THE ROLE OF CD4 T CELL HELP IN EFFECTIVE CD8 T CELL RESPONSES DURING *MYCOBACTERIUM TUBERCULOSIS* INFECTION

A Dissertation Presented

By

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Abstract

Tuberculosis (TB), a transmissible disease caused by *Mycobacterium tuberculosis* (Mtb), is a global health threat. To design an effective vaccine, we need to better understand how different elements of our immune system collaborate to fight against Mtb. CD4 T cells are crucial in protective immunity to Mtb because they produce cytokines including interferon-γ. In contrast, CD8 T cells are thought to play a modest role. Whether CD4 T cells act as "helper" cells to promote optimal CD8 T cell responses during TB is unknown. We argue CD8 T cells' role are likely underestimated because CD8 T cell functions are compromised without CD4 T cells. Here, using two independent models, I show that CD4 T cell help promotes CD8 T cell effector functions and prevents CD8 T cell exhaustion. I demonstrate CD4 and CD8 T cells synergistically enhance the survival of infected mice. Purified helped, but not helpless, CD8 T cells effectively restrict intracellular Mtb growth. Thus, CD4 T cell help is indispensable for generating protective CD8 T cell responses. In addition, I investigate the mechanisms of CD4 T cell help. Signals from CD4 T cells, and signals relayed by antigen presenting cells collectively shape CD8 T cell responses. We infer that vaccines aimed for eliciting both CD4 and CD8 T cells, in which CD8 T cells are properly helped by CD4 T cells, are more likely to be successful.

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CHAPTER I: INTRODUCTION

Global Tuberculosis Burden

Tuberculosis (TB), caused by pathogen *Mycobacterium tuberculosis* (Mtb), is a transmissible disease. It's estimated that a quarter of global population is latently infected [1]. In 2019, about 10 million people worldwide fell ill with TB, and cases occurring in South-East Asia and Africa accounted for 70% of the new cases [1]. TB was the leading cause of death from a single infectious agent before the start of the COVID-19 pandemic. It claimed 1.4 million deaths in 2019, and the number is estimated to increase in 2020 because of health service disruption during COVID-19 pandemic [1].

TB is transmitted via aerosol droplets that contain Mtb. Once patients with pulmonary TB cough, sneeze, or shout, these infectious droplets are expelled, and inhaled by exposed individuals to establish infection. Most infected people don't develop symptoms, a stage called latent tuberculosis infection (LTBI) [2-4]. It is characterized by a contained infection, and it's not transmissible during this state. However, 10% of infected people will progress into active, symptomatic disease, and experience chronic cough, coughing with bloody mucus, weight loss, and fatigue. Active TB disease can transmit among people. TB mostly affects the lungs, although extrapulmonary TB can occur [5, 6]. Risk factors to develop active TB include HIV infection, diabetes, immunocompromised conditions, and

undernutrition. The first two years after initial infection has the highest likelihood of progressing to active disease [7].

Despite all the resources and efforts that have worked to put an end to the TB epidemic, we have yet to succeed. The World Health Organization (WHO) developed a strategy to reduce 90% of new cases and 95% of TB deaths by 2035 [1]. TB incidence rates has indeed slowly decreased each year, but the decrease is not enough to achieve the set goal [1]. Additionally, the reduction in TB deaths is even slower. This perhaps reflects the challenge of TB treatment. Although TB is a curable disease, only about 85% of people who develop TB can be successfully treated, and it takes months to complete the medicine regimen. Depending on the individual case, the regimen could take six to nine months with four different antibiotics: Isoniazid, Rifampin, Ethambutol, and Pyrazinamide. Patient non-adherence to the regimen has been the major difficulty to treating TB. Moreover, the emergence of multidrug-resistant tuberculosis (MDR-TB) further complicates the situation. In fact, the cases of MDR-TB increase each year and have become a public health crisis in itself [1].

Prophylactic vaccination is an alternative strategy to reduce global TB burden and combat the challenge of treatment. There is one approved vaccine for TB, Bacille Calmette-Guerin (BCG), an attenuated strain derived from *Mycobacterium bovis*. BCG has been introduced almost a century ago, and it's the most widely used vaccine worldwide, but it seems to have minimal effect on reducing the global TB burden. Indeed, the efficacy of BCG is controversial.

Although BCG consistently confers great protection in infants and toddlers to prevent severe meningeal TB and miliary TB, its efficacy against adult pulmonary disease varies among populations [8]. It's thought that prior sensitization with other Mycobacteria, which often occurs in people living in low latitude areas, and the heterogeneity of BCG strains used in vaccination attributes to the inconsistent efficacy [9-11]. Thus, a more effective vaccine is needed to halt the TB pandemic. Researchers didn't hesitate in searching for alternative vaccines, and in fact, several candidates are currently in clinical trials [12]. However, we've yet to have a vaccine that can replace BCG. The biggest hurdle remains the incomplete understanding of protective immunity during both primary infection and vaccine induced protection [13]. A better delineation of protective immune responses to Mtb is essential to facilitate vaccine development.

Immunity to Tuberculosis

Early events

Mtb travels with aerosol droplets and accesses distal lung alveoli, where it establishes infection. Like during all the other infections, innate immune cells are the first line of defense against Mtb. Despite the early innate immune responses, Mtb burden increases rapidly in lung during the first few weeks after infection, an observation from experimental animal models, suggesting these cells are unable to control infection on their own. Nevertheless, these early events occurred before

adaptive immune responses have been shown to enormously impact the pathogenesis of TB. Mtb first infects and replicates in alveolar macrophages (AMs) in lung alveoli, and then spreads to other resident and recruited myeloid cells including macrophages, dendritic cells (DCs), and neutrophils. Not until recently did we start to appreciate the heterogenicity of myeloid cells in infected lungs and the complex responses they have to Mtb. Using multi-color flow cytometry and the fixable fluorochrome Mtb, several groups studied the distribution of Mtb in myeloid cells early during infection [14-16]. It is now clear that some populations harbor more Mtb compared to others. Depending on the dose, route, and timing of infection, and the flow cytometry panel used to distinguish myeloid cells, $CD11c^{hi}$ monocyte derived cells, neutrophils, and $CD64^{+}Mertk^{+}$ interstitial macrophages are reportedly the major infected cells [14-16]. This unequal distribution of Mtb supports the idea that Mtb establishes a niche for its replication. Whether the niches form because certain cells are intrinsically more permissive or/and they are less responsive to adaptive immunity remains unknown.

The established infection then leads to Mtb dissemination, through infected DCs or extracellular Mtb, to lung draining mediastinal lymph nodes (MLN), which initiate T cell responses. T cell priming occurs 8-9 days after infection. Once Mtb is found in MLN, Mtb-specific IFNγ responses can be detected [17]. Naïve T cell activation, measured by proliferation and CD69 expression, is also detectable in MLN [18, 19]. The activated effector cells then migrate toward the lung, which

occurs 15-18 days after infection, and finally reach the lung with sufficient effective responses to stop the rapid replication of Mtb 18-20 days after infection.

The delayed T cell priming and arrival to lungs contributes to the establishment of Mtb infection. This is supported by the observation that dissemination occurs earlier in resistant C57BL/6 mice, generating faster Mtb-specific responses, than in susceptible C3H mice [17]. Why T cell priming is delayed is not entirely clear. This may be because of the slow growing nature of Mtb, which doubles after/in about 24 hours *in vitro*, leading to slow dissemination and delayed priming. However, even with high dose infection, the dissemination is only accelerated for 1-2 days [18]. It's thought that the poor migratory ability of DCs residing in lung alveoli, and the more tolerogenic environment contribute to the slow dissemination [20].

Despite arriving late, T cells contain Mtb replication and the bacterial loads in lungs reach a stationary phase. This restriction is associated with T cells, along with recruited B cells and myeloid cells, to "wall off" Mtb by forming a unique structure called granuloma, the hallmark of TB. The granuloma is an aggregate of organized immune cells, which has a necrotic center composed of dead and dying macrophages containing Mtb. Surrounding cells of this necrotic center are transformed cells of macrophages, including foam cells, multinucleated giant cells, and epithelioid cells. T cells, B cells, and DCs are at the outer edge. This compact structure creates a harsh environment that is low of oxygen and nutrients for the Mtb inside. However, being a pathogen that coevolved with humans for thousands

of years, Mtb can enter a “dormancy” state in response [21]. Although it normally replicates slowly, Mtb can even switch to a non-replicating and metabolic stable phase to persist in the host. Therefore, a chronic infection is established, in which T cells can effectively contain, but cannot eliminate persistent Mtb.

The role of CD4 T cells in immunity to Mtb

CD4 T cells’ crucial role in immunity to TB has been established back in the 1980s. T cells, but not antibodies, are found to mediate protection against Mtb, and these cells are then identified as CD4 T cells [22, 23]. This is supported by later findings that mice deficient in CD4 T cells succumb early after infection [24], and an “experiment of nature” that AIDS patients have higher risk of developing active tuberculosis [25]. Moreover, the ability to produce IFNγ is thought to be the mechanism of CD4 T cell mediated protection. IFNγ activates infected macrophages. Activated macrophages then trigger several antimicrobial pathways, including inducible nitric oxide synthase (iNOS), IFNγ-inducible GTPases, phagosome maturation and acidification, autophagy, and vitamin D receptor signaling [26-31]. Indeed, IFNγ deficient mice are extremely susceptible to infection [32, 33]. Therefore, CD4 T cells producing IFNγ and the consequential activation of macrophages is the “central dogma” of protective immunity to Mtb [4].

Because of the strong correlation between IFNγ producing CD4 T cells and protection, vaccines are designed to generate strong $IFN\gamma^{+}$ CD4 T cell responses (i.e., Th1 responses) [34, 35]. However, MVA85A, a vaccine that produced durable

Th1 responses, failed to enhance protection in the BCG-vaccinated population [36]. Recent studies in animal models reveal that IFNγ producing CD4 T cells, although they are necessary, are not sufficient for protection. In mice, IFNγ has modest effect on controlling Mtb burden in the lung, despite it mediating great control in the spleen [37]. Increased ability of IFNγ production by CD4 T cells exacerbates lung infection and leads to early mortality of mice [37]. This “required”, but not “sufficient” role of IFNγ producing CD4 T cells perhaps explains the conflicted results in searching for immune correlates of protection after BCG vaccination in humans. While some studies indicate BCG induced IFNγ production correlates with protection [38], other studies don’t find association between Th1 cytokine producing T cells and the reduced risk for TB [35, 36, 39].

It’s clear that explanations beyond the narrow focus on IFNγ producing CD4 T cells are necessary to understand protective immunity, and to identify new biomarkers needed for vaccine development. Therefore, other subsets of CD4 T cells and their contribution to protection have been studied. Th17 cells can inhibit bacterial growth when adoptively transferred into mice [40]. Th17 cells are associated with vaccine induced protection [41-44]. They populate lungs after vaccination and enhance the recruitment of IFNγ producing CD4 T cells upon challenge [45]. In addition, T helper cells expressing both Th1 and Th17 features, the Th1* or ex-Th17 cells, are found during Mtb infection [46-48]. Th1* cells are $IFN\gamma^{+}CXCR3^{+}CCR6^{+}$ cells that co-expressing T-bet and RORgt [49], whereas ex-Th17 cells are tissue resident memory cells that derived from previous IL-17

producing cells during infection [50]. The $CXCR3^{+}CCR6^{+}CCR4^{-}$ cells are found in peripheral blood of latently infected patient, suggesting a protective role during TB [47, 48]. Moreover, CD4 T cells that produced multiple cytokines, polyfunctional CD4 T cells, are shown to be protective. In a mucosal BCG study in non-human primate (NHP), the induction of polyfunctional CD4 T cells producing IFNγ, TNFa, IL-2, and IL-17 are associated with protection [51]. Interestingly, MVA85A induced CD4 T cells producing IFNγ, TNFa, and IL-2, but this vaccine didn't confer protection [36]. The search for new biomarkers that correlated with protection remains a direction for future study.

