahmed M, Das S, Gopal R, Horne W, Connell TD, et al. Targeting dendritic cells to accelerate T-cell activation overcomes a bottleneck in tuberculosis vaccine efficacy. *Nat Commun.* 2016;7:13894. Epub 2016/12/23. doi: 10.1038/ncomms13894. PubMed PMID: 28004802; PubMed Central PMCID: PMC5192216.

67. Leveton C, Barnass S, Champion B, Lucas S, De Souza B, Nicol M, et al. T-cell-mediated protection of mice against virulent *Mycobacterium tuberculosis*. *Infect Immun*. 1989;57(2):390-5. Epub 1989/02/01. PubMed PMID: 2492259; PubMed Central PMCID: PMCPMC313109.
68. Muller I, Cobbold SP, Waldmann H, Kaufmann SH. Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. *Infect Immun*. 1987;55(9):2037-41. PubMed PMID: 3114143; PubMed Central PMCID: PMCPMC260652.
69. Orme IM. Protection against *Mycobacterium tuberculosis* infection by adoptive immunotherapy. Requirement for T cell-deficient recipients. *Journal of Experimental Medicine*. 1983;158(1):74-83. doi: 10.1084/jem.158.1.74.
70. Mogues T, Goodrich ME, Ryan L, LaCourse R, North RJ. The Relative Importance of T Cell Subsets in Immunity and Immunopathology of Airborne *Mycobacterium tuberculosis* Infection in Mice. *The Journal of Experimental Medicine*. 2001;193(3):271-80. doi: 10.1084/jem.193.3.271.
71. Shimokata K, Kishimoto H, Takagi E, Tsunekawa H. Determination of the T-Cell Subset Producing γ -Interferon in Tuberculous Pleural Effusion. *Microbiology and Immunology*. 1986;30(4):353-61. doi: 10.1111/j.1348-0421.1986.tb00952.x.
72. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med*. 1993;178(6):2243-7. Epub 1993/12/01. PubMed PMID: 8245795; PubMed Central PMCID: PMCPMC2191280.
73. Casanova JL, Abel L. Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol*. 2002;20:581-620. Epub 2002/02/28. doi: 10.1146/annurev.immunol.20.081501.125851. PubMed PMID: 11861613.
74. Bustamante J, Boisson-Dupuis S, Abel L, Casanova JL. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-gamma immunity. *Semin Immunol*. 2014;26(6):454-70. doi: 10.1016/j.smim.2014.09.008. PubMed PMID: 25453225; PubMed Central PMCID: PMCPMC4357480.
75. Orme IM, Roberts AD, Griffin JP, Abrams JS. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. *J Immunol*. 1993;151(1):518-25. Epub 1993/07/01. PubMed PMID: 8100846.
76. Green AM, Difazio R, Flynn JL. IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during *Mycobacterium tuberculosis* infection. *J Immunol*. 2013;190(1):270-7. Epub 2012/12/13. doi: 10.4049/jimmunol.1200061. PubMed PMID: 23233724; PubMed Central PMCID: PMCPMC3683563.
77. Sakai S, Mayer-Barber KD, Barber DL. Defining features of protective CD4 T cell responses to *Mycobacterium tuberculosis*. *Curr Opin Immunol*. 2014;29:137-42. Epub 2014/07/08. doi: 10.1016/j.coi.2014.06.003. PubMed PMID: 25000593; PubMed Central PMCID: PMCPMC4122329.

78. Cowley SC, Elkins KL. CD4+ T cells mediate IFN-gamma-independent control of *Mycobacterium tuberculosis* infection both in vitro and in vivo. *J Immunol*. 2003. PubMed PMID: 14568944.
79. Sakai S, Kauffman KD, Sallin MA, Sharpe AH, Young HA, Ganusov VV, et al. CD4 T Cell-Derived IFN- γ Plays a Minimal Role in Control of Pulmonary *Mycobacterium tuberculosis* Infection and Must Be Actively Repressed by PD-1 to Prevent Lethal Disease. *PLoS Pathog*. 2016;12(5):e1005667. doi: 10.1371/journal.ppat.1005667.
80. Serbina NV, Flynn JL. Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of *Mycobacterium tuberculosis*-infected mice. *Infect Immun*. 1999;67(8):3980-8. Epub 1999/07/23. PubMed PMID: 10417164; PubMed Central PMCID: PMC96683.
81. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. *Annu Rev Immunol*. 2013;31:475-527. Epub 2013/03/23. doi: 10.1146/annurev-immunol-032712-095939. PubMed PMID: 23516984.
82. Dwivedi VP, Bhattacharya D, Chatterjee S, Prasad DVR, Chattopadhyay D, Van Kaer L, et al. *Mycobacterium tuberculosis* Directs T Helper 2 Cell Differentiation by Inducing Interleukin-1 β Production in Dendritic Cells. *The Journal of biological chemistry*. 2012;287(40):33656-63. doi: 10.1074/jbc.M112.375154.
83. Pitt JM, Stavropoulos E, Redford PS, Beebe AM, Bancroft GJ, Young DB, et al. Blockade of IL-10 signaling during bacillus Calmette-Guerin vaccination enhances and sustains Th1, Th17, and innate lymphoid IFN-gamma and IL-17 responses and increases protection to *Mycobacterium tuberculosis* infection. *J Immunol*. 2012;189(8):4079-87. Epub 2012/09/14. doi: 10.4049/jimmunol.1201061. PubMed PMID: 22972927; PubMed Central PMCID: PMC3467194.
84. Baliko Z, Szereday L, Szekeres-Bartho J. Th2 biased immune response in cases with active *Mycobacterium tuberculosis* infection and tuberculin anergy. *FEMS Immunol Med Microbiol*. 1998;22(3):199-204. PubMed PMID: 9848680.
85. Dlugovitzky D. Circulating profile of Th1 and Th2 cytokines in tuberculosis patients with different degrees of pulmonary involvement. *FEMS Immunology and Medical Microbiology*. 1997;18(3):203-7. doi: 10.1111/j.1574-695X.1997.tb01046.x.
86. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol*. 2007;8(4):369-77. Epub 2007/03/14. doi: 10.1038/ni1449. PubMed PMID: 17351619.
87. Khader SA, Gopal R. IL-17 in protective immunity to intracellular pathogens. *Virulence*. 2010;1(5):423-7. Epub 2010/12/24. doi: 10.4161/viru.1.5.12862. PubMed PMID: 21178483; PubMed Central PMCID: PMC32953849.

88. Jasenosky LD, Scriba TJ, Hanekom WA, Goldfeld AE. T cells and adaptive immunity to *Mycobacterium tuberculosis* in humans. *Immunol Rev.* 2015;264(1):74-87. Epub 2015/02/24. doi: 10.1111/imr.12274. PubMed PMID: 25703553.
89. Shafiani S, Tucker-Heard G, Kariyone A, Takatsu K, Urdahl KB. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J Exp Med.* 2010;207(7):1409-20. Epub 2010/06/16. doi: 10.1084/jem.20091885. PubMed PMID: 20547826; PubMed Central PMCID: PMC2901066.
90. Scott-Browne JP, Shafiani S, Tucker-Heard G, Ishida-Tsubota K, Fontenot JD, Rudensky AY, et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med.* 2007;204(9):2159-69. Epub 2007/08/22. doi: 10.1084/jem.20062105. PubMed PMID: 17709423; PubMed Central PMCID: PMC2118702.
91. Orme IM, Collins FM. Adoptive protection of the *Mycobacterium tuberculosis*-infected lung. Dissociation between cells that passively transfer protective immunity and those that transfer delayed-type hypersensitivity to tuberculin. *Cell Immunol.* 1984;84(1):113-20. PubMed PMID: 6421492.
92. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci U S A.* 1992;89(24):12013-7. Epub 1992/12/15. PubMed PMID: 1465432; PubMed Central PMCID: PMC250688.
93. Schaible UE, Collins HL, Priem F, Kaufmann SHE. Correction of the Iron Overload Defect in β -2-Microglobulin Knockout Mice by Lactoferrin Abolishes Their Increased Susceptibility to Tuberculosis. *The Journal of Experimental Medicine.* 2002;196(11):1507-13. doi: 10.1084/jem.20020897.
94. Rolph MS, Raupach B, Kobernick HH, Collins HL, Perarnau B, Lemonnier FA, et al. MHC class Ia-restricted T cells partially account for beta2-microglobulin-dependent resistance to *Mycobacterium tuberculosis*. *Eur J Immunol.* 2001;31(6):1944-9. PubMed PMID: 11433392.
95. Behar SM, Dascher CC, Grusby MJ, Wang CR, Brenner MB. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis*. *J Exp Med.* 1999;189(12):1973-80. Epub 1999/06/22. PubMed PMID: 10377193; PubMed Central PMCID: PMC2192974.
96. Zhang S, Zhang H, Zhao J. The role of CD4 T cell help for CD8 CTL activation. *Biochem Biophys Res Commun.* 2009;384(4):405-8. Epub 2009/05/05. doi: 10.1016/j.bbrc.2009.04.134. PubMed PMID: 19410556.
97. Feau S, Garcia Z, Arens R, Yagita H, Borst J, Schoenberger SP. The CD4(+) T-cell help signal is transmitted from APC to CD8(+) T-cells via CD27-CD70 interactions. *Nat Commun.* 2012;3:948. doi: 10.1038/ncomms1948. PubMed PMID: 22781761; PubMed Central PMCID: PMC3606886.