The role of CD8 T cells in immunity to Mtb

The essential role of CD8 T cells

CD8 T cells are recognized as an integral part of immunity to Mtb. Mtb specific CD8 T cells are detected in people with prior BCG vaccination and LTBI, and even patients with active disease [52-55]. Early studies derived CD8 T cell lines from these samples for functional studies and showed specific CD8 T cell responses are generated. Nevertheless, the first evidence that CD8 T cells contribute to protective immunity to Mtb came from murine models. I.M. Orme in the 1980s showed lymphocytes from BCG-immunized mice were able to transfer protection to immunodeficient mice [56]. Using this approach, he further examined CD8 T cells' role with fractionized lymphocytes by depleting CD4 or CD8 T cells before transfer [57, 58]. Adoptively transferred CD8 T cells can mediate protection

in lungs and spleens in immunodeficient mice against subsequent infection [57-60]. Antibody mediated depletion of CD8 T cells *in vivo* is another approach to determine whether CD8 T cells transfer protection. Indeed, mice depleted of CD8 T cells have higher bacterial burden in spleens after iv infection [61]. Although CD8 T cells are proved to play a role in immunity to Mtb, the magnitude of protection they mediate varies between models where different strains and routes of infection were used [61-63].

The use of knockout (KO) mice in the 1990s confirms the indispensable role of CD8 T cells in response to Mtb. By disruption of the expression of MHCI molecules on cell surface, and disabling the development of MHCI-restricted CD8 T cells, β2-microglobulin (β2m), transporter associated with antigen processing-1 (TAP-1), and MHCI heavy chain (K^bD^b) KO mice were used to determine the role of CD8 T cells [64-66]. These mice die prematurely after Mtb infection, highlighting the role of CD8 T cells. Interestingly, the susceptibilities of these mice to Mtb are different. The β2m KO mice are the most susceptible and they succumb rapidly after iv infection [66]. Although suggesting the role of CD8 T cells, the susceptibility of β2m KO mice is complicated by the involvement of β2m in the expression of CD1 and class Ib MHC molecules, which also presents Mtb antigens. To test whether the absence of CD1-restricted, or MHCI-restricted CD8 T cells contribute to the susceptibility of β2m KO mice, Behar and colleagues compared the susceptibility of CD1 KO mice to TAP-1 KO mice, and found TAP-1 KO mice, but

not CD1d KO mice had impaired resistance to Mtb [64]. This result again evidenced the crucial role of MHCI-restricted CD8 T cells.

Although it's clear that CD8 T cells play a role in protective immunity to Mtb, their relative importance compared to CD4 T cells is questioned. In the adoptive transfer models, where CD8 T cells mediate various magnitudes of protection in spleens of immunodeficient mice, CD4 T cells consistently confer significant protection in both lungs and spleens of recipient mice [61-63]. In a well-controlled study performed by Mogues and colleagues, mice depleted of CD4 T cells succumbed soon after low dose aerosol infection, while mice depleted of CD8 T cells survived much longer than mice deficient in CD4 T cells [24]. However, CD4 T cells have shown to be necessary for optimal CD8 responses in many infections and cancers; what is called CD4 T cell help [67, 68]. CD8 T cells' relative contribution could be underestimated in the models in which they were assayed alone, because CD8 T cells' function could be compromised without CD4 T cells. This part is addressed in later sections.

The lack of evidence in humans for the requirement of CD8 T cells in immunity to TB makes the contention even more complicated. The higher risk of primary and reactivated TB in AIDS patient strongly argues the importance of CD4 T cells [69-71], while no similar situation of humans specifically deficient in CD8 T cells exposed to TB to provide the argument for CD8 T cells. Non-human primates (NHP) are thought to be more relevant models for TB study because NHP capture a diverse spectrum of disease (i.e., active and latent tuberculosis), and form

granulomas that are not typically found in murine models. NHP also share immune features with humans that mice or rodents don't have, including orthologues of genes for group 1 CD1 antigen presenting molecules and cytotoxic protein granulysin [72, 73]. These features make NHP strong candidates to study CD8 T cells. Indeed, cytokine producing CD8 T cells are found within granulomas in NHP [74].

Recent data from NHP emphasized the crucial role of CD8 T cells in vaccine induced protection and primary infection [75-78]. The strongest evidence came from Chen and colleagues' study in which they conducted antibody mediated depletion of CD8 T cells in BCG vaccinated rhesus macaques. BCG induced protection was largely compromised in anti-CD8 treated, BCG immunized macaques [78]. In addition, several vaccine strategies induce strong memory CD8 T cell responses, including intravenous administration of BCG (iv BCG) [76], CMV vectors expressing TB antigen [77], and aerosolized MtbΔsigH [79]. These vaccines confer significant protection to challenge in rhesus macaques. Although it's hard to establish the immune correlates of protection from these studies, these results shed light on the role of CD8 T cells in vaccine mediated protection. Fewer studies address CD8 T cells' role in primary infection in NHP models. Nevertheless, Gideon and Hughes recently identified protective features by comparing cellular composition in restrictive vs. permissive granulomas using scRNA-seq. In this study, CD8 T cells with cytotoxic features were correlated with restriction of Mtb

growth in granulomas. This study indicated that CD8 T cells are also essential in primary infection [80].

CD8 T cell effector functions

Despite evidence showing that CD8 T cells play a vital/significant role in protective immunity to Mtb, how CD8 T cells mediate protection is not clear. CD8 T cells can make cytokines that are known to contribute to immunity against Mtb, including IFNγ, TNF, IL-17, and IL-2. However, CD4 T cells can also secrete these cytokines, and are the main producers in most cases. This raises a question whether CD8 T cells have a unique role during Mtb infection. Cytotoxicity function is thought to be a distinct function of CD8 T cells in many viral infections and cancer, but it hasn't been appreciated in Mtb infection until recently [75].

IFNγ has a well-established role in host resistance to Mtb. IFNγ and IFNγR KO mice are extremely susceptible in Mtb infection [32, 33]. CD8 T cells derived from infected experimental animals and TB patients produce IFNγ *in vitro* [81-84]. In fact, IFNγ secretion by CD8 T cells is regularly used as a readout for screening the responses to Mtb antigens, including the use of interferon-gamma release assay (IGRAs) for LTBI diagnosis. Using a direct intracellular staining technique, Bold and colleagues assayed IFNγ *ex vivo* without stimulation, and showed CD8 T cells were a major producer of IFNγ in the lungs of Mtb infected mice, with IFNγ production second only to CD4 T cells [85]. Similarly, Caruso and colleagues showed the amount of IFNγ in lungs only transiently decreased in CD4 KO mice,

and CD8 T cells were the producers of IFNγ in these mice [86]. Interestingly, two other studies found that CD8 T cells' ability to produce IFNγ depended on CD4 T cells [85, 87]. Whether IFNγ produced by CD8 T cells contributes to their protection against Mtb is a harder question to answer. Tascon and colleagues found adoptively transferred WT CD8 T cells, but not IFNγ KO CD8 T cells protected nude mice from Mtb infection [59]. However, whether IFNγ production is required for CD8 T cell transferred protection in intact mice remains unanswered.

TNFa is another effective cytokine during Mtb infection. In addition to data acquired from experimental animals, the increased incidence of TB in autoimmune disease patients that received TNFa blocking treatment also highlights the role of TNFa in immunity to Mtb [88, 89]. TNFa can act synergistically with IFNγ to activate infected macrophages for Mtb killing [90, 91]. Infected macrophages, CD4 T cells, and CD8 T cells isolated from infected experimental animal and TB patient can produce TNFa [92, 93]. As discussed for IFNγ, whether CD8 T cells mediate protection *in vivo* through TNFa production is unknown.

CD8 T cells are called cytotoxic T lymphocytes (CTL), highlighting their unique function of cytolysis. Cytolysis induced apoptosis can restrict Mtb growth by promoting the engulfment of dead cells by uninfected macrophages (i.e., efferocytosis) [94, 95]. Cytolysis has been shown *in vitro* when cell lines derived from Mtb infected patient or experimental animal recognize and kill target cells [53, 81, 96]. In addition, by differential fluorescent-labeling of target cells that were Mtb peptide pulsed or non-pulsed, and transferred into infected or uninfected mice, the

cytotoxicity function of CD8 T cells can be measured based on the ratio of peptide pulsed vs. non-pulsed target cells in infected versus uninfected. Using this CTL assay, Mtb specific CD8 T cells' cytotoxic activity *in vivo* was detected [83, 97]. CD8 T cells can lyse target cells through several molecular mechanisms: 1. Release of cytotoxic granules; 2. CD95-CD95L (Fas-FasL) dependent cytotoxicity; 3. TNFa induced apoptosis. Although the usage of these cytotoxic pathways by CD8 T cells has been studied using derived CD8 T cell lines [53, 98, 99], the relative importance of these mechanisms *in vivo* are controversial. Both granzyme and perforin KO mice show no impairment in Mtb growth control [100], although perforin KO mice succumb earlier than WT mice in a study using iv infection [65]. The mice deficient in CD95-CD92L pathway don't have a defect in controlling Mtb growth at early timepoints after infection, but have higher bacterial burden in lung at later timepoints [101]. Although showing limited effects in mice, these results are confounded by the redundancy of these cytotoxic pathways, and perhaps by the increased IFNγ in these KO mice [98].

Granulysin is another important cytotoxic granule expressed by human and NHP CD8 T and NK cells. Granulysin has been shown to directly lyse extracellular Mtb and kill intracellular Mtb through a perforin dependent mechanism [73]. Because of the lack of granulysin gene ortholog in rodents, NHP may provide a better experimental model for accessing cytotoxic function of CD8 T cells. Recently, Gideon and Hughes tried to define protective cellular responses in cynomolgus macaques by comparing the cellular component in restrictive vs. permissive

granulomas using scRNA-seq [80]. They found granulomas with greater Mtb control were enriched with cytotoxic CD8 T cells. Cytotoxic CD8 T cells expressing granzyme B, perforin, and granulysin are also found in human leprosy, an infection caused by *Mycobacterium leprae* [75]. Interestingly, these CD8 T cells express various NK receptors that are typically found on NK cells [75].

Despite this challenge to prove the importance of a specific effector function, it reflects the complexity of mechanisms CD8 T cells used to confer protection. Both cytokine and cytotoxic dependent functions can be involved. The mechanisms CD8 T cells used to mediate protection *in vivo* still needs to be elucidated.

CD8 T cells recognition of infected cells

With the effector functions mentioned above, we would expect CD8 T cell target infected cells in lungs that presented Mtb peptides on the MHCI molecules. This occurs via the cognate recognition of peptide-MHCI by the TCR on CD8 T cells. However, CD8 T cells don't always recognize infected cells. In our study led by Jason Yang, we showed CD8 T cells specific for the immunodominant epitope, $TB10.4_{4-11}$ didn't efficiently recognize Mtb infected macrophages [102]. TB10.4 is encoded by gene *esxH*. After Mtb infection, 30-50% of pulmonary CD8 T cells in C57BL/6J mice are specific for $TB10.4_{4-11}$. Why $TB10.4_{4-11}$ specific CD8 T cell responses are so immunodominant is not clear. Immunodominance describes a phenomenon that despite thousands of peptides generated from pathogen associated antigens during infections, only a few of the trimmed peptides can be

loaded on MHCI or II molecules by APCs, and presented to CD8 and CD4 T cells, respectively. In addition, the dominant T cells can actively suppress the subdominant T cells, in part through competing for APCs; a process called immunodomination. Immunodominant Mtb antigens are often used in TB diagnosis tests, and are included as components for TB vaccines.

In our study, the ability of TB10.$4_{4\text{-}11}$ specific CD8 T cells to recognize infected macrophages, measured by Nur77 upregulation and IFNγ secretion, were compared to Ag85b specific CD4 T cells. TB10.$4_{4\text{-}11}$ specific CD8 T cells were less efficient in recognizing infected macrophages, and failed to control Mtb growth in macrophages. In contrast, Ag85b specific CD4 T cells effectively recognized infected cells and inhibited intracellular Mtb growth [102]. This provided a potential mechanism by which vaccines eliciting immunodominant CD8 T cell responses fail to confer protection [36]. This result is consistent with a previous finding that CD8 T cells derived from recipients of an adenoviral vector vaccine, AERAS-402 didn't recognize infected cells [103]. The discrepancy between the overwhelming TB10.$4_{4\text{-}11}$ specific CD8 T cell responses generated *in vivo*, but the failure of these cells to restrict Mtb growth because of inefficient recognition raises a possibility: whether Mtb actively drives the immunodominant response as a strategy to evade immune surveillance by CD8 T cells. This theory may be supported by the evolutionary conserve of Mtb genes that contained T cell epitopes [104, 105]. We therefore hypothesized that TB10.4 is a "decoy antigen" and the

immunodominance of TB10.4$_{4-11}$ specific CD8 T cells were generated by Mtb for its fitness.

To test this decoy hypothesis, a study led by Rujapak Sutiwisesak in our lab leveraged a naturally occurring polymorphism in TB10.4$_{4-11}$ epitope, esxH^{A10T} [106]. An isogenic strain of Erdman that expressed esxH^{A10T} epitope was generated. They found polymorphism EsxH^{A10T} altered immune hierarchy of CD8 T cells. The frequency of TB10.4$_{4-11}$ specific CD8 T cells was decreased, but the frequency of subdominant Mtb32A$_{309-318}$ specific CD8 T cells was increased. Moreover, the abilities of Erd.EsxH^{WT} and Erd.EsxH^{A10T} elicited CD8 T cells to recognize infected macrophages were tested. For this purpose, the Mtb-infected macrophages ELISPOT (MIME) assay was developed by Yash Patankar and colleagues [107]. Purified CD8 T cells from Erd.EsxH^{WT} or Erd.EsxH^{A10T} infected mice were cultured with infected macrophages, and the frequency of CD8 T cells recognizing infected macrophages were calculated by the spots developed in IFNγ ELISPOT. They found Erd.EsxH^{A10T} elicited CD8 T cell recognized infected macrophages differently. This result indicates immune hierarchy is altered when the immunodominant response is disrupted, and supports the hypothesis that a non-protective dominant response suppressed subdominant responses [106].

Despite TB10.4$_{4-11}$ specific CD8 T cells not recognizing infected macrophages, there are some pulmonary CD8 T cells from a polyclonal pool that can recognize infected macrophages [107]. This recognition of infected cells by polyclonal CD8 T cells has been indirectly studied for years when CD8 T cells

derived from Mtb infected experimental animals and/or TB patients were assayed for their cytotoxic functions. Interestingly, when T cells were cultured with infected cells that have different multiplicity of infection (MOI), CD8 T cells responded better to infected cells with high MOI, while CD4 T cells can respond to infected cells with low MOI and the responses plateau at high MOI. This result that CD8 T cells preferentially recognized heavily infected cells was also observed by Lewinsohn and colleagues [108]. Direction for future studies includes the identification of antigens presented on infected cells that can be recognized by CD8 T cells, and to test whether vaccines that contain these antigens confer better protection.

A Lesson from Other Infectious Diseases and Tumor: CD4 T Cells are Required for Optimal CD8 T cell Responses

Helped vs. helpless CD8 T cells

Through antibody mediated depletion, adoptive transfer, and genetic KO mice, CD8 T cells' role in immunity to Mtb was assessed in murine models. Despite being recognized as an integral part of immunity, the relative contribution of CD8 T cells has been questioned. However, the approaches mentioned above only examined CD8 T cells as an independent cell subset, and can't assess the synergy between CD4 and CD8 T cells. CD4 T cells are essential for optimal CD8 responses in many viral infections and cancer, which is called CD4 T cell help. CD8 T cell function is likely compromised without CD4 T cell help.

The importance of CD4 T cell help was first established in generation of functional CD8 T cell memory responses following both immunization and primary infection. The lack of CD4 T cell help results in memory responses that are defective in magnitude and function [109-111]. Despite most studies reporting the effective memory responses were “programmed” during CD8 T cell priming [109, 110, 112], one study demonstrated the need of CD4 T cell help to maintain, in addition to generate, the effective memory responses [113]. Moreover, it’s known that inflammation and antigen load play key roles for the generation of memory CD8 T cells, and CD4 T cells regulate this process [114-116]. For example, helpless CD8 T cells have increased T-bet expression [115], which could compromise memory formation. Treg cells also modulate in this process through the production of IL-10 [117, 118].

The role of CD4 T cell help in primary infection is more controversial. In some studies, the magnitude and function of CD8 T cell responses were comparable with or without CD4 T cells, while other studies showed defective CD8 T cell responses in the absence of CD4 T cells. The fact that innate signals can activate APCs in addition to CD4 T cells reconciles the conflicts [119]. Thus, CD4 T cell help is more crucial in immunizations or infections in which type I IFN production is modest. In contrast, CD4 T cell help is bypassed in infections when strong inflammation is induced. The conflicting results from the same pathogens may also reflect the difference in dose and virulence of inoculums. However, CD4 T cells are shown to be crucial to maintain CD8 T cell function in chronic viral

infections. Without CD4 T cells, CD8 T cells lose antiviral function, resulting in uncontrolled infection [120-124]. Moreover, IL-21 secreted by CD4 T cells are associated with supporting CD8 T cell function in chronic infections [125-127].

As mentioned above, whether CD8 T cell effector functions during primary infection depend on CD4 T cell help needs to be clarified. Recently, RNA-seq and scRNA-seq have used to assess the impact of CD4 T cell help on CD8 T cell transcriptional profiles, suggesting a global impact on CD8 T effector cells. Using both vaccine and acute LCMV infection models, Ahrends and colleagues uncovered that CD4 T cell help turned on transcriptional networks of CD8 T cells, promoting both effector and memory CD8 T cell formation [128]. Moreover, helped CD8 T cells had greater migratory potential through upregulating chemokine receptors. In another study, Zander and colleagues identified a CD4 help- and IL-21-dependent subset of CD8 T cells in chronic LCMV infection using scRNA-seq, and they showed this subset had cytotoxic function [129].

T cell exhaustion are associated with the lack of CD4 T cell help. The loss of antiviral function on helpless CD8 T cell during chronic virus infection implies these CD8 T cells may be exhausted, though the term “T cell exhaustion” was not used in these early studies [121, 124]. Nevertheless, recent studies showed helpless CD8 T cells expressed multiple inhibitory receptors, and through transcriptional analysis, helpless CD8 T cells have been shown to have features resembling exhausted T cells [128, 130, 131]. In summary, CD4 T cell help is indispensable in many aspects of the CD8 T cell response.

Molecular nature of CD4 T cell help

Considerable studies address the molecular mechanisms of how CD4 T cells provide help to CD8 T cells. CD4 and CD8 T cells differ in the ways they are activated. CD8 T cells recognize intracellular antigens that are processed by the proteasome and presented on MHC I molecules, while CD4 T cells recognize antigens that entered the endocytic pathway and are loaded on the MHC II molecules after degradation in the phagosome. Thus, the antigen presenting cells (APC) that have both MHC I and II molecules, and are efficient in "cross-presentation" (loading the endocytic antigens on MHC I molecules) become the bridge to connect the CD4 and CD8 T cell immunity against pathogens. The "help" signals from CD4 T cells are also delivered through this bridge. It's shown that $XCR1^+$ DCs are the platform that relay the "help" to CD8 T cells [132, 133]. $XCR1^+$ DCs are "licensed" when they received signals through CD40 that are engaged with CD40L on activated CD4 T cells [134, 135]. The activating signals, combined with innate signals $XCR1^+$ DCs received through pattern recognition receptors, upregulate the expression of MHC molecules and co-stimulatory receptor CD70 and CD80/86, which interacts with CD27 and CD28 on CD8 T cells, respectively. Activated $XCR1^+$ DCs also produce more cytokines, including IL-12, IL-15, and Type 1 IFN. These cytokines and co-stimulatory signals then integrate as an output that $XCR1^+$ DCs present to CD8 T cells, dictating their differentiation and effector functions. In addition, IL-2 and IL-21 produced by CD4 T cells are also reported to be help signals that CD4 T cells directly deliver to CD8 T cells without DCs.

For several years, scientists were puzzled by the low likelihood that CD4 and CD8 T cells specific for same antigen were cognate engaged with the same DCs. Recently, two groups answered this question by a two-step priming model using intravital 2-photon microscopy [132, 133]. In this model, CD4 and CD8 T cells are first activated by different subsets of migratory DCs in separate locations. The activated CD8 T cells then secrete XCL1 to recruit $XCR1^+$ resident DCs. Finally, $XCR1^+$ DCs migrate deeper into the paracortical area of lymph nodes, where they cognate engage with pre-activated CD4 and CD8 T cells and relay help to pre-activated CD8 T cells.

CD4 T cell Help during Tuberculosis

How CD4 T cell help shapes CD8 T cell responses during Mtb infection is not fully elucidated. Nevertheless, a few studies suggest CD8 T cells' effector functions may depend on CD4 T cells. Serbina and colleagues first showed CD8 T cells from infected CD4 KO mice had impaired ability to lyse target cells *in vitro* [136]. However, this result was compromised by later studies reported that CD4 KO mice have MHCII restricted T cells [137, 138]. The ability to produce IFNγ by CD8 T cells in the absence of CD4 T cells was addressed, but conflicting results were generated. Two studies showed that IFNγ production by CD8 T cells depend on CD4 T cells. Using direct intracellular staining without *in vitro* stimulation, Bold and colleagues found that in lungs of infected mice, the frequency of IFN^+ CD8 T

cells correlated with the frequency of IFN^+ CD4 T cells, and the percentage of IFN^+ CD8 T cells was decreased in mice following antibody mediated depletion of CD4 T cells [85]. In addition, Green and colleagues showed impaired CD8 responses, including IFNγ production, in the absence of CD4 T cells in a model where RAG KO mice were reconstituted with splenocytes that were depleted or non-depleted of CD4 T cells [87]. In contrast, Carusa and colleagues reported IFNγ produced by CD8 T cells was only transiently diminished in the absence of CD4 T cells [86].

As discussed previously, several pathways have shown to play roles in CD4 T cells providing help to CD8 T cells during viral infection. Deficiency in these signalings during Mtb infection leads to impaired CD8 T cell responses, which implies CD4 T cells play an essential role in effective CD8 T cell responses. Booty and Barreira-Silva et al showed impaired priming in lymph nodes and accumulation in lungs of $TB10.4_{4\text{-}11}$ specific IL-21R KO CD8 T cells, with experiments in which $TB10.4_{4\text{-}11}$ specific IL-21R KO or competent CD8 T cells were co-transferred into infected C57BL/6 mice [139]. CD8 T cells from IL-21R KO mice had decreased cytokine production, but increased inhibitory receptor expression compared to CD8 T cells from WT mice [139]. Moreover, compromised primary and secondary CD8 T cell responses in IL-15 KO mice are also reported [140, 141]. Interestingly, while Lazarevic and colleagues reported the limited role of IL-15 signaling in generating effective primary CD8 T responses, compared to its significant role in recall responses [140], Rausch and colleagues found impaired expansion, proliferation, and effector functions of CD8 T cells from IL-15 KO mice in primary

Mtb infection [141]. Overall, Mtb specific CD8 T cell responses in the quality and quantity of Mtb specific CD8 T cell responses in the presence or absence of CD4 T cell help needs further investigation to resolve the conflicts.

Whether helped CD8 T cells mediate enhanced protection is unknown. This question is challenging to address because it requires the dissociation of CD4 T cells' role as regulatory cells from the direct protection they mediate. In the study mentioned above, Booty and Barreira-Silva et al indirectly studied this question [139]. They showed IL-21R KO T cells mediated less protection when adoptively transferred into TCRα KO mice before challenge, while purified CD4 T cells from WT or IL-21R KO mice conferred comparable protection. However, they failed to show the protection mediated by WT or IL-21R KO CD8 T cells because an unexpected wasting syndrome developed in TCRα KO mice infused with IL-21R KO CD8 T cells. This study may imply that "helpless" CD8 T cell responses contribute to the susceptibility of IL-21R KO mice, but further studies are needed to be conclusive. Thus, whether CD4 T cell help is essential to protective CD8 T cell immunity remains unknown.

Thesis Objectives

A better understanding of how different elements of our immune system collaborate to fight Mtb is the foundation for developing effective vaccines. The relative contribution of CD8 T cells in immunity to TB is contested mainly because

of previous findings in murine models. In light of emerging data implying the essential role of CD8 T cells, we argue CD8 T cells are an important arm in protective immunity and their role needs further investigation. Recent studies focus on the immunodominant CD8 T cells, and the possibility that Mtb exploits immunodominance to evade immune surveillance. However, some CD8 T cells can recognize infected cells. To better understand protective CD8 T cell responses, this dissertation focuses on whether CD4 T cell help is involved in shaping CD8 T cell responses, and the molecular mechanisms of such CD4 T cell help. More importantly, I address whether helped CD8 T cells mediate better protection against Mtb.