98. Amigorena S. Helping the Help for CD8+ T Cell Responses. *Cell*. 2015;162(6):1210-2. Epub 2015/09/12. doi: 10.1016/j.cell.2015.08.051. PubMed PMID: 26359981.
99. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature*. 2003;421:852. doi: 10.1038/nature01441.
100. Bevan MJ. Helping the CD8(+) T-cell response. *Nat Rev Immunol*. 2004;4(8):595-602. Epub 2004/08/03. doi: 10.1038/nri1413. PubMed PMID: 15286726.
101. Bold TD, Ernst JD. CD4+ T cell-dependent IFN-gamma production by CD8+ effector T cells in *Mycobacterium tuberculosis* infection. *J Immunol*. 2012;189(5):2530-6. Epub 2012/07/28. doi: 10.4049/jimmunol.1200994. PubMed PMID: 22837486; PubMed Central PMCID: PMC3424308.
102. Serbina NV, Lazarevic V, Flynn JL. CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during *Mycobacterium tuberculosis* infection. *J Immunol*. 2001;167(12):6991-7000. Epub 2001/12/12. doi: 10.4049/jimmunol.167.12.6991. PubMed PMID: 11739519.
103. Silva CL, Silva MF, Pietro RC, Lowrie DB. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. *Immunology*. 1994;83(3):341-6. Epub 1994/11/01. PubMed PMID: 7835957; PubMed Central PMCID: PMC1415049.
104. Serbina NV, Flynn JL. CD8(+) T cells participate in the memory immune response to *Mycobacterium tuberculosis*. *Infect Immun*. 2001;69(7):4320-8. Epub 2001/06/13. doi: 10.1128/IAI.69.7.4320-4328.2001. PubMed PMID: 11401969; PubMed Central PMCID: PMC98502.
105. Wang J, Santosuosso M, Ngai P, Zganiacz A, Xing Z. Activation of CD8 T Cells by Mycobacterial Vaccination Protects against Pulmonary Tuberculosis in the Absence of CD4 T Cells. *The Journal of Immunology*. 2004;173(7):4590-7. doi: 10.4049/jimmunol.173.7.4590.
106. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol*. 1999;162(9):5407-16. PubMed PMID: 10228018.
107. Lazarevic V, Nolt D, Flynn JL. Long-Term Control of *Mycobacterium tuberculosis* Infection Is Mediated by Dynamic Immune Responses. *The Journal of Immunology*. 2005;175(2):1107-17. doi: 10.4049/jimmunol.175.2.1107.
108. van Pinxteren LA, Cassidy JP, Smedegaard BH, Agger EM, Andersen P. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. *Eur J Immunol*. 2000;30(12):3689-98. doi: 10.1002/1521-4141(200012)30:12<3689::AID-IMMU3689>3.0.CO;2-4. PubMed PMID: 11169412.
109. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, et al. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS Pathog*.

- 2009;5(4):e1000392. Epub 2009/04/22. doi: 10.1371/journal.ppat.1000392. PubMed PMID: 19381260; PubMed Central PMCID: PMC2663842.
110. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor- α is required in the protective immune response against mycobacterium tuberculosis in mice. *Immunity*. 1995;2(6):561-72. doi: 10.1016/1074-7613(95)90001-2.
111. Cooper AM, D'Souza C, Frank AA, Orme IM. The course of Mycobacterium tuberculosis infection in the lungs of mice lacking expression of either perforin- or granzyme-mediated cytolytic mechanisms. *Infect Immun*. 1997;65(4):1317-20. doi: CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. PubMed PMID: 9119468; PubMed Central PMCID: PMC175134.
112. Laochumroonvorapong P, Wang J, Liu CC, Ye W, Moreira AL, Elkon KB, et al. Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection in mice. *Infect Immun*. 1997;65(1):127-32. PubMed PMID: 8975902; PubMed Central PMCID: PMC174566.
113. Serbina NV, Liu CC, Scanga CA, Flynn JL. CD8+ CTL from lungs of Mycobacterium tuberculosis-infected mice express perforin in vivo and lyse infected macrophages. *J Immunol*. 2000;165(1):353-63. Epub 2000/06/22. doi: 10.4049/jimmunol.165.1.353. PubMed PMID: 10861072.
114. Kamath AB, Woodworth J, Xiong X, Taylor C, Weng Y, Behar SM. Cytolytic CD8+ T Cells Recognizing CFP10 Are Recruited to the Lung after Mycobacterium tuberculosis Infection. *The Journal of Experimental Medicine*. 2004;200(11):1479-89. doi: 10.1084/jem.20041690.
115. Silva CL, Lowrie DB. Identification and Characterization of Murine Cytotoxic T Cells That Kill Mycobacterium tuberculosis. *Infection and Immunity*. 2000;68(6):3269-74. doi: 10.1128/iai.68.6.3269-3274.2000.
116. Woodworth JS, Wu Y, Behar SM. Mycobacterium tuberculosis-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo. *J Immunol*. 2008;181(12):8595-603. Epub 2008/12/04. doi: 10.4049/jimmunol.181.12.8595. PubMed PMID: 19050279; PubMed Central PMCID: PMC133658.
117. Sousa AO, Mazzaccaro RJ, Russell RG, Lee FK, Turner OC, Hong S, et al. Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. *Proc Natl Acad Sci U S A*. 2000;97(8):4204-8. Epub 2000/04/13. doi: 10.1073/pnas.97.8.4204. PubMed PMID: 10760288; PubMed Central PMCID: PMC18197.
118. Turner J, D'Souza CD, Pearl JE, Marietta P, Noel M, Frank AA, et al. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. *Am J Respir Cell Mol Biol*. 2001;24(2):203-9. doi: 10.1165/ajrcmb.24.2.4370. PubMed PMID: 11159055.
119. Silva BDS, Trentini MM, da Costa AC, Kipnis A, Junqueira-Kipnis AP. Different phenotypes of CD8+ T cells associated with bacterial load in active

- tuberculosis. *Immunol Lett.* 2014;160(1):23-32. Epub 2014/04/04. doi: 10.1016/j.imlet.2014.03.009. PubMed PMID: 24694750.
120. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science.* 1998;282(5386):121-5. PubMed PMID: 9756476.
121. Lin PL, Flynn JL. CD8 T cells and *Mycobacterium tuberculosis* infection. *Semin Immunopathol.* 2015;37(3):239-49. Epub 2015/04/29. doi: 10.1007/s00281-015-0490-8. PubMed PMID: 25917388; PubMed Central PMCID: PMC4439333.
122. Flynn JL, Chan J. Immune evasion by *Mycobacterium tuberculosis*: living with the enemy. *Current Opinion in Immunology.* 2003;15(4):450. doi: 10.1016/s0952-7915(03)00075-x.
123. Deretic V, Fratti RA. *Mycobacterium tuberculosis* phagosome. *Mol Microbiol.* 1999;31(6):1603-9. Epub 1999/04/21. PubMed PMID: 10209735.
124. Ehrt S, Schnappinger D. *Mycobacterial* survival strategies in the phagosome: defence against host stresses. *Cell Microbiol.* 2009;11(8):1170-8. Epub 2009/05/15. doi: 10.1111/j.1462-5822.2009.01335.x. PubMed PMID: 19438516; PubMed Central PMCID: PMC3170014.
125. Vandal OH, Nathan CF, Ehrt S. Acid resistance in *Mycobacterium tuberculosis*. *J Bacteriol.* 2009;191(15):4714-21. Epub 2009/05/26. doi: 10.1128/JB.00305-09. PubMed PMID: 19465648; PubMed Central PMCID: PMC2715723.
126. Sturgill-Koszycki S, Schlesinger P, Chakraborty P, Haddix P, Collins H, Fok A, et al. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science.* 1994;263(5147):678-81. doi: 10.1126/science.8303277.
127. Russell DG, Purdy GE, Owens RM, Rohde K, Yates RM. *Mycobacterium tuberculosis* and the four-minute phagosome. *ASM News.* 2005;71(10):459-63.
128. Clemens DL, Horwitz MA. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med.* 1995;257-70
PubMed Central PMCID: PMC2191842.
129. Hart PD, Armstrong JA, Brown CA, Draper P. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. *Infect Immun.* 1972;5(5):803-7. PubMed PMID: 4629254; PubMed Central PMCID: PMC422443.
130. Orme IM. Vaccines to prevent tuberculosis infection rather than disease: Physiological and immunological aspects. *Tuberculosis (Edinb).* 2016. doi: 10.1016/j.tube.2014.10.008. PubMed PMID: 25500316.
131. Wang J, Li BX, Ge PP, Li J, Wang Q, Gao GF, et al. *Mycobacterium tuberculosis* suppresses innate immunity by coopting the host ubiquitin system. *Nat Immunol.* 2015;16(3):237-45. Epub 2015/02/03. doi: 10.1038/ni.3096. PubMed PMID: 25642820.