Using a series of unbiased approaches, we uncover the “helped” vs. “helpless” CD8 T cell features in chapter II. In this chapter, we also address whether helped CD8 T cells are more protective through both *in vitro* and *in vivo* methods. Furthermore, how the CD4 T cell help delivers to CD8 T cells is addressed in chapter III. The requirements of several pathways in shaping effective CD8 T cells responses are determined. I then report an unexpected role of MHCII molecules in regulating CD8 T cells’ behavior in chapter IV. Finally, I discuss how this current dissertation broadens our knowledge of the role CD8 T cells play in immunity to Mtb, focusing on the impact of CD4 T cell help, the new findings of CD8 T cell in NHP models, and the implication on vaccine design. The data presented in this dissertation will enhance our understanding of protective immune responses in TB, and provide insights to vaccine development.

CHAPTER II. CD4 T CELL HELP PREVENTS CD8 T CELL EXHUASTION AND PROMOTES CONTROL OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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Abstract

CD4 T cells are essential for immunity to tuberculosis because they produce cytokines including interferon-γ. Whether CD4 T cells act as “helper” cells to promote optimal CD8 T cell responses during *Mycobacterium tuberculosis* is unknown. Using two independent models, we show that CD4 T cell help enhances CD8 effector functions and prevents CD8 T cell exhaustion. We demonstrate synergy between CD4 and CD8 T cells in promoting the survival of infected mice. Purified helped, but not helpless, CD8 T cells efficiently restrict intracellular bacterial growth *in vitro*. Thus, CD4 T cell help plays an essential role in generating protective CD8 T cell responses against *M. tuberculosis* infection *in vitro* and *in vivo*. We infer that vaccines which elicit both CD4 and CD8 T cells are more likely to be successful than vaccines that elicit only CD4 or CD8 T cells.

Introduction

Tuberculosis (TB) is a serious global health issue. BCG is the only approved TB vaccine and despite achieving clinically significant protection in infants and toddlers, it has variable efficacy against pulmonary disease, which is the form of disease that leads to transmission [8]. More effective vaccines are required to stop transmission and reduce global TB burden. To develop more effective vaccines, we need a better understanding of how *Mycobacterium tuberculosis* (Mtb) evades immunity and how the different elements of our immune system interact to contain and eliminate Mtb infection.

T cells are required for containment of primary Mtb infection and most TB vaccines in development elicit T cell responses [13, 61, 142]. While Mtb infection elicits both CD4 and CD8 T cells, the increased risk of TB in HIV-infected persons highlights a crucial role for CD4 T cells [69-71, 143]. The essential role for CD4 T cells in immunity to Mtb is supported experimentally by the early mortality of mice lacking CD4 T cells [24]. While mice lacking CD8 T cells also die prematurely after Mtb infection, they survive longer than mice without CD4 T cells, which has led to the conclusion that CD4 T cells are more important for protection than CD8 T cells [24]. The inability of CD8 T cells to transfer significant protection to immunodeficient mice supports this assertion [144]. However, there are other interpretations of these data. For example, studies on people with AIDS have demonstrated the importance of CD4 T cells in regulating virtually every arm of the

immune response. The contribution of CD4 T cells in generating optimal CD8 T cell and B cell responses is called CD4 T cell help. We hypothesize that CD8 T cells require CD4 T cell help to develop into effector CD8 T cells capable of controlling Mtb infection.

CD4 T cell help enables CD8 T cells to respond more efficiently to viruses and cancer [68]. CD4 T cells promote CD8 T cell expression of effector molecules such as IFNγ, TNF, granzyme A and B, and facilitate greater migration of CD8 T cells by upregulating several chemokine receptors [128]. The development of $CX3CR1^{+}$ CD8 T cells, noted for their cytolytic function, requires CD4 T cell help [129]. Helped CD8 T cells express transcriptional networks that promote effector and memory cell development [128]. Indeed, CD4 T cell help during CD8 T cell priming promotes memory development and imprints a quick effector recall response upon challenge [109-112]. In contrast, helpless CD8 T cells upregulate inhibitory receptors characteristic of exhausted CD8 T cells [128].

If CD4 T cells mediate both effector and regulatory functions during TB, the question arises whether the lack of CD4 T cell help compromises CD8 T cell responses and contributes to the susceptibility of CD4 T cell deficient mice. This is a difficult hypothesis to test as it requires dissociating the effector and regulatory functions of CD4 T cells. Nevertheless, other studies have addressed how CD4 T cells affect CD8 T cell immunity during TB. CD8 T cell priming and recruitment to the lung occurs in CD4-deficient mice, although the quantity and quality of Mtb-specific CD8 T cells elicited without CD4 T cells hasn't been assessed [86, 136].

During TB, CD4 T cell help promotes CD8 T cell IFNγ production and cytolytic activity, but whether helped CD8 T cells mediate greater protection is unknown [85, 87, 136]. To answer these questions, we compared CD8 T cell responses in infected WT or MHCII KO mice and confirmed our results in a novel adoptive transfer model in which purified CD8 T cells from uninfected mice were transferred with or without Ag85b-specific CD4 T cells into TCRα knockout (KO) mice. We found that helped and helpless Mtb-specific CD8 T cell responses differed in effector molecule and coinhibitory receptor expressions. Importantly, we found that helped CD8 T cells mediate better Mtb control than helpless CD8 T cells, leading us to predict that vaccines that elicit both CD4 and CD8 T cell responses are likely to confer the greatest protection.

Results

Mtb-specific CD8 T cell responses are generated independently of CD4 T cells

MHCII KO mice were used to study the CD8 T cell response to Mtb in the absence of CD4 T cell help. The "helpless" CD8 T cells (i.e., from MHCII KO mice) were compared to "helped" CD8 T cells from Mtb-infected C57BL/6 mice (hereafter, WT). MHCII KO mice had significantly higher bacterial burden in lungs and spleens (Figure 2.1 A right) and succumbed early after infection as previously described (Figure 2.1 A left) [24]. To determine whether CD4 T cell help is required to generate CD8 T cell responses in the lung, we measured Mtb-specific CD8 T cell responses for two dominant epitopes, $TB10.4_{4-11}$ and $32A_{309-318}$ [106, 145]. These CD8 T cell responses were elicited by Mtb infection without apparent delay in the absence of CD4 T cells (Figure 2.1 B). The frequencies and absolute numbers of $TB10.4_{4-11}$ specific CD8 T cells were higher in MHCII KO than WT mice early during infection, which may have been driven by the greater bacterial load in MHCII KO mice (Figure 2.1 B,C). CD8 T cells differentiate into $KLRG1^{+}CD127^{-}$ short-lived effector cells (SLEC) or $KLRG1^{-}CD127^{+}$ memory precursor cells (MPEC) upon activation. The frequencies of SLEC and MPEC CD8 T cells in the lung of WT and MHCII KO mice were similar. However, the frequencies of both SLEC and MPEC $TB10.4_{4-11}$ -specific CD8 T cells were greater in WT mice than in MHCII KO mice (Figure 2.1 D,E). These results indicate that CD8 T cell responses to Mtb are generated independently of CD4 T cell help. However, CD4 T cells promote the

differentiation of CD8 T cells into SLEC and MPEC cells, consistent with results previously shown in a vaccination model [128].

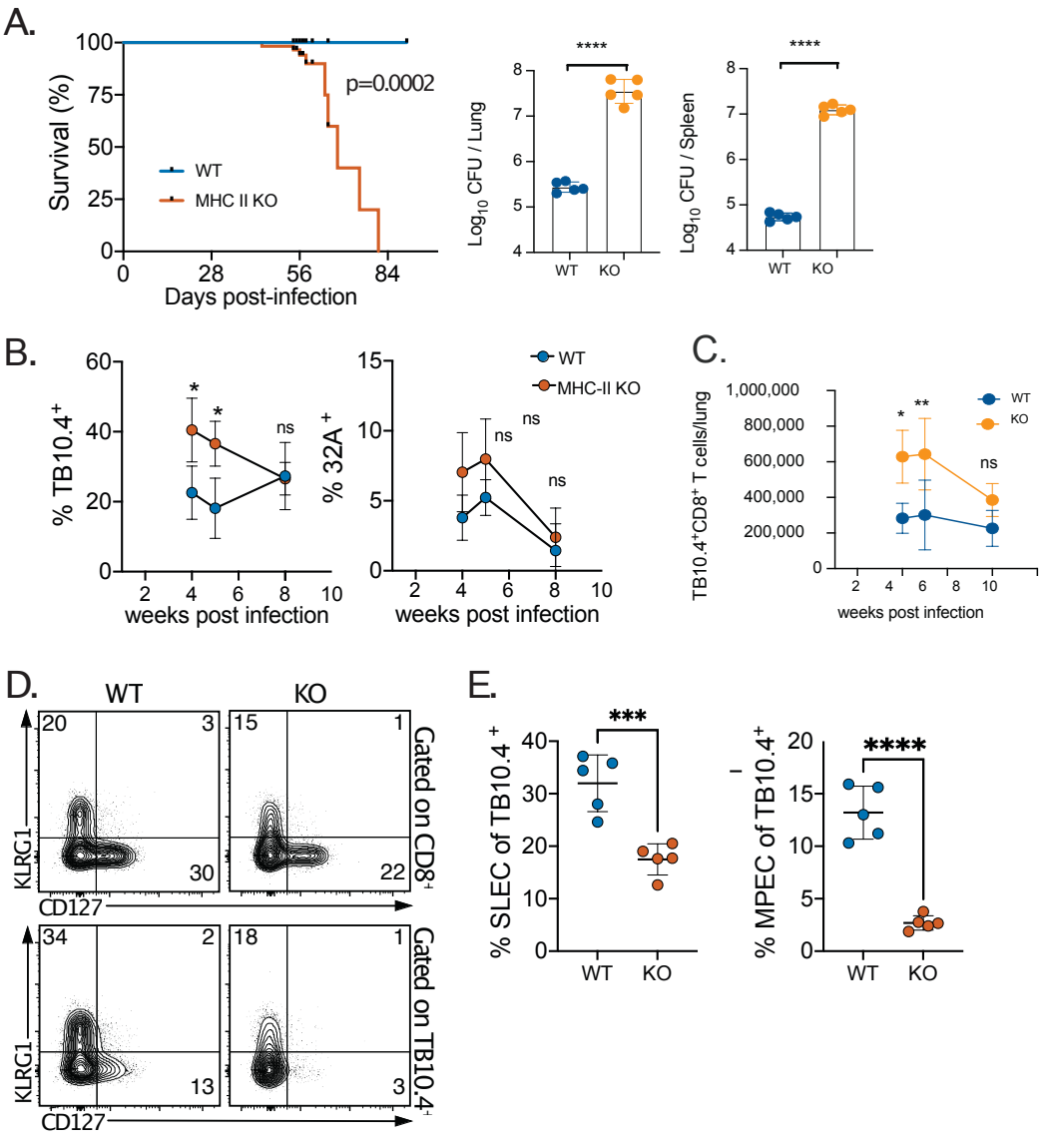

A.
Survival (%)
Days post-infection
p=0.0002
WT
MHC II KO
Log10 CFU / Lung
Log10 CFU / Spleen
****
B.
% TB10.4+
% 32A+
weeks post infection
ns
WT
MHC-II KO
C.
TB10.4+CD8+ T cells/lung
weeks post infection
D.
WT
KO
KLRG1
CD127
Gated on CD8+
Gated on TB10.4+
E.
% SLEC of TB10.4+
% MPEC of TB10.4+
***
****

Figure 2. 1 Mtb-specific CD8 T cell responses are generated in MHCII KO mice.