132. Ehrt S, Shiloh MU, Ruan J, Choi M, Gunzburg S, Nathan C, et al. A novel antioxidant gene from *Mycobacterium tuberculosis*. *J Exp Med*. 1997;186(11):1885-96. PubMed PMID: 9382887; PubMed Central PMCID: PMC2199150.
133. Behar SM, Martin CJ, Booty MG, Nishimura T, Zhao X, Gan HX, et al. Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*. *Mucosal Immunol*. 2011;4(3):279-87. Epub 2011/02/11. doi: 10.1038/mi.2011.3. PubMed PMID: 21307848; PubMed Central PMCID: PMC2199150.
134. Oddo M, Renno T, Attinger A, Bakker T, MacDonald HR, Meylan PR. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. *J Immunol*. 1998;160(11):5448-54. PubMed PMID: 9605147.
135. Divangahi M, Chen M, Gan H, Desjardins D, Hickman TT, Lee DM, et al. *Mycobacterium tuberculosis* evades macrophage defenses by inhibiting plasma membrane repair. *Nat Immunol*. 2009;10(8):899-906. Epub 2009/06/30. doi: 10.1038/ni.1758. PubMed PMID: 19561612; PubMed Central PMCID: PMC2730354.
136. Behar SM, Divangahi M, Remold HG. Evasion of innate immunity by *Mycobacterium tuberculosis*: is death an exit strategy? *Nat Rev Microbiol*. 2010;8(9):668-74. Epub 2010/08/03. doi: 10.1038/nrmicro2387. PubMed PMID: 20676146; PubMed Central PMCID: PMC2730354.
137. Baena A, Porcelli SA. Evasion and subversion of antigen presentation by *Mycobacterium tuberculosis*. *Tissue Antigens*. 2009;74(3):189-204. Epub 2009/07/01. doi: 10.1111/j.1399-0039.2009.01301.x. PubMed PMID: 19563525; PubMed Central PMCID: PMC2730354.
138. Divangahi M, Desjardins D, Nunes-Alves C, Remold HG, Behar SM. Eicosanoid pathways regulate adaptive immunity to *Mycobacterium tuberculosis*. *Nat Immunol*. 2010;11(8):751-8. Epub 2010/07/14. doi: 10.1038/ni.1904. PubMed PMID: 20622882; PubMed Central PMCID: PMC2730354.
139. Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, Hsu T, et al. *Mycobacterium tuberculosis* nuoG is a virulence gene that inhibits apoptosis of infected host cells. *PLoS Pathog*. 2007;3(7):e110. Epub 2007/07/31. doi: 10.1371/journal.ppat.0030110. PubMed PMID: 17658950; PubMed Central PMCID: PMC1924871.
140. Blomgran R, Desvignes L, Briken V, Ernst JD. *Mycobacterium tuberculosis* inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host Microbe*. 2012;11(1):81-90. Epub 2012/01/24. doi: 10.1016/j.chom.2011.11.012. PubMed PMID: 22264515; PubMed Central PMCID: PMC3266554.
141. Moreira-Teixeira L, Redford PS, Stavropoulos E, Ghilardi N, Maynard CL, Weaver CT, et al. T Cell-Derived IL-10 Impairs Host Resistance to *Mycobacterium tuberculosis* Infection. *The Journal of Immunology*. 2017;ji1601340. doi: 10.4049/jimmunol.1601340.

142. de la Barrera S, Aleman M, Musella R, Schierloh P, Pasquinelli V, Garcia V, et al. IL-10 down-regulates costimulatory molecules on Mycobacterium tuberculosis-pulsed macrophages and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients. *Clin Exp Immunol*. 2004;138(1):128-38. Epub 2004/09/18. doi: 10.1111/j.1365-2249.2004.02577.x. PubMed PMID: 15373915; PubMed Central PMCID: PMC1809183.
143. Russell MS, Iskandar M, Mykytczuk OL, Nash JHE, Krishnan L, Sad S. A Reduced Antigen Load In Vivo, Rather Than Weak Inflammation, Causes a Substantial Delay in CD8+ T Cell Priming against Mycobacterium bovis (Bacillus Calmette-Guerin). *The Journal of Immunology*. 2007;179(1):211-20. doi: 10.4049/jimmunol.179.1.211.
144. Bold TD, Banaei N, Wolf AJ, Ernst JD. Suboptimal activation of antigen-specific CD4+ effector cells enables persistence of M. tuberculosis in vivo. *PLoS Pathog*. 2011;7(5):e1002063. Epub 2011/06/04. doi: 10.1371/journal.ppat.1002063. PubMed PMID: 21637811; PubMed Central PMCID: PMC3102708.
145. Moguche AO, Musvosvi M, Penn-Nicholson A, Plumlee CR, Mearns H, Geldenhuys H, et al. Antigen Availability Shapes T Cell Differentiation and Function during Tuberculosis. *Cell Host Microbe*. 2017;21(6):695-706 e5. doi: 10.1016/j.chom.2017.05.012. PubMed PMID: 28618268; PubMed Central PMCID: PMC5533182.
146. Wang X, Barnes PF, Huang F, Alvarez IB, Neuenschwander PF, Sherman DR, et al. Early secreted antigenic target of 6-kDa protein of Mycobacterium tuberculosis primes dendritic cells to stimulate Th17 and inhibit Th1 immune responses. *J Immunol*. 2012;189(6):3092-103. Epub 2012/08/21. doi: 10.4049/jimmunol.1200573. PubMed PMID: 22904313; PubMed Central PMCID: PMC3436987.
147. Samten B, Wang X, Barnes PF. Mycobacterium tuberculosis ESX-1 system-secreted protein ESAT-6 but not CFP10 inhibits human T-cell immune responses. *Tuberculosis*. 2009;89:S74-S6. doi: 10.1016/s1472-9792(09)70017-4.
148. Mariotti S, Sargentini V, Pardini M, Giannoni F, De Spirito M, Gagliardi MC, et al. Mycobacterium tuberculosis may escape helper T cell recognition by infecting human fibroblasts. *Human Immunology*. 2013;74(6):722-9. doi: 10.1016/j.humimm.2013.02.005.
149. Noss EH, Harding CV, Boom WH. Mycobacterium tuberculosis inhibits MHC class II antigen processing in murine bone marrow macrophages. *Cell Immunol*. 2000;201(1):63-74. Epub 2000/05/12. doi: 10.1006/cimm.2000.1633. PubMed PMID: 10805975.
150. Athman JJ, Sande OJ, Groft SG, Reba SM, Nagy N, Wearsch PA, et al. Mycobacterium tuberculosis Membrane Vesicles Inhibit T Cell Activation. *J Immunol*. 2017;198(5):2028-37. Epub 2017/01/27. doi: 10.4049/jimmunol.1601199. PubMed PMID: 28122965; PubMed Central PMCID: PMC5322216.

151. Srivastava S, Grace PS, Ernst JD. Antigen Export Reduces Antigen Presentation and Limits T Cell Control of *M. tuberculosis*. *Cell Host Microbe*. 2016;19(1):44-54. Epub 2016/01/15. doi: 10.1016/j.chom.2015.12.003. PubMed PMID: 26764596; PubMed Central PMCID: PMC4715867.
152. Rock KL, Reits E, Neefjes J. Present Yourself! By MHC Class I and MHC Class II Molecules. *Trends Immunol*. 2016;37(11):724-37. Epub 2016/09/12. doi: 10.1016/j.it.2016.08.010. PubMed PMID: 27614798; PubMed Central PMCID: PMC4715867.
153. Heemels MT, Ploegh H. Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu Rev Biochem*. 1995;64:463-91. doi: 10.1146/annurev.bi.64.070195.002335. PubMed PMID: 7574490.
154. Rock KL, Shen L. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol Rev*. 2005. doi: 10.1111/j.0105-2896.2005.00301.x. PubMed PMID: 16181335.
155. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *Journal of Experimental Medicine*. 1976;143(5):1283-8. doi: 10.1084/jem.143.5.1283.
156. McDonnell AM, Robinson BW, Currie AJ. Tumor antigen cross-presentation and the dendritic cell: where it all begins? *Clin Dev Immunol*. 2010;2010:539519. Epub 2010/10/27. doi: 10.1155/2010/539519. PubMed PMID: 20976125; PubMed Central PMCID: PMC2957101.
157. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol*. 2012;12(8):557-69. Epub 2012/07/14. doi: 10.1038/nri3254. PubMed PMID: 22790179.
158. Houde M, Bertholet S, Gagnon E, Brunet S, Goyette G, Laplante A, et al. Phagosomes are competent organelles for antigen cross-presentation. *Nature*. 2003;425(6956):402-6. Epub 2003/09/26. doi: 10.1038/nature01912. PubMed PMID: 14508490.
159. Lanzavecchia A. Mechanisms of antigen uptake for presentation. *Curr Opin Immunol*. 1996;8(3):348-54. PubMed PMID: 8794000.
160. Booty MG, Nunes-Alves C, Carpenter SM, Jayaraman P, Behar SM. Multiple Inflammatory Cytokines Converge To Regulate CD8+ T Cell Expansion and Function during Tuberculosis. *J Immunol*. 2016;196(4):1822-31. doi: 10.4049/jimmunol.1502206.
161. Okamoto I, Kohno K, Tanimoto T, Ikegami H, Kurimoto M. Development of CD8+ effector T cells is differentially regulated by IL-18 and IL-12. *J Immunol*. 1999;162(6):3202-11. Epub 1999/03/27. PubMed PMID: 10092771.
162. Akdis M, Burgler S, Cramer R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. *J Allergy Clin Immunol*. 2011;127(3):701-21 e1-70. Epub 2011/03/08. doi: 10.1016/j.jaci.2010.11.050. PubMed PMID: 21377040.
163. Bastidas S, Graw F, Smith MZ, Kuster H, Gunthard HF, Oxenius A. CD8+ T cells are activated in an antigen-independent manner in HIV-infected

- individuals. *J Immunol.* 2014;192(4):1732-44. Epub 2014/01/22. doi: 10.4049/jimmunol.1302027. PubMed PMID: 24446519.
164. Giacomini E, Iona E, Ferroni L, Miettinen M, Fattorini L, Orefici G, et al. Infection of Human Macrophages and Dendritic Cells with *Mycobacterium tuberculosis* Induces a Differential Cytokine Gene Expression That Modulates T Cell Response. *The Journal of Immunology.* 2001;166(12):7033-41. doi: 10.4049/jimmunol.166.12.7033.
165. Anikeeva N, Sykulev Y. Mechanisms controlling granule-mediated cytolytic activity of cytotoxic T lymphocytes. *Immunol Res.* 2011;51(2-3):183-94. Epub 2011/11/08. doi: 10.1007/s12026-011-8252-8. PubMed PMID: 22058021; PubMed Central PMCID: PMC3244531.
166. Muller AJ, Filipe-Santos O, Eberl G, Aebischer T, Spath GF, Bousso P. CD4⁺ T cells rely on a cytokine gradient to control intracellular pathogens beyond sites of antigen presentation. *Immunity.* 2012;37(1):147-57. Epub 2012/06/26. doi: 10.1016/j.immuni.2012.05.015. PubMed PMID: 22727490.
167. McSorley SJ. The Role of Non-Cognate T Cell Stimulation during Intracellular Bacterial Infection. *Front Immunol.* 2014;5:319. Epub 2014/07/30. doi: 10.3389/fimmu.2014.00319. PubMed PMID: 25071779; PubMed Central PMCID: PMC4089505.
168. O'Donnell H, McSorley SJ. Salmonella as a model for non-cognate Th1 cell stimulation. *Front Immunol.* 2014;5:621. Epub 2014/12/30. doi: 10.3389/fimmu.2014.00621. PubMed PMID: 25540644; PubMed Central PMCID: PMC4261815.
169. Martin MD, Badovinac VP. Antigen-dependent and -independent contributions to primary memory CD8 T cell activation and protection following infection. *Sci Rep.* 2015;5:18022. Epub 2015/12/15. doi: 10.1038/srep18022. PubMed PMID: 26658291; PubMed Central PMCID: PMC4675085.
170. Srivastava S, Ernst JD. Cutting edge: Direct recognition of infected cells by CD4 T cells is required for control of intracellular *Mycobacterium tuberculosis* in vivo. *J Immunol.* 2013;191(3):1016-20. Epub 2013/07/03. doi: 10.4049/jimmunol.1301236. PubMed PMID: 23817429; PubMed Central PMCID: PMC3725655.
171. Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL, Jr., Eissa NT. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med.* 2009;15(3):267-76. Epub 2009/03/03. doi: 10.1038/nm.1928. PubMed PMID: 19252503.
172. Singh CR, Moulton RA, Armitage LY, Bidani A, Snuggs M, Dhandayuthapani S, et al. Processing and Presentation of a *Mycobacterial* Antigen 85B Epitope by Murine Macrophages Is Dependent on the Phagosomal Acquisition of Vacuolar Proton ATPase and In Situ Activation of Cathepsin D. *The Journal of Immunology.* 2006;177(5):3250-9. doi: 10.4049/jimmunol.177.5.3250.
173. Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, Golenbock DT, et al. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression

- and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J Immunol*. 2001;167(2):910-8. Epub 2001/07/07. doi: 10.4049/jimmunol.167.2.910. PubMed PMID: 11441098.
174. Singh CR, Bakhru P, Khan A, Li QB, Jagannath C. Cutting edge: Nicastrin and related components of gamma-secretase generate a peptide epitope facilitating immune recognition of intracellular mycobacteria, through MHC class II-dependent priming of T cells. *J Immunol*. 2011;187(11):5495-9. Epub 2011/11/01. doi: 10.4049/jimmunol.1100521. PubMed PMID: 22039303; PubMed Central PMCID: PMC3221744.
175. Ramachandra L, Noss E, Boom WH, Harding CV. Processing of *Mycobacterium tuberculosis* Antigen 85B Involves Intraphagosomal Formation of Peptide-Major Histocompatibility Complex II Complexes and Is Inhibited by Live Bacilli that Decrease Phagosome Maturation. *The Journal of Experimental Medicine*. 2001;194(10):1421-32. doi: 10.1084/jem.194.10.1421.
176. Gonzalez-Juarrero M, Orme IM. Characterization of Murine Lung Dendritic Cells Infected with *Mycobacterium tuberculosis*. *Infection and Immunity*. 2001;69(2):1127-33. doi: 10.1128/IAI.69.2.1127-1133.2001.
177. Bastian M, Braun T, Bruns H, Rollinghoff M, Stenger S. Mycobacterial lipopeptides elicit CD4⁺ CTLs in *Mycobacterium tuberculosis*-infected humans. *J Immunol*. 2008;180(5):3436-46. Epub 2008/02/23. doi: 10.4049/jimmunol.180.5.3436. PubMed PMID: 18292570.
178. Klucar P, Barnes PF, Kong Y, Samten B, Tvinnereim A, Spallek R, et al. Characterization of effector functions of human peptide-specific CD4⁺ T-cell clones for an intracellular pathogen. *Human Immunology*. 2008;69(8):475-83. doi: 10.1016/j.humimm.2008.05.008.
179. Tanchot C, Lemonnier FA, Pérarnau B, Freitas AA, Rocha B. Differential Requirements for Survival and Proliferation of CD8 Naïve or Memory T Cells. *Science*. 1997;276(5321):2057-62. doi: 10.1126/science.276.5321.2057.
180. Sagerström CG, Kerr EM, Allison JP, Davis MM. Activation and differentiation requirements of primary T cells in vitro. *Proceedings of the National Academy of Sciences of the United States of America*. 1993;90(19):8987-91. PubMed PMID: PMC47486.
181. Lozza L, Farinacci M, Bechtle M, Staber M, Zedler U, Baiocchi A, et al. Communication between Human Dendritic Cell Subsets in Tuberculosis: Requirements for Naive CD4(+) T Cell Stimulation. *Front Immunol*. 2014;5:324. Epub 2014/07/30. doi: 10.3389/fimmu.2014.00324. PubMed PMID: 25071784; PubMed Central PMCID: PMC4094910.
182. Balaji KN, Boom WH. Processing of *Mycobacterium tuberculosis* bacilli by human monocytes for CD4⁺ alpha beta and gamma delta T cells: role of particulate antigen. *Infect Immun*. 1998;66(1):98-106. Epub 1998/01/10. PubMed PMID: 9423845; PubMed Central PMCID: PMC107864.
183. Carpenter SM, Yang JD, Lee J, Barreira-Silva P, Behar SM. Vaccine-elicited memory CD4⁺ T cell expansion is impaired in the lungs during