(A) Left, cumulative survivals of WT and MHCII KO mice after infection with aerosolized Mtb. Data were compiled from 9 experiments with 56 WT and 57 MHCII KO mice. The black ticks represent censored subjects (56 WT and 48 MHCII KO mice) that were analyzed at defined time points. The difference between the strains was statistically significant (p=0.0002) as determined by the log-rank test. Right, bacterial burden in lungs and spleens of WT and MHCII KO mice were determined at 8 weeks post infection (wpi). Data are representative of two independent experiments, 5 mice/group. Bars, mean ± SD. (B-C) Lung cells from WT and MHCII KO mice were analyzed at 4, 5, and 8 wpi to determine the frequencies of $TB10.4_{4-11}$ and $32A_{309-318}$ -specific CD8 T cells and the number of $TB10.4_{4-11}$ specific CD8 T cells. The frequencies of $KLRG1^{+}CD127^{-}$ (SLEC) and $KLRG1^{-}CD127^{+}$ (MPEC) among total CD8 T cells and $TB10.4_{4-11}$ -specific CD8 T cells was determined 8 wpi by flow cytometry (D, representative data) and analyzed statistically (E). (B, C, E) Representative data of three independent experiments, 4-5 mice/group. Data represent mean ± SD. Statistical significance was analyzed by multiple t test (B, C) or unpaired t test (A, E). p-values: *, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Helped vs helpless CD8 T cells: transcriptional signatures of cytotoxic vs. exhausted CD8 T cells

To comprehensively identify differences between “helped” and “helpless” CD8 T cells, we purified lung CD8 T cells from WT or MHCII KO mice 8 weeks post infection (wpi) and performed RNA-seq. We found 1,072 genes differed in their expression between WT and KO CD8 T cells (padj<0.05 and $|\log_2$ fold-change$|\geq 1$). Among these genes, 173 were upregulated in WT cells, while 899 genes were upregulated in KO cells (Figure 2.2 A). Among the genes upregulated in WT cells, MHCII molecules were the ones with the largest fold changes. Enrichment analysis using Gene Ontology (GO) terms revealed WT CD8 T cells expressed pathways involved in IFNγ production and cell killing (Figure 2.2 B). These data show that IFNγ production and cytotoxicity by CD8 T cells is augmented by CD4 T cells during Mtb infection. Gene sets associated with NK cell immunity were detected in the enrichment analysis, including expression of NK receptors *Klrk1* (NKG2D) and *CD244* (2B4) (Figure 2.2 B, C). These receptors are associated with secretion of multiple cytotoxic granules, indicating enhanced cytotoxicity of helped CD8 T cells [75]. Since the RNA-seq analysis was performed using total lung CD8 T cells, which contain approximately 50% intravascular cells and could dilute the signal attributable to Mtb-specific CD8 T cells, we changed the criteria to “padj <0.05” without restrictions on fold-change. More genes associated with an effector state were identified as upregulated in WT cells including transcription factors that drive effector CD8 T cell differentiation (e.g., *Tbx21*, *Zeb2*,

and *Id2*), activation markers (e.g., *Il2ra*, *CD69*, and *Itgal*), and cytotoxic molecules (e.g., *Gzma* and *Gzmb*) (Figure 2.2 C).

Gene set enrichment analysis (GSEA) found that the MHCII KO CD8 T cells upregulated genes associated with T cell exhaustion and were enriched for genes from the signature of CD8 T cells from mice with chronic LCMV infection [146] (Figure 2.2 D, top row). Genes upregulated in KO cells were enriched in genes differentially expressed by exhausted cells compared to effector or memory CD8 T cells (Figure 2.2 D, top row). Indeed, MHCII KO CD8 T cells upregulated many genes encoding inhibitory receptors, including *Pdcd1*(PD-1), *Havcr2* (Tim-3), *Lag3*, and *Entpd1* (CD39) (Figure 2.2 C). Transcription factors *Eomes, c-Maf,* and *Tox* were all upregulated in MHCII KO CD8 T cells (Figure 2.2 C). In contrast, WT CD8 T cells upregulated genes from the effector and memory signatures (Figure 2.2 D, bottom row). Thus, helped WT CD8 T cells express an effector transcriptional program that is remarkable for greater expression of molecules associated with cytotoxicity including TNF, granzyme A and B, and NK receptors. In contrast, helpless MHCII KO CD8 T cells have an exhaustion signature including PD-1, Tim-3, Tox, CD39 and Lag-3.

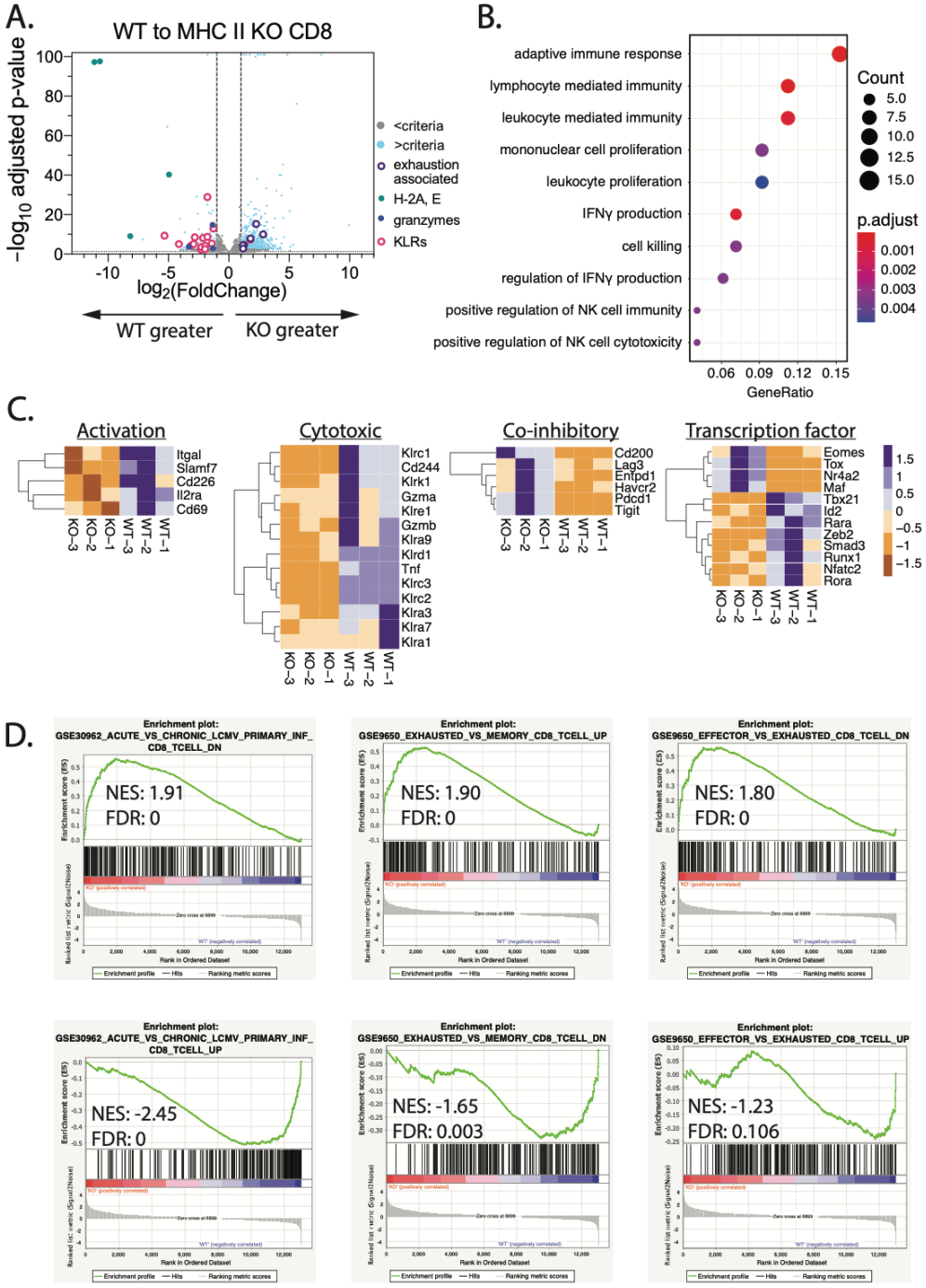

A.
WT to MHC II KO CD8
−log10 adjusted p-value
log2(FoldChange)
WT greater
KO greater
<criteria
>criteria
exhaustion associated
H-2A, E
granzymes
KLRs
B.
adaptive immune response
lymphocyte mediated immunity
leukocyte mediated immunity
mononuclear cell proliferation
leukocyte proliferation
IFNγ production
cell killing
regulation of IFNγ production
positive regulation of NK cell immunity
positive regulation of NK cell cytotoxicity
GeneRatio
Count
p.adjust
C.
Activation
Cytotoxic
Co-inhibitory
Transcription factor
Itgal
Slamf7
Cd226
Il2ra
Cd69
Klrc1
Cd244
Klrk1
Gzma
Klre1
Gzmb
Klra9
Klrd1
Tnf
Klrc3
Klrc2
Klra3
Klra7
Klra1
Cd200
Lag3
Entpd1
Havcr2
Pdcd1
Tigit
Eomes
Tox
Nr4a2
Maf
Tbx21
Id2
Rara
Zeb2
Smad3
Runx1
Nfatc2
Rora
KO−3
KO−2
KO−1
WT−3
WT−2
WT−1
D.
Enrichment plot: GSE30962_ACUTE_VS_CHRONIC_LCMV_PRIMARY_INF_CD8_TCELL_DN
NES: 1.91
FDR: 0
Enrichment plot: GSE9650_EXHAUSTED_VS_MEMORY_CD8_TCELL_UP
NES: 1.90
FDR: 0
Enrichment plot: GSE9650_EFFECTOR_VS_EXHAUSTED_CD8_TCELL_DN
NES: 1.80
FDR: 0
Enrichment plot: GSE30962_ACUTE_VS_CHRONIC_LCMV_PRIMARY_INF_CD8_TCELL_UP
NES: -2.45
FDR: 0
Enrichment plot: GSE9650_EXHAUSTED_VS_MEMORY_CD8_TCELL_DN
NES: -1.65
FDR: 0.003
Enrichment plot: GSE9650_EFFECTOR_VS_EXHAUSTED_CD8_TCELL_UP
NES: -1.23
FDR: 0.106
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Figure 2. 2 Transcriptional features of WT vs. MHCII KO CD8 T cells.

(A) Volcano plot of transcriptome comparison. Transcripts of purified MHCII KO CD8 T cells were compared to WT CD8 T cells from Mtb-infected mice 8 wpi. Blue dots indicate differentially expressed genes (p-adjusted value <0.05 and $|\log_2$ fold change$|\geq 1$). (B) Gene Ontology analysis for genes significantly upregulated in WT CD8 T cells. Bubble plot presents top 10 pathways that were most significantly enriched. (C) Heatmap displaying differentially expressed genes in each category. Color indicates row z scores. (D) GSEA. Data were compared to published datasets available at Gene Expression Omnibus (GEO): GSE30962 and GSE9650. NES, normalized enrichment score. FDR, false discovery rate.