- tuberculosis. *PLoS Pathog.* 2017;13(11):e1006704. Epub 2017/11/28. doi: 10.1371/journal.ppat.1006704. PubMed PMID: 29176787.
184. Lai R, Jeyanathan M, Afkhami S, Zganiacz A, Hammill JA, Yao Y, et al. CD11b(+) Dendritic Cell-Mediated Anti-Mycobacterium tuberculosis Th1 Activation Is Counterregulated by CD103(+) Dendritic Cells via IL-10. *J Immunol.* 2018. Epub 2018/01/28. doi: 10.4049/jimmunol.1701109. PubMed PMID: 29374077.
185. Egen JG, Rothfuchs AG, Feng CG, Horwitz MA, Sher A, Germain RN. Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity.* 2011;34(5):807-19. doi: 10.1016/j.immuni.2011.03.022.
186. Das G, Vohra H, Saha B, Mishra GC. Th1-specific Bystander Costimulation Imparts Resistance against Mycobacterium tuberculosis Infection. *Scandinavian Journal of Immunology.* 2000;52(5):515-8. doi: 10.1046/j.1365-3083.2000.00808.x.
187. Gercken J, Pryjma J, Ernst M, Flad HD. Defective antigen presentation by Mycobacterium tuberculosis-infected monocytes. *Infect Immun.* 1994;62(8):3472-8. Epub 1994/08/01. PubMed PMID: 8039918; PubMed Central PMCID: PMC302980.
188. Grace PS, Ernst JD. Suboptimal Antigen Presentation Contributes to Virulence of Mycobacterium tuberculosis In Vivo. *J Immunol.* 2016;196(1):357-64. Epub 2015/11/18. doi: 10.4049/jimmunol.1501494. PubMed PMID: 26573837; PubMed Central PMCID: PMC4684992.
189. Portal-Celhay C, Tufariello JM, Srivastava S, Zahra A, Klevorn T, Grace PS, et al. Mycobacterium tuberculosis EsxH inhibits ESCRT-dependent CD4(+) T-cell activation. *Nature microbiology.* 2016;2:16232. doi: 10.1038/nmicrobiol.2016.232.
190. Einarsdottir T, Lockhart E, Flynn JL. Cytotoxicity and secretion of gamma interferon are carried out by distinct CD8 T cells during Mycobacterium tuberculosis infection. *Infect Immun.* 2009;77(10):4621-30. Epub 2009/08/12. doi: 10.1128/IAI.00415-09. PubMed PMID: 19667047; PubMed Central PMCID: PMC2747936.
191. Li L, Yang B, Zhang X, Lao S, Changyou W. Mycobacterium tuberculosis-specific polyfunctional cytotoxic CD8+ T cells express CD69. *Tuberculosis (Edinb).* 2014;94(3):219-25. doi: 10.1016/j.tube.2013.12.007. PubMed PMID: 24566284.
192. Canaday DH, Ziebold C, Noss EH, Chervenak KA, Harding CV, Boom WH. Activation of human CD8+ alpha beta TCR+ cells by Mycobacterium tuberculosis via an alternate class I MHC antigen-processing pathway. *J Immunol.* 1999;162(1):372-9. Epub 1999/01/14. PubMed PMID: 9886409.
193. Mazzaccaro RJ, Gedde M, Jensen ER, van Santen HM, Ploegh HL, Rock KL, et al. Major histocompatibility class I presentation of soluble antigen facilitated by Mycobacterium tuberculosis infection. *Proc Natl Acad Sci U S A.*

- 1996;93(21):11786-91. Epub 1996/10/15. PubMed PMID: 8876215; PubMed Central PMCID: PMCPMC38136.
194. Bodnar KA, Serbina NV, Flynn JL. Fate of *Mycobacterium tuberculosis* within murine dendritic cells. *Infect Immun*. 2001;69(2):800-9. Epub 2001/02/13. doi: 10.1128/IAI.69.2.800-809.2001. PubMed PMID: 11159971; PubMed Central PMCID: PMCPMC97955.
195. Feng CG, Demangel C, Kamath AT, Macdonald M, Britton WJ. Dendritic cells infected with *Mycobacterium bovis* bacillus Calmette Guérin activate CD8(+) T cells with specificity for a novel mycobacterial epitope. *Int Immunol*. 2001;13(4):451-8. Epub 2001/04/03. PubMed PMID: 11282984.
196. Majlessi L, Rojas MJ, Brodin P, Leclerc C. CD8+-T-Cell Responses of *Mycobacterium*-Infected Mice to a Newly Identified Major Histocompatibility Complex Class I-Restricted Epitope Shared by Proteins of the ESAT-6 Family. *Infection and Immunity*. 2003;71(12):7173-7. doi: 10.1128/iai.71.12.7173-7177.2003.
197. Hess J, Miko D, Catic A, Lehmsiek V, Russell DG, Kaufmann SH. *Mycobacterium bovis* Bacille Calmette-Guérin strains secreting listeriolysin of *Listeria monocytogenes*. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95(9):5299-304.
198. Simmons DP, Canaday DH, Liu Y, Li Q, Huang A, Boom WH, et al. *Mycobacterium tuberculosis* and TLR2 agonists inhibit induction of type I IFN and class I MHC antigen cross processing by TLR9. *J Immunol*. 2010;185(4):2405-15. Epub 2010/07/28. doi: 10.4049/jimmunol.0904005. PubMed PMID: 20660347; PubMed Central PMCID: PMCPMC2990778.
199. Lewinsohn DA, Winata E, Swarbrick GM, Tanner KE, Cook MS, Null MD, et al. Immunodominant tuberculosis CD8 antigens preferentially restricted by HLA-B. *PLoS Pathog*. 2007;3(9):1240-9. Epub 2007/09/26. doi: 10.1371/journal.ppat.0030127. PubMed PMID: 17892322; PubMed Central PMCID: PMCPMC2323292.
200. Flyer DC. Identification by Mass Spectrometry of CD8+-T-Cell *Mycobacterium tuberculosis* Epitopes within the Rv0341 Gene Product. *Infection and Immunity*. 2002;70(6):2926-32. doi: 10.1128/iai.70.6.2926-2932.2002.
201. Caccamo N, Guggino G, Meraviglia S, Gelsomino G, Di Carlo P, Titone L, et al. Analysis of *Mycobacterium tuberculosis*-specific CD8 T-cells in patients with active tuberculosis and in individuals with latent infection. *PLoS One*. 2009;4(5):e5528. Epub 2009/05/14. doi: 10.1371/journal.pone.0005528. PubMed PMID: 19436760; PubMed Central PMCID: PMCPMC2678250.
202. Harrieff MJ, Burgdorf S, Kurts C, Wiertz EJHJ, Lewinsohn DA, Lewinsohn DM. TAP Mediates Import of *Mycobacterium tuberculosis*-Derived Peptides into Phagosomes and Facilitates Loading onto HLA-I. *PLoS ONE*. 2013;8(11):e79571. doi: 10.1371/journal.pone.0079571.
203. Cho S, Mehra V, Thoma-Uszynski S, Stenger S, Serbina N, Mazzaccaro RJ, et al. Antimicrobial activity of MHC class I-restricted CD8+ T cells in human

- tuberculosis. *Proc Natl Acad Sci U S A*. 2000;97(22):12210-5. doi: 10.1073/pnas.210391497.
204. Mohagheghpour N, Gammon D, Kawamura LM, van Vollenhoven A, Benike CJ, Engleman EG. CTL response to *Mycobacterium tuberculosis*: identification of an immunogenic epitope in the 19-kDa lipoprotein. *J Immunol*. 1998;161(5):2400-6. Epub 1998/09/02. PubMed PMID: 9725236.
205. Bruns H, Meinken C, Schauenberg P, Harter G, Kern P, Modlin RL, et al. Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against *Mycobacterium tuberculosis* in humans. *J Clin Invest*. 2009. doi: 10.1172/JCI38482. PubMed PMID: 19381021; PubMed Central PMCID: PMC2673881.
206. Lewinsohn DM, Briden AL, Reed SG, Grabstein KH, Alderson MR. *Mycobacterium tuberculosis*-reactive CD8+ T lymphocytes: the relative contribution of classical versus nonclassical HLA restriction. *J Immunol*. 2000;165(2):925-30. Epub 2000/07/06. doi: 10.4049/jimmunol.165.2.925. PubMed PMID: 10878367.
207. Grotzke JE, Harriff MJ, Siler AC, Nolt D, Delepine J, Lewinsohn DA, et al. The *Mycobacterium tuberculosis* phagosome is a HLA-I processing competent organelle. *PLoS Pathog*. 2009;5(4):e1000374. Epub 2009/04/11. doi: 10.1371/journal.ppat.1000374. PubMed PMID: 19360129; PubMed Central PMCID: PMC2661020.
208. Lewinsohn DA, Heinzl AS, Gardner JM, Zhu L, Alderson MR, Lewinsohn DM. *Mycobacterium tuberculosis*-specific CD8+ T cells preferentially recognize heavily infected cells. *Am J Respir Crit Care Med*. 2003;168(11):1346-52. Epub 2003/09/13. doi: 10.1164/rccm.200306-837OC. PubMed PMID: 12969871.
209. Harriff MJ, Cansler ME, Toren KG, Canfield ET, Kwak S, Gold MC, et al. Human lung epithelial cells contain *Mycobacterium tuberculosis* in a late endosomal vacuole and are efficiently recognized by CD8(+) T cells. *PLoS One*. 2014;9(5):e97515. Epub 2014/05/16. doi: 10.1371/journal.pone.0097515. PubMed PMID: 24828674; PubMed Central PMCID: PMC4020835.
210. Hwang SA, Actor JK. Lactoferrin modulation of BCG-infected dendritic cell functions. *International Immunology*. 2009;21(10):1185-97. doi: 10.1093/intimm/dxp084.
211. Koh KW, Lehming N, Seah GT. Degradation-resistant protein domains limit host cell processing and immune detection of mycobacteria. *Molecular Immunology*. 2009;46(7):1312-8. doi: 10.1016/j.molimm.2008.11.008.
212. Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med*. 2003;9(8):1039-46. doi: 10.1038/nm906. PubMed PMID: 12872166.
213. Geffner L, Basile JI, Yokobori N, Kviatcovsky D, Sabio y Garcia C, Ritacco V, et al. *Mycobacterium tuberculosis* multidrug resistant strain M induces an altered activation of cytotoxic CD8+ T cells. *PLoS One*. 2014;9(5):e97837. Epub

- 2014/05/20. doi: 10.1371/journal.pone.0097837. PubMed PMID: 24836916; PubMed Central PMCID: PMC4024032.
214. Bhat P, Leggatt G, Matthaei KI, Frazer IH. The kinematics of cytotoxic lymphocytes influence their ability to kill target cells. *PLoS One*. 2014;9(5):e95248. Epub 2014/05/08. doi: 10.1371/journal.pone.0095248. PubMed PMID: 24801876; PubMed Central PMCID: PMC4011687.
215. Smith SM, Malin AS, Pauline T, Lukey, Atkinson SE, Content J, et al. Characterization of human *Mycobacterium bovis* bacille Calmette-Guérin-reactive CD8⁺ T cells. *Infection and Immunity*. 1999;67(10):5223-30.
216. Lewinsohn DM, Zhu L, Madison VJ, Dillon DC, Fling SP, Reed SG, et al. Classically restricted human CD8⁺ T lymphocytes derived from *Mycobacterium tuberculosis*-infected cells: definition of antigenic specificity. *J Immunol*. 2001;166(1):439-46. Epub 2000/12/21. doi: 10.4049/jimmunol.166.1.439. PubMed PMID: 11123322.
217. Ginsberg AM, Ruhwald M, Mearns H, McShane H. TB vaccines in clinical development. *Tuberculosis*. 2016;99:S16-S20. doi: 10.1016/j.tube.2016.05.013.
218. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol*. 1999. doi: 10.1146/annurev.immunol.17.1.51. PubMed PMID: 10358753.
219. Lindestam Arlehamn CS, Lewinsohn D, Sette A, Lewinsohn D. Antigens for CD4 and CD8 T cells in tuberculosis. *Cold Spring Harb Perspect Med*. 2014;4(7):a018465. Epub 2014/05/24. doi: 10.1101/cshperspect.a018465. PubMed PMID: 24852051; PubMed Central PMCID: PMC4066646.
220. Pai M, Riley LW, Colford JM. Interferon- γ assays in the immunodiagnosis of tuberculosis: a systematic review. *The Lancet Infectious Diseases*. 2004;4(12):761-76. doi: 10.1016/s1473-3099(04)01206-x.
221. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *The Lancet*. 2013;381(9871):1021-8. doi: 10.1016/s0140-6736(13)60177-4.
222. Kashangura R, Sena ES, Young T, Garner P. Effects of MVA85A vaccine on tuberculosis challenge in animals: systematic review. *Int J Epidemiol*. 2015;44(6):1970-81. Epub 2015/09/10. doi: 10.1093/ije/dyv142. PubMed PMID: 26351306; PubMed Central PMCID: PMC4689998.
223. Pawlowski A, Jansson M, Skold M, Rottenberg ME, Kallenius G. Tuberculosis and HIV co-infection. *PLoS Pathog*. 2012;8(2):e1002464. Epub 2012/03/01. doi: 10.1371/journal.ppat.1002464. PubMed PMID: 22363214; PubMed Central PMCID: PMC3280977.
224. Serafini A, Pisu D, Palu G, Rodriguez GM, Manganeli R. The ESX-3 secretion system is necessary for iron and zinc homeostasis in *Mycobacterium tuberculosis*. *PLoS One*. 2013;8(10):e78351. Epub 2013/10/25. doi: 10.1371/journal.pone.0078351. PubMed PMID: 24155985; PubMed Central PMCID: PMC3796483.

225. Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS Pathog.* 2011;7(9):e1002251. Epub 2011/10/08. doi: 10.1371/journal.ppat.1002251. PubMed PMID: 21980284; PubMed Central PMCID: PMC3182942.
226. Woodworth JS, Shin D, Volman M, Nunes-Alves C, Fortune SM, Behar SM. Mycobacterium tuberculosis directs immunofocusing of CD8+ T cell responses despite vaccination. *J Immunol.* 2011;186(3):1627-37. Epub 2010/12/24. doi: 10.4049/jimmunol.1002911. PubMed PMID: 21178003; PubMed Central PMCID: PMC3133636.
227. Nunes-Alves C, Booty MG, Carpenter SM, Rothchild AC, Martin CJ, Desjardins D, et al. Human and Murine Clonal CD8+ T Cell Expansions Arise during Tuberculosis Because of TCR Selection. *PLoS Pathog.* 2015;11(5):e1004849. Epub 2015/05/07. doi: 10.1371/journal.ppat.1004849. PubMed PMID: 25945999; PubMed Central PMCID: PMC4422591.
228. Skjot RLV, Brock I, Arend SM, Munk ME, Theisen M, Ottenhoff THM, et al. Epitope Mapping of the Immunodominant Antigen TB10.4 and the Two Homologous Proteins TB10.3 and TB12.9, Which Constitute a Subfamily of the *esat-6* Gene Family. *Infection and Immunity.* 2002;70(10):5446-53. doi: 10.1128/iai.70.10.5446-5453.2002.
229. Skjot RLV, Oettinger T, Rosenkrands I, Ravn P, Brock I, Jacobsen S, et al. Comparative Evaluation of Low-Molecular-Mass Proteins from Mycobacterium tuberculosis Identifies Members of the ESAT-6 Family as Immunodominant T-Cell Antigens. *Infection and Immunity.* 2000;68(1):214-20. doi: 10.1128/iai.68.1.214-220.2000.
230. Lewinsohn DA, Swarbrick GM, Park B, Cansler ME, Null MD, Toren KG, et al. Comprehensive definition of human immunodominant CD8 antigens in tuberculosis. *NPJ Vaccines.* 2017;2(1). doi: 10.1038/s41541-017-0008-6. PubMed PMID: 28775896; PubMed Central PMCID: PMC5538316.
231. Carpenter SM, Nunes-Alves C, Booty MG, Way SS, Behar SM. A Higher Activation Threshold of Memory CD8+ T Cells Has a Fitness Cost That Is Modified by TCR Affinity during Tuberculosis. *PLoS Pathog.* 2016;12(1):e1005380. Epub 2016/01/09. doi: 10.1371/journal.ppat.1005380. PubMed PMID: 26745507; PubMed Central PMCID: PMC4706326.
232. Tamura T, Ariga H, Kinashi T, Uehara S, Kikuchi T, Nakada M, et al. The role of antigenic peptide in CD4+ T helper phenotype development in a T cell receptor transgenic model. *Int Immunol.* 2004;16(12):1691-9. Epub 2004/10/13. doi: 10.1093/intimm/dxh170. PubMed PMID: 15477229.
233. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med.* 2011;208(6):1279-89. Epub 2011/05/25. doi: 10.1084/jem.20110308. PubMed PMID: 21606508; PubMed Central PMCID: PMC3173240.