Helped vs helpless CD8 T cells: cytotoxic vs. exhausted CD8 T cells

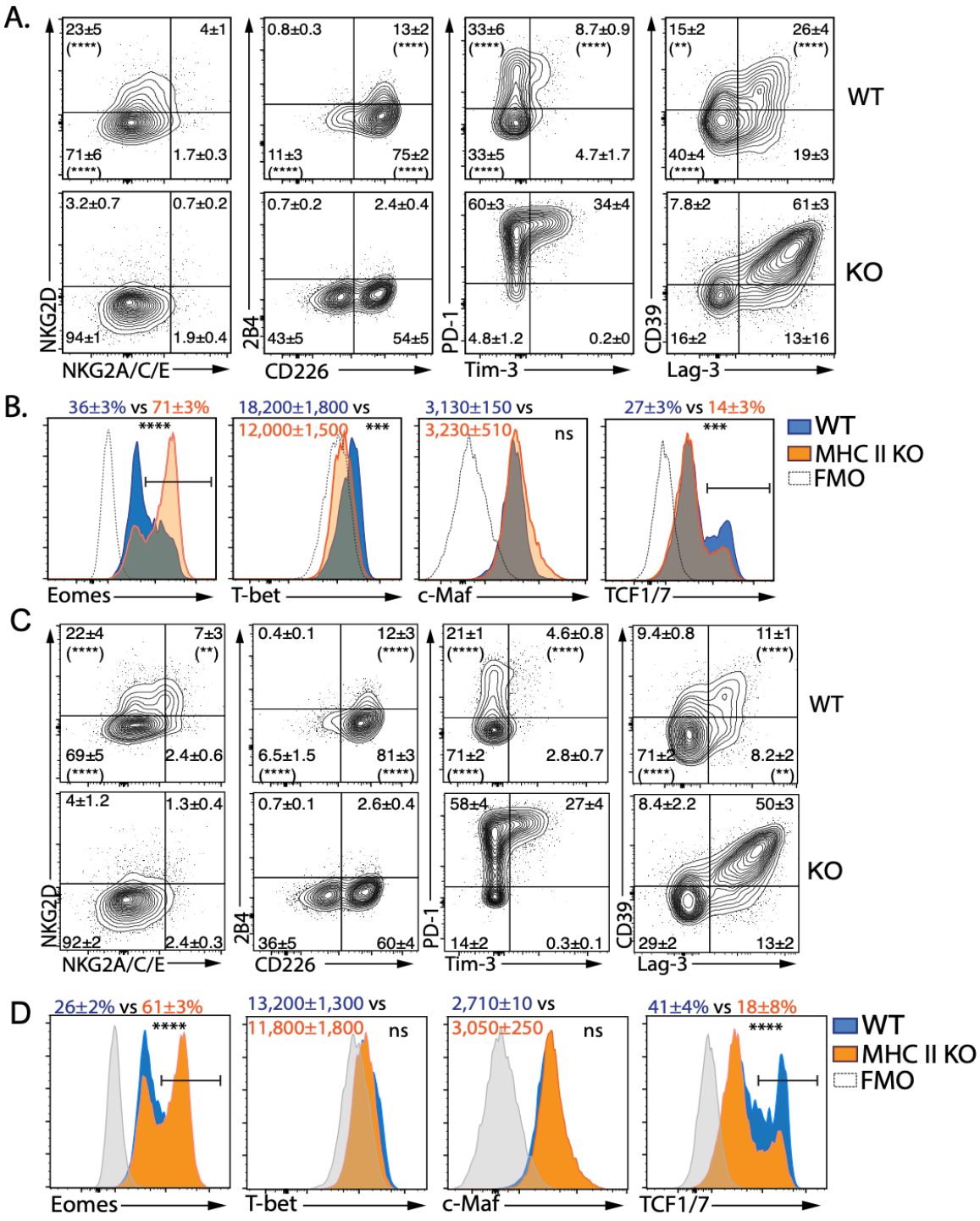
To validate the transcriptional signatures, we performed single cell analysis of CD8 T cells from infected WT or MHCII KO mice using 19-parameter flow cytometry. Since cells located in lung parenchyma are more likely to interact with infected cells, an established approach was used to distinguish intravascular from parenchymal CD8 T cells [147, 148]. More parenchymal WT CD8 T cells expressed NK receptors including NKG2D and 2B4 than parenchymal MHCII KO CD8 T cells. In contrast, MHCII KO CD8 T cells expressed significantly more co-inhibitory receptors, including PD-1, Tim-3, Lag-3, and CD39, typical of exhausted CD8 T cells (Figure 2.3 A). Differences in transcription factor expression were consistent with our RNA-seq analysis, with a dramatic increase in Eomes expression by MHCII KO CD8 T cells (Figure 2.3 B). WT CD8 T cells expressed higher levels of T-bet and Tcf-1. These differences between WT and MHCII KO CD8 T cells were also identified in total lung CD8 T cells (Figure 2.3 C,D).

To facilitate the flow cytometric analysis, UMAP projections were created to visualize differences between the WT and MHCII KO CD8 T cells during Mtb infection (https://arxiv.org/abs/1802.03426). Intravascular and parenchymal CD8 T cells were easily distinguished in the UMAP projection (Figure 2.3 E). In the lung parenchyma, most CD8 T cells were antigen-experienced ($CD44^{+}$), but a minor population of naïve cells ($CD62L^{hi}CD44^{lo}$) was also identified. The majority of MHCII KO CD8 T cells localized to the lung parenchyma, perhaps driven by the higher bacterial burden in MHCII KO mice (Figure 2.3 E, F).

Antigen-experienced ($CD44^+CD62L^-$) lung parenchymal WT and MHCII KO CD8 T cells were clustered into eight distinct populations on the UMAP projection using PhenoGraph™[149] (Figure 2.3 H, I). Clusters A-D were dominated by WT CD8 T cells (Figure 2.3 J), which were T-$bet^+Slamf7^+PD1^{lo}$, suggesting an effector state [150-152]. Cells in cluster A expressed Klrg1, Tcf1, and Eomes. While Tcf1 and Eomes can regulate the generation of memory and exhausted T cells, both populations arise from $Klrg1^-$ populations [146, 153]. Thus, Cluster A may represent cells in transition. Cluster B and C expressed T-bet and Slamf7, which defined these as effector cells. Cluster B are terminal effector cells based on their KLRG1 expression. As both $KLRG1^+$ and $KLRG1^-$ populations expressed NKG2D, NKG2A/C/E, and 2B4, cluster B and C might vary in their differentiation states rather than having distinct effector functions (Figure 2.3 G). Cluster D expressed Tcf1, Eomes, and CD127, consistent with memory precursor cells (see Figure 1, MPECs) [154].

Clusters E-H were predominantly MHCII KO CD8 T cells and were distinguished by their expression of PD-1 (Figure 2.3 H-J). The PD-$1^{low}TCF1^+Eomes^{low}$ phenotype of cluster E was typical of exhausted progenitor CD8 T cells, while cluster H appeared to be terminally exhausted CD8 T cells based on expression of the inhibitory receptors Tim-3, Lag-3, and CD39, and the transcription factors Eomes and c-Maf. Cluster F and G likely represent exhausted states as well, since both expressed high PD-1 levels and Eomes. Cluster F didn't express as many inhibitory receptors as cluster H, and cluster G was the only

cluster that expressed KLRG1 and T-bet among clusters E-H. Recent studies suggest CD8 T cells with an effector-like profile can represent a transition state before terminal exhaustion [155]. Similarly, KLRG1 is expressed on partially exhausted cells [156, 157]. In summary, we extend the results of our RNA-seq analysis by showing that helped WT CD8 T cells included subsets of cells that were effector cells with cytotoxic features. In contrast, helpless MHCII KO CD8 T cells appeared to be in various states of exhaustion.


A.
23±5 (****)
4±1
71±6 (****)
1.7±0.3
3.2±0.7
0.7±0.2
94±1
1.9±0.4
NKG2D
NKG2A/C/E
0.8±0.3
13±2 (****)
11±3 (****)
75±2 (****)
0.7±0.2
2.4±0.4
43±5
54±5
2B4
CD226
33±6 (****)
8.7±0.9 (****)
33±5 (****)
4.7±1.7
60±3
34±4
4.8±1.2
0.2±0
PD-1
Tim-3
15±2 (**)
26±4 (****)
40±4 (****)
19±3
7.8±2
61±3
16±2
13±16
CD39
Lag-3
WT
KO
B.
36±3% vs 71±3%
****
18,200±1,800 vs
12,000±1,500
***
3,130±150 vs
3,230±510
ns
27±3% vs 14±3%
***
Eomes
T-bet
c-Maf
TCF1/7
WT
MHC II KO
FMO
C
22±4 (****)
7±3 (**)
69±5 (****)
2.4±0.6
4±1.2
1.3±0.4
92±2
2.4±0.3
NKG2D
NKG2A/C/E
0.4±0.1
12±3 (****)
6.5±1.5 (****)
81±3 (****)
0.7±0.1
2.6±0.4
36±5
60±4
2B4
CD226
21±1 (****)
4.6±0.8 (****)
71±2 (****)
2.8±0.7
58±4
27±4
14±2
0.3±0.1
PD-1
Tim-3
9.4±0.8
11±1 (****)
71±2 (****)
8.2±2 (**)
8.4±2.2
50±3
29±2
13±2
CD39
Lag-3
WT
KO
D
26±2% vs 61±3%
****
13,200±1,300 vs
11,800±1,800
ns
2,710±10 vs
3,050±250
ns
41±4% vs 18±8%
****
Eomes
T-bet
c-Maf
TCF1/7
WT
MHC II KO
FMO

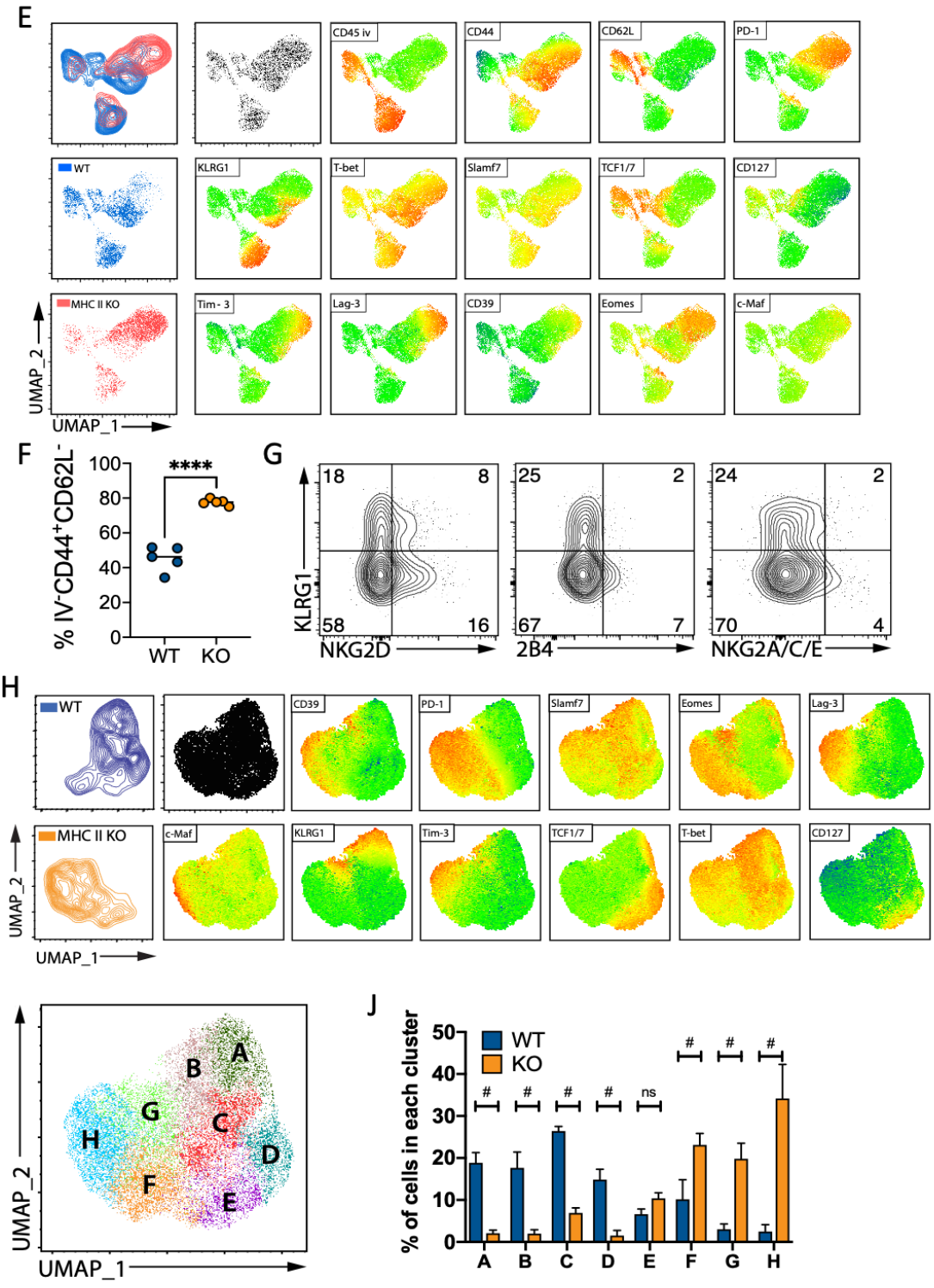

E
CD45 iv
CD44
CD62L
PD-1
WT
KLRG1
T-bet
Slamf7
TCF1/7
CD127
MHC II KO
Tim - 3
Lag-3
CD39
Eomes
c-Maf
UMAP_2
UMAP_1
F
% IV-CD44+CD62L-
****
WT
KO
G
KLRG1
NKG2D
2B4
NKG2A/C/E
18
8
58
16
25
2
67
7
24
2
70
4
H
WT
MHC II KO
CD39
PD-1
Slamf7
Eomes
Lag-3
c-Maf
KLRG1
Tim-3
TCF1/7
T-bet
CD127
UMAP_2
UMAP_1
I
A
B
C
D
E
F
G
H
UMAP_2
UMAP_1
J
% of cells in each cluster
WT
KO
#
ns
A
B
C
D
E
F
G
H

Figure 2. 3 WT CD8 T cells are cytotoxic effectors, while MHCII KO CD8 T cells are exhausted cells.

WT and MHCII KO mice were infected with aerosolized Mtb for 8 weeks. (A-B) Anti-CD45-AF647 was injected intravenously 3 minutes before euthanasia. (A) Lung parenchymal ($CD45iv^{-}CD44^{+}CD62L^{-}$) CD8 T cells were analyzed by flow cytometry to determine the frequencies of NK and inhibitory receptors. The mean % ± SD for each quadrant is indicated (n=5/group). The percentage of WT vs. KO cells in each quadrant was compared, and if statistically significant, is indicated in the WT quadrant. (B) The median fluorescence intensity (MFI) or average percentage of cells expressing each transcription factors, ± SD, are indicated. (C-D) Total lung CD8 T cells were analyzed by flow cytometry to determine the percentage of NK and inhibitory receptors, and the median fluorescence intensity (MFI) or average percentage of cells expressing each transcription factors. The mean % ± SD are indicated (n=5/group). UMAP projections from the flow cytometric analysis of lung total CD8 T cells (E) or parenchymal CD8 T cells (H) were overlayed with expression of the indicated markers. (F) Frequencies of CD8 T cells located in parenchyma were analyzed statistically. Bar, mean. (G) Frequencies of KLRG1 and NKG2D, NKG2A/C/E, or 2B4 in lung parenchymal ($CD45iv^{-}CD44^{+}CD62L^{-}$) CD8 T cells were determined. (I-J) Eight clusters were identified using PhenoGraph and the percentage of each cluster among WT or MHCII KO lung parenchymal CD8 T cells were analyzed statistically. Bars, mean ± SD. (A-I) Representative data of two independent experiments, 5 mice/group. Statistical significance was analyzed by two-way ANOVA (A, C, J) or unpaired t

test (B, D, F). p-values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; #, $p<0.0001$. ns, no significant differences.

Validation of the "helped" and "helpless" CD8 T cell states using an independent model

We considered whether a developmental or intrinsic defect in MHCII KO CD8 T cells could explain the differences we observed between "helped" and "helpless" CD8 T cells. We analyzed splenic CD8 T cells from uninfected WT and MHCII KO mice and found higher expression of Lag-3 and PD-1 on MHCII KO CD8 T cells. (Figure 2.4 A-C). Although these coinhibitory receptors were expressed at very low frequencies of CD8 T cells from uninfected mice compared to cells from the lungs of infected mice, it indicated that MHCII KO CD8 T cells differ from WT CD8 T cells [158]. Therefore, we developed an independent model to validate our findings. Purified splenic CD8 T cells from naïve C57BL/6J mice were transferred into TCRα KO recipients with or without CD4 T cells. As CD4 T cells can transfer protection, which would be a potential confounder, we sought to minimize their contribution to protection by using CD4 T cells from the P25 TCRtg mouse. P25 TCRtg CD4 T cells (hereafter, P25 cells) are specific for Ag85b, a protein that is downregulated by Mtb within weeks of infection [159, 160]. The ability of P25 cells to transfer protection was largely independent of their number. By restricting the diversity and number of transferred CD4 T cells, and selecting an antigen that is transiently expressed, we hoped to limit the impact of CD4 T cells on protection while still providing CD4 T cell help. TCRα KO mice received 5 million CD8 T cells and 100,000 P25 cells (hereafter, helped mice) or only 5 million CD8 T cells (hereafter, helpless mice).

We compared the survival of helped and helpless mice to recipient TCRα KO mice that received only P25 cells or no cells after Mtb infection (Figure 2.5 A). TCRα KO mice have a median survival time (MST) of 39 days. Helpless mice (CD8 T cells only) have a MST of 46 days, which shows that CD8 T cells alone conferred little protection. TCRα KO mice that only received P25 cells survived 88 days (MST). In contrast, helped mice survived 136 days (MST). We determined whether P25 and CD8 T cells synergized to promote survival using the statistical models developed by Demidenko and Miller and based on the Bliss definition of drug independence [161]. The additive effect of P25 and CD8 T cells on survival (i.e., independent effect) can be calculated as $1 - (1-S_A(t))x(1-S_B(t))$, where $S_A(t)$ and $S_B(t)$ are the survival curves of mice received P25 and CD8 T cells, respectively. The curve generated by this function largely overlaps with the survival curve of mice received P25 cells, indicating that CD8 T cells by themselves, contribute little to the overall survival (Figure 2.5 B). The actual survival curve of helped mice is significantly greater than their predicted independent effects, establishing synergy between P25 and CD8 T cells (Figure 2.5 B). We infer that the prolonged survival of mice that received both polyclonal CD8 T cells and P25 cells arose from the action of helped CD8 T cells.

Like the MHCII KO mice, antigen-specific CD8 T cell responses ($TB10.4^+$) were generated in both helped and helpless mice (Figure 2.5 C). Very few $32A_{309-318}$ -specific CD8 T cells were detected. The frequency of $TB10.4_{4-11}$ -specific CD8 T cells was comparable between helped and helpless mice. More CD8 T cells

differentiated into SLECs in helped than in helpless mice at 7 wpi (Figure 2.5 D, E). In contrast, the MPEC frequencies were similar. Thus, this transfer model shows that CD8 responses to Mtb could be generated in the absence of CD4 T cells, but CD4 T cells helped CD8 T cells differentiated into SLECs and together, had a synergistic effect on survival.

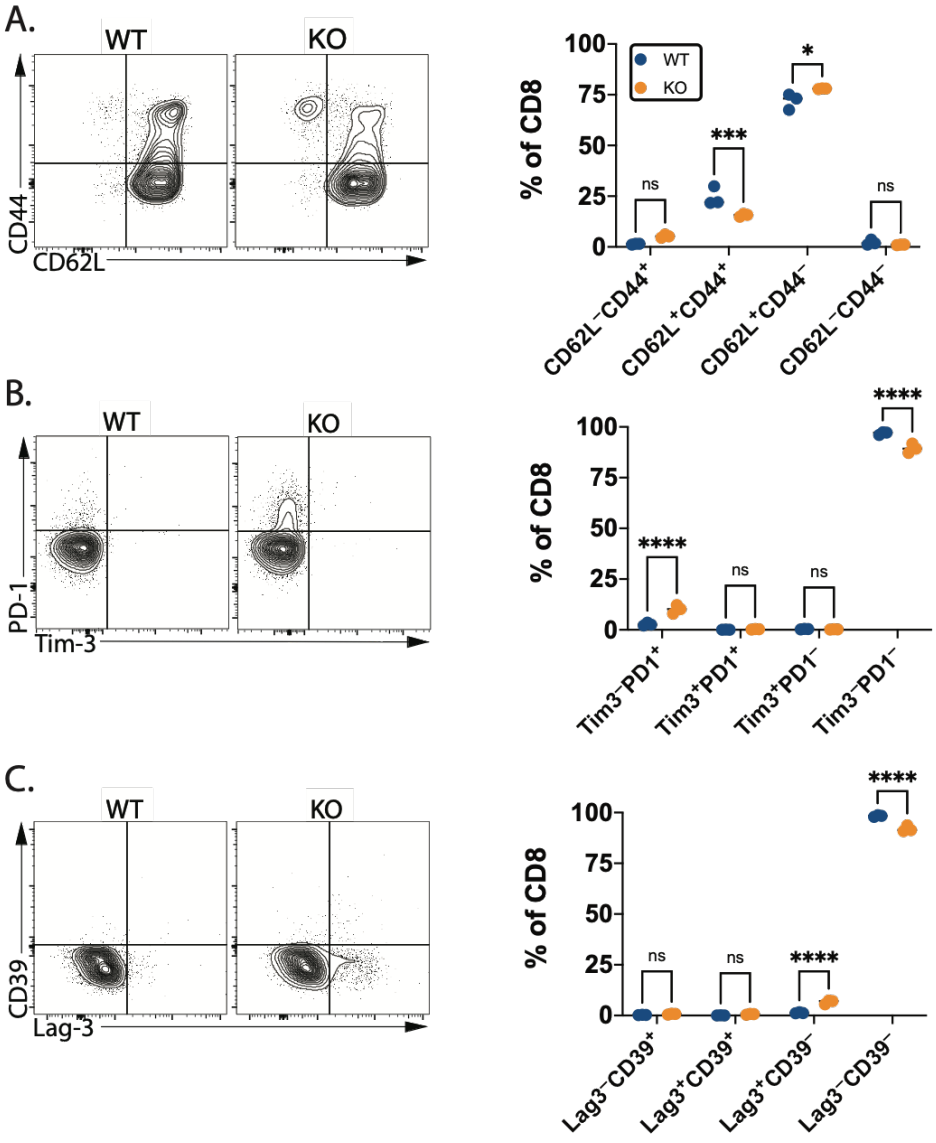

A.
WT
KO
CD44
CD62L
% of CD8
WT
KO
ns
***
*
ns
CD62L⁻CD44⁺
CD62L⁺CD44⁺
CD62L⁺CD44⁻
CD62L⁻CD44⁻
B.
WT
KO
PD-1
Tim-3
% of CD8
****
ns
ns
****
Tim3⁻PD1⁺
Tim3⁺PD1⁺
Tim3⁺PD1⁻
Tim3⁻PD1⁻
C.
WT
KO
CD39
Lag-3
% of CD8
ns
ns
****
****
Lag3⁻CD39⁺
Lag3⁺CD39⁺
Lag3⁺CD39⁻
Lag3⁻CD39⁻

Figure 2. 4 Phenotypic differences between naïve splenic WT and MHCII KO CD8 T cells.

CD8 T cells in spleens of naïve WT or MHCII KO mice were analyzed by flow cytometry. (A) Expressions and percentages of $CD44^{+}CD62L^{-}$, $CD44^{+}CD62L^{+}$, $CD44^{-}CD62L^{+}$, and $CD44^{-}CD62L^{-}$ cells were determined. (B) Expressions and percentages of $PD\text{-}1^{+}Tim\text{-}3^{-}$, $PD\text{-}1^{+}Tim\text{-}3^{+}$, $PD\text{-}1^{-}Tim\text{-}3^{+}$, and $PD\text{-}1^{-}Tim\text{-}3^{-}$ cells were determined. (C) Expression and percentages of $CD39^{+}Lag\text{-}3^{-}$, $CD39^{+}Lag\text{-}3^{+}$, $CD39^{-}Lag\text{-}3^{+}$, and $CD39^{-}Lag\text{-}3^{-}$ are shown. (A-C) Bar, mean. Statistical significance was analyzed by two-way ANOVA. p-values: *, $p<0.05$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