234. Mariani F, Cappelli G, Riccardi G, Colizzi V. Mycobacterium tuberculosis H37Rv comparative gene-expression analysis in synthetic medium and human macrophage. *Gene*. 2000. PubMed PMID: 10940566.
235. Repasy T, Lee J, Marino S, Martinez N, Kirschner DE, Hendricks G, et al. Intracellular bacillary burden reflects a burst size for Mycobacterium tuberculosis in vivo. *PLoS Pathog*. 2013;9(2):e1003190. Epub 2013/02/26. doi: 10.1371/journal.ppat.1003190. PubMed PMID: 23436998; PubMed Central PMCID: PMC3578792.
236. Srivastava S, Ernst JD, Desvignes L. Beyond macrophages: the diversity of mononuclear cells in tuberculosis. *Immunological Reviews*. 2014;262(1):179-92. doi: 10.1111/imr.12217.
237. Skold M, Behar SM. Tuberculosis Triggers a Tissue-Dependent Program of Differentiation and Acquisition of Effector Functions by Circulating Monocytes. *The Journal of Immunology*. 2008;181(9):6349-60. doi: 10.4049/jimmunol.181.9.6349.
238. Gonzalez-Juarrero M, Shim TS, Kipnis A, Junqueira-Kipnis AP, Orme IM. Dynamics of Macrophage Cell Populations During Murine Pulmonary Tuberculosis. *The Journal of Immunology*. 2003;171(6):3128-35. doi: 10.4049/jimmunol.171.6.3128.
239. Gallegos AM, Pamer EG, Glickman MS. Delayed protection by ESAT-6-specific effector CD4+ T cells after airborne M. tuberculosis infection. *J Exp Med*. 2008;205(10):2359-68. Epub 2008/09/10. doi: 10.1084/jem.20080353. PubMed PMID: 18779346; PubMed Central PMCID: PMC2556792.
240. Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, et al. Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity*. 2006;24(1):105-17. Epub 2006/01/18. doi: 10.1016/j.immuni.2005.12.001. PubMed PMID: 16413927.
241. Smith VL, Cheng Y, Bryant BR, Schorey JS. Exosomes function in antigen presentation during an in vivo Mycobacterium tuberculosis infection. *Sci Rep*. 2017;7:43578. Epub 2017/03/07. doi: 10.1038/srep43578. PubMed PMID: 28262829; PubMed Central PMCID: PMC5338015.
242. Srivastava S, Ernst JD. Cell-to-cell transfer of M. tuberculosis antigens optimizes CD4 T cell priming. *Cell Host Microbe*. 2014;15(6):741-52. Epub 2014/06/13. doi: 10.1016/j.chom.2014.05.007. PubMed PMID: 24922576; PubMed Central PMCID: PMC4098643.
243. Jayaraman P, Sada-Ovalle I, Nishimura T, Anderson AC, Kuchroo VK, Remold HG, et al. IL-1beta promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J Immunol*. 2013;190(8):4196-204. Epub 2013/03/15. doi: 10.4049/jimmunol.1202688. PubMed PMID: 23487424; PubMed Central PMCID: PMC3622150.
244. Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity*. 2008;28(2):271-84. Epub 2008/02/12. doi:

- 10.1016/j.immuni.2007.12.010. PubMed PMID: 18261937; PubMed Central PMCID: PMC2390753.
245. Nepal RM, Vesosky B, Turner J, Bryant P. DM, but not cathepsin L, is required to control an aerosol infection with *Mycobacterium tuberculosis*. *Journal of Leukocyte Biology*. 2008;84(4):1011-8. doi: 10.1189/jlb.1207852.
246. Caccamo N, Pietra G, Sullivan LC, Brooks AG, Prezzemolo T, La Manna MP, et al. Human CD8 T lymphocytes recognize *Mycobacterium tuberculosis* antigens presented by HLA-E during active tuberculosis and express type 2 cytokines. *Eur J Immunol*. 2015;45(4):1069-81. doi: 10.1002/eji.201445193. PubMed PMID: 25631937.
247. D'Souza S, Rosseels V, Romano M, Tanghe A, Denis O, Jurion F, et al. Mapping of Murine Th1 Helper T-Cell Epitopes of Mycolyl Transferases Ag85A, Ag85B, and Ag85C from *Mycobacterium tuberculosis*. *Infection and Immunity*. 2003;71(1):483-93. doi: 10.1128/iai.71.1.483-493.2003.
248. Meng L, Tong J, Wang H, Tao C, Wang Q, Niu C, et al. PPE38 Protein of *Mycobacterium tuberculosis* Inhibits Macrophage MHC Class I Expression and Dampens CD8+ T Cell Responses. *Front Cell Infect Microbiol*. 2017;7:68. Epub 2017/03/30. doi: 10.3389/fcimb.2017.00068. PubMed PMID: 28348981; PubMed Central PMCID: PMC5346565.
249. Rock KL, Rothstein L, Gamble S. Generation of class I MHC-restricted T-T hybridomas. *The Journal of Immunology*. 1990;145(3):804-11.
250. Ilghari D, Lightbody KL, Veverka V, Waters LC, Muskett FW, Renshaw PS, et al. Solution structure of the *Mycobacterium tuberculosis* EsxG.EsxH complex: functional implications and comparisons with other *M. tuberculosis* Esx family complexes. *J Biol Chem*. 2011;286(34):29993-30002. Epub 2011/07/07. doi: 10.1074/jbc.M111.248732. PubMed PMID: 21730061; PubMed Central PMCID: PMC3191040.
251. Flesch I, Kaufmann SH. *Mycobacterial* growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. *J Immunol*. 1987;138(12):4408-13. PubMed PMID: 3108389.
252. Flynn JL. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *Journal of Experimental Medicine*. 1993;178(6):2249-54. doi: 10.1084/jem.178.6.2249.
253. Chan J. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. *Journal of Experimental Medicine*. 1992;175(4):1111-22. doi: 10.1084/jem.175.4.1111.
254. Lee J, Kornfeld H. Interferon-gamma Regulates the Death of *M. tuberculosis*-Infected Macrophages. *J Cell Death*. 2010. PubMed PMID: 21072140; PubMed Central PMCID: PMC2975577.
255. Montoya D, Inkeles MS, Liu PT, Realegeno S, Teles RM, Vaidya P, et al. IL-32 is a molecular marker of a host defense network in human tuberculosis. *Sci Transl Med*. 2014;6(250):250ra114. Epub 2014/08/22. doi:

- 10.1126/scitranslmed.3009546. PubMed PMID: 25143364; PubMed Central PMCID: PMC4175914.
256. Bai X, Shang S, Henao-Tamayo M, Basaraba RJ, Ovrutsky AR, Matsuda JL, et al. Human IL-32 expression protects mice against a hypervirulent strain of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2015;112(16):5111-6. Epub 2015/03/31. doi: 10.1073/pnas.1424302112. PubMed PMID: 25820174; PubMed Central PMCID: PMC4413346.
257. Bai X, Kim SH, Azam T, McGibney MT, Huang H, Dinarello CA, et al. IL-32 is a host protective cytokine against *Mycobacterium tuberculosis* in differentiated THP-1 human macrophages. *J Immunol*. 2010;184(7):3830-40. Epub 2010/03/02. doi: 10.4049/jimmunol.0901913. PubMed PMID: 20190143.
258. van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, et al. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell*. 2007;129(7):1287-98. Epub 2007/07/03. doi: 10.1016/j.cell.2007.05.059. PubMed PMID: 17604718.
259. Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, Brosch R, et al. Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. *PLoS Pathog*. 2012;8(2):e1002507. Epub 2012/02/10. doi: 10.1371/journal.ppat.1002507. PubMed PMID: 22319448; PubMed Central PMCID: PMC3271072.
260. Lindenstrom T, Aagaard C, Christensen D, Agger EM, Andersen P. High-frequency vaccine-induced CD8(+) T cells specific for an epitope naturally processed during infection with *Mycobacterium tuberculosis* do not confer protection. *Eur J Immunol*. 2014. doi: 10.1002/eji.201344358. PubMed PMID: 24677089; PubMed Central PMCID: PMC4112357.
261. Grotzke JE, Siler AC, Lewinsohn DA, Lewinsohn DM. Secreted immunodominant *Mycobacterium tuberculosis* antigens are processed by the cytosolic pathway. *J Immunol*. 2010;185(7):4336-43. Epub 2010/08/31. doi: 10.4049/jimmunol.1000801. PubMed PMID: 20802151; PubMed Central PMCID: PMC2988655.
262. Woodworth JS, Fortune SM, Behar SM. Bacterial protein secretion is required for priming of CD8+ T cells specific for the *Mycobacterium tuberculosis* antigen CFP10. *Infect Immun*. 2008;76(9):4199-205. Epub 2008/07/02. doi: 10.1128/IAI.00307-08. PubMed PMID: 18591224; PubMed Central PMCID: PMC2519428.
263. Smith J, Manoranjan J, Pan M, Bohsali A, Xu J, Liu J, et al. Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in *Mycobacterium marinum* escape from the vacuole. *Infect Immun*. 2008;76(12):5478-87. Epub 2008/10/15. doi: 10.1128/IAI.00614-08. PubMed PMID: 18852239; PubMed Central PMCID: PMC2583575.
264. Gudmundsdottir H, Wells AD, Turka LA. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J Immunol*. 1999;162(9):5212-23. PubMed PMID: 10227995.

265. Jayaraman P, Jacques MK, Zhu C, Steblenko KM, Stowell BL, Madi A, et al. TIM3 Mediates T Cell Exhaustion during Mycobacterium tuberculosis Infection. *PLoS Pathogens*. 2016;12(3):e1005490. doi: 10.1371/journal.ppat.1005490.
266. Peddireddy V, Doddam SN, Ahmed N. Mycobacterial Dormancy Systems and Host Responses in Tuberculosis. *Front Immunol*. 2017;8(Pt 12):84. Epub 2017/03/07. doi: 10.3389/fimmu.2017.00084. PubMed PMID: 28261197; PubMed Central PMCID: PMC5309233.
267. Medina, North. Resistance ranking of some common inbred mouse strains to Mycobacterium tuberculosis and relationship to major histocompatibility complex haplotype and Nramp1 genotype. *Immunology*. 2001;93(2):270-4. doi: 10.1046/j.1365-2567.1998.00419.x.
268. Smith CM, Proulx MK, Olive AJ, Laddy D, Mishra BB, Moss C, et al. Tuberculosis Susceptibility and Vaccine Protection Are Independently Controlled by Host Genotype. *MBio*. 2016;7(5):e01516-16. Epub 2016/09/22. doi: 10.1128/mBio.01516-16. PubMed PMID: 27651361; PubMed Central PMCID: PMC5030360.
269. Winau F, Kaufmann SH, Schaible UE. Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria. *Cell Microbiol*. 2004;6(7):599-607. Epub 2004/06/10. doi: 10.1111/j.1462-5822.2004.00408.x. PubMed PMID: 15186397.
270. Ngai P, McCormick S, Small C, Zhang X, Zganiacz A, Aoki N, et al. Gamma interferon responses of CD4 and CD8 T-cell subsets are quantitatively different and independent of each other during pulmonary Mycobacterium bovis BCG infection. *Infect Immun*. 2007;75(5):2244-52. Epub 2007/02/20. doi: 10.1128/IAI.00024-07. PubMed PMID: 17307945; PubMed Central PMCID: PMC1865770.
271. Houben D, Demangel C, van Ingen J, Perez J, Baldeon L, Abdallah AM, et al. ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. *Cell Microbiol*. 2012;14(8):1287-98. Epub 2012/04/25. doi: 10.1111/j.1462-5822.2012.01799.x. PubMed PMID: 22524898.
272. Conrad WH, Osman MM, Shanahan JK, Chu F, Takaki KK, Cameron J, et al. Mycobacterial ESX-1 secretion system mediates host cell lysis through bacterium contact-dependent gross membrane disruptions. *Proc Natl Acad Sci U S A*. 2017;114(6):1371-6. Epub 2017/01/26. doi: 10.1073/pnas.1620133114. PubMed PMID: 28119503; PubMed Central PMCID: PMC5307465.
273. Simeone R, Sayes F, Song O, Groschel MI, Brodin P, Brosch R, et al. Cytosolic access of Mycobacterium tuberculosis: critical impact of phagosomal acidification control and demonstration of occurrence in vivo. *PLoS Pathog*. 2015;11(2):e1004650. Epub 2015/02/07. doi: 10.1371/journal.ppat.1004650. PubMed PMID: 25658322; PubMed Central PMCID: PMC4450080.
274. Quigley J, Hughitt VK, Velikovsky CA, Mariuzza RA, El-Sayed NM, Briken V. The Cell Wall Lipid PDIM Contributes to Phagosomal Escape and Host Cell Exit of Mycobacterium tuberculosis. *MBio*. 2017;8(2):e00148-17. Epub

- 2017/03/09. doi: 10.1128/mBio.00148-17. PubMed PMID: 28270579; PubMed Central PMCID: PMC5340868.
275. Mehra A, Zahra A, Thompson V, Sirisaengtaksin N, Wells A, Porto M, et al. Mycobacterium tuberculosis type VII secreted effector EsxH targets host ESCRT to impair trafficking. PLoS Pathog. 2013;9(10):e1003734. Epub 2013/11/10. doi: 10.1371/journal.ppat.1003734. PubMed PMID: 24204276; PubMed Central PMCID: PMC3814348.
276. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, et al. Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest. 2005;115(9):2472-9. Epub 2005/08/20. doi: 10.1172/JCI24617. PubMed PMID: 16110326; PubMed Central PMCID: PMC1187936.
277. Verherstraeten S, Goossens E, Valgaeren B, Pardon B, Timbermont L, Haesebrouck F, et al. Perfringolysin O: The Underrated Clostridium perfringens Toxin? Toxins (Basel). 2015;7(5):1702-21. Epub 2015/05/27. doi: 10.3390/toxins7051702. PubMed PMID: 26008232; PubMed Central PMCID: PMC4448169.
278. Sun R, Skeiky YA, Izzo A, Dheenadhayalan V, Imam Z, Penn E, et al. Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with Mycobacterium tuberculosis. Vaccine. 2009;27(33):4412-23. Epub 2009/06/09. doi: 10.1016/j.vaccine.2009.05.048. PubMed PMID: 19500523.
279. Sassetti CM, Boyd DH, Rubin EJ. Genes required for mycobacterial growth defined by high density mutagenesis. Molecular Microbiology. 2003;48(1):77-84. Epub 2003/03/27. PubMed PMID: 12657046.
280. Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A. 2003;100(22):12989-94. doi: 10.1073/pnas.2134250100.
281. Smittipat N, Billamas P, Palittapongarnpim M, Thong-On A, Temu MM, Thanakijcharoen P, et al. Polymorphism of variable-number tandem repeats at multiple loci in Mycobacterium tuberculosis. J Clin Microbiol. 2005;43(10):5034-43. Epub 2005/10/07. doi: 10.1128/JCM.43.10.5034-5043.2005. PubMed PMID: 16207958; PubMed Central PMCID: PMC1248453.
282. Subbramanian RA, Moriya C, Martin KL, Peyerl FW, Hasegawa A, Naoi A, et al. Engineered T-cell receptor tetramers bind MHC-peptide complexes with high affinity. Nature biotechnology. 2004;22(11):1429-34. doi: 10.1038/nbt1024.
283. Huang Y, Huang Y, Fang Y, Wang J, Li Y, Wang N, et al. Relatively Low Level of Antigen-specific Monocytes Detected in Blood from Untreated Tuberculosis Patients Using CD4+ T-cell Receptor Tetramers. PLoS Pathogens. 2012;8(11):e1003036. doi: 10.1371/journal.ppat.1003036.
284. Kamath A, Woodworth JS, Behar SM. Antigen-specific CD8+ T cells and the development of central memory during Mycobacterium tuberculosis infection. J Immunol. 2006;177(9):6361-9. Epub 2006/10/24. doi:

10.4049/jimmunol.177.9.6361. PubMed PMID: 17056567; PubMed Central PMCID: PMC3133654.

285. Elvang T, Christensen JP, Billeskov R, Thi Kim Thanh Hoang T, Holst P, Thomsen AR, et al. CD4 and CD8 T cell responses to the M. tuberculosis Ag85B-TB10.4 promoted by adjuvanted subunit, adenovector or heterologous prime boost vaccination. *PLoS One*. 2009;4(4):e5139. Epub 2009/04/10. doi: 10.1371/journal.pone.0005139. PubMed PMID: 19357780; PubMed Central PMCID: PMC2663846.

286. Billeskov R, Vingsbo-Lundberg C, Andersen P, Dietrich J. Induction of CD8 T cells against a novel epitope in TB10.4: correlation with mycobacterial virulence and the presence of a functional region of difference-1. *J Immunol*. 2007;179(6):3973-81. Epub 2007/09/06. doi: 10.4049/jimmunol.179.6.3973. PubMed PMID: 17785835.

287. Weinrich Olsen A, van Pinxteren LA, Meng Okkels L, Birk Rasmussen P, Andersen P. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85b and esat-6. *Infect Immun*. 2001;69(5):2773-8. Epub 2001/04/09. doi: 10.1128/IAI.69.5.2773-2778.2001. PubMed PMID: 11292688; PubMed Central PMCID: PMC98224.

288. Brandt L, Elhay M, Rosenkrands I, Lindblad EB, Andersen P. ESAT-6 subunit vaccination against Mycobacterium tuberculosis. *Infect Immun*. 2000. PubMed PMID: 10639447; PubMed Central PMCID: PMC97206.

289. Lee J, Martinez N, West K, Kornfeld H. Differential adjuvant activities of TLR7 and TLR9 agonists inversely correlate with nitric oxide and PGE2 production. *PLoS One*. 2015;10(4):e0123165. Epub 2015/04/16. doi: 10.1371/journal.pone.0123165. PubMed PMID: 25875128; PubMed Central PMCID: PMC4395302.

290. Rock JM, Hopkins FF, Chavez A, Diallo M, Chase MR, Gerrick ER, et al. Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nature Microbiology*. 2017;2:16274. doi: 10.1038/nmicrobiol.2016.274.