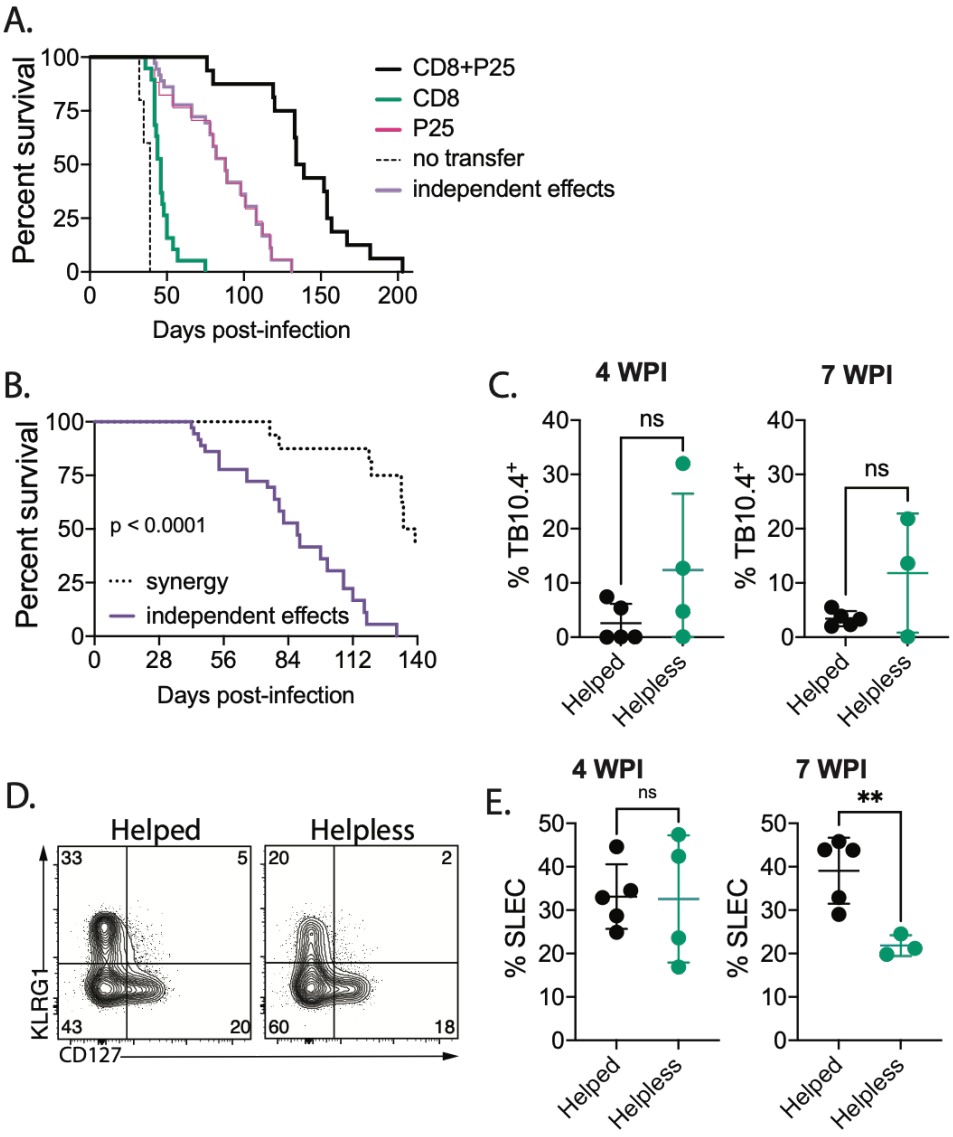

A.
Percent survival
100
75
50
25
0
0
50
100
150
200
Days post-infection
CD8+P25
CD8
P25
no transfer
independent effects
B.
Percent survival
100
75
50
25
0
p < 0.0001
synergy
independent effects
0
28
56
84
112
140
Days post-infection
C.
4 WPI
7 WPI
% TB10.4+
40
30
20
10
0
ns
ns
Helped
Helpless
D.
Helped
Helpless
KLRG1
CD127
33
5
43
20
20
2
60
18
E.
4 WPI
7 WPI
% SLEC
50
40
30
20
10
0
ns
**
Helped
Helpless

Figure 2. 5 An adoptive transfer model confirms the helped vs. helpless CD8 T cell phenotype.

Purified polyclonal CD8 T cells from C57BL/6J mice were transferred to TCRα KO mice with or without Ag85-specific CD4 T cells (P25) (i.e., helped and helpless, respectively), and then infected with aerosolized Mtb. (A) The survival of helped, helpless, TCRα KO mice that received only P25 cells, and TCRα KO mice that didn't receive any cells was monitored. Data were combined from 3 experiments using a total of 17-19 transferred mice per group and 5 TCRα KO mice that received no cells. The difference between the groups was statistically significant ($p<0.0001$) as determined using the log-rank test for trend. (B) Determination of synergy between P25 and CD8 T cells as described in the text. The theoretical additive benefit is calculated by the "independent effects" function, while the "synergy" group is the actual survival observed after transfer of P25 and CD8 T cells (i.e., from (A)). These two scenarios differed statistically ($p<0.0001$) by the log-rank test. (C) Lungs from TCRα KO mice that received P25 and CD8 T cells (helped) vs. only CD8 T cells (helpless) were analyzed at 4 and 7 wpi by flow cytometry to determine the frequencies of $TB10.4_{4-11}$-specific CD8 T cells. (D) The proportion of lung $KLRG1^{+}CD127^{-}$(SLEC) and $KLRG1^{-}CD127^{+}$(MPEC) CD8 T cells was determined 7 wpi. Quadrant numbers represent percentages. (E) The frequencies of lung $KLRG1^{+}CD127^{-}$ (SLEC) among total CD8 T cells was determined 4 and 7 wpi by flow cytometry and analyzed statistically. (C, E) Bars, mean ± SD. Data are representative of three independent experiments, 3-5

mice/group. Statistical significance was analyzed by unpaired t test. p-values: **, $p<0.01$. ns, no significant differences.

Common features of "helped" and "helpless" CD8 T cell in the WT/KO and the adoptive transfer model

We analyzed helped and helpless lung parenchymal CD8 T cells from the adoptive transfer model to determine whether they shared phenotypic characteristics with CD8 T cells from WT and MHCII KO mice. Importantly, we found that in the adoptive transfer model, helped CD8 T cells expressed more of the NK cell receptors 2B4 and NKG2D and transcription factor T-bet. In contrast, helpless CD8 T cells had higher expression of Eomes (Figure 2.6 A).

Next, we created UMAP projections and performed PhenoGraph™ analysis using WT, KO, helped, and helpless CD8 T cells (Figure 2.6 B-E). In this analysis, we compared differences between helped vs. helpless CD8 T cells, as well as differences between the two models (i.e., WT/KO vs. adoptive transfer). The clusters were similar to those identified in the WT/KO model (Figure 2.6 C-E), with one additional cluster ("I", Figure 2.6 B). Helped and helpless CD8 T cells differed in many clusters (Figure 2.6 C-E). Helped CD8 T cells were significantly enriched in cluster A and C, which contained most of the effector cells. In contrast, helpless CD8 T cells were more abundant in cluster F, G, and H, which contained exhausted cells in various states (Figure 2.6 D, E). Thus, the differences between "helped" vs. "helpless" CD8 T cells in the adoptive transfer model were concordant with those identified in the WT/KO model, indicating that the development of T cell exhaustion arises from a lack of T cell help and not from developmental defects in MHCII KO mice.

We then compared WT to helped, and KO to helpless CD8 T cells to visualize differences between these two models (Figure 2.6 C, E). WT and helped CD8 T cells were similar except for an increased frequency of helped CD8 T cells in cluster E, which are progenitor exhausted CD8 T cells. Helpless CD8 T cells were also enriched in cluster E, indicating cluster E was characteristic of the adoptive transfer model. When compared to WT and helped CD8 T cells, KO and helpless CD8 T cells were significantly enriched in cluster F and H, which appear to be exhausted and terminally exhausted cells. The differences in the exhausted states of CD8 T cells from the WT/KO and adoptive transfer models might have arisen from their analysis at different time points, 8 and 6 wpi, respectively. Nevertheless, these data demonstrate shared features of helped and helpless CD8 T cells from the two models. “Helped” CD8 T cells were more likely to express NK receptors and resemble effector CD8 T cells, while “helpless” CD8 T cells were exhausted.

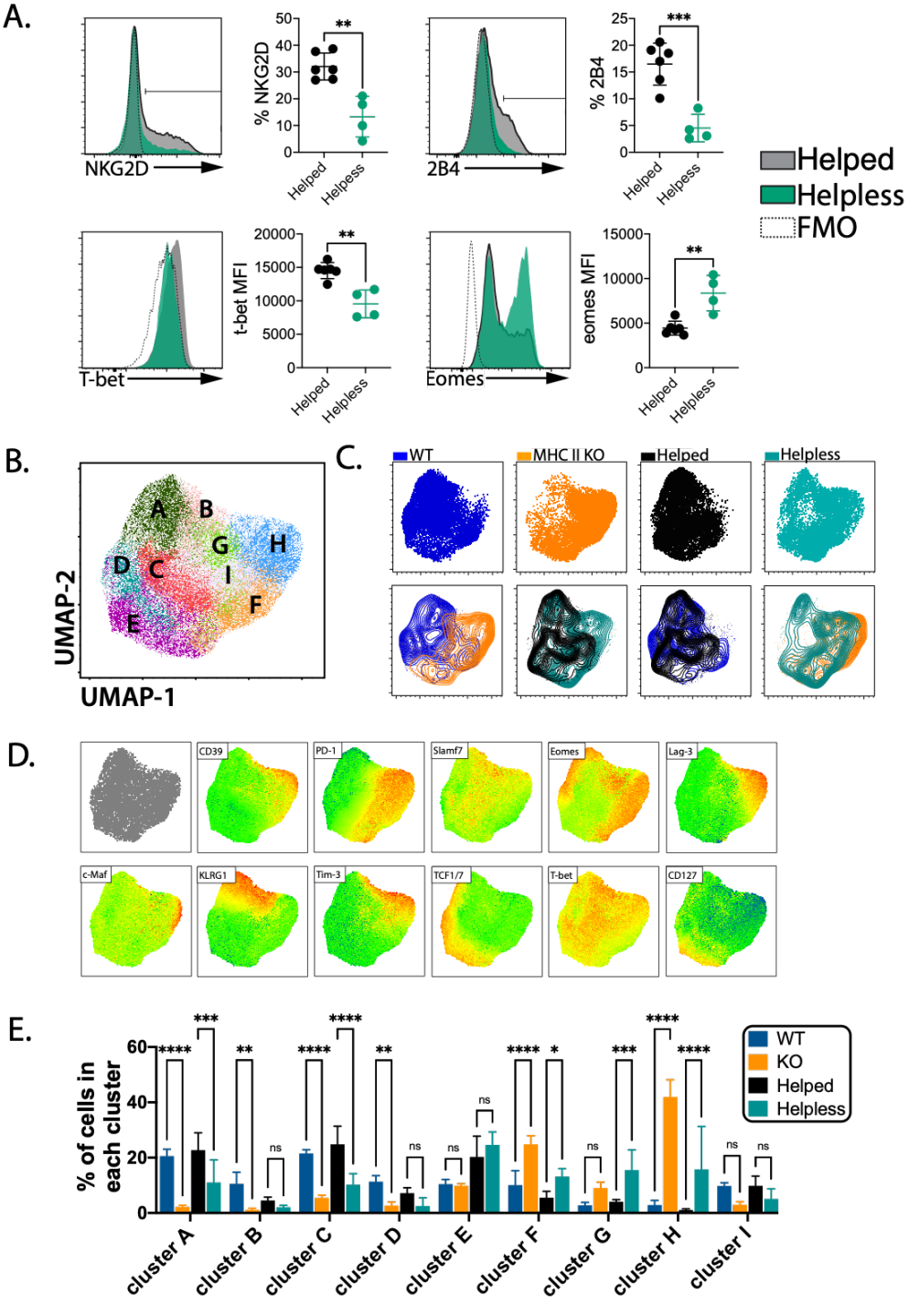

A.
NKG2D
% NKG2D
2B4
% 2B4
T-bet
t-bet MFI
Eomes
eomes MFI
Helped
Helpless
Helped
Helpless
FMO
B.
UMAP-2
UMAP-1
A
B
C
D
E
F
G
H
I
C.
WT
MHC II KO
Helped
Helpless
D.
CD39
PD-1
Slamf7
Eomes
Lag-3
c-Maf
KLRG1
Tim-3
TCF1/7
T-bet
CD127
E.
% of cells in each cluster
WT
KO
Helped
Helpless
cluster A
cluster B
cluster C
cluster D
cluster E
cluster F
cluster G
cluster H
cluster I

Figure 2. 6 Comparison of WT/KO and adoptive transfer model finds shared "helped" and "helpless" characters.

(A) Helped and helpless mice (i.e., the adoptive transfer model) were infected with aerosolized Mtb for 6 weeks. Anti-CD45-AF647 was injected intravenously 3 minutes before euthanasia. Lung parenchymal ($CD45iv^{-}CD44^{+}CD62L^{-}$) CD8 T cells were analyzed by flow cytometry to determine the expression of NK receptors (percentage) and transcription factors (MFI). The histograms show representative data and the accompanying graphs represent the statistical analysis. Bars, mean ± SD. (B-E) Lung parenchymal CD8 T cells from WT/KO mice 8wpi and helped/helpless mice 6wpi were used to create UMAP projections and analyzed with PhenoGraph. (B) Nine clusters were identified with PhenoGraph analysis. (C) Pairwise comparisons of WT, KO, helped, and helpless lung parenchymal CD8 T cells were overlayed onto the UMAP projection. (D) UMAP projections were overlayed with expression of each indicated marker. (E) The percentage of each cluster among WT, KO, helped or helpless lung parenchymal CD8 T cells were compared statistically. Bars, mean ± SD. (A-E) Representative data of two independent experiments, 4-5 mice/group. Statistical significance was analyzed by unpaired t test (A) or two-way ANOVA (E). p values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

CD4 T cell help promotes IFNγ and IL-2 production by CD8 T cells that recognize Mtb-infected macrophages

To gain insight into the functional differences between "helped" vs. "helpless" CD8 T cells, we measured their ability to produce cytokines when stimulated. We showed previously that TB10.$4_{4\text{-}11}$-specific CD8 T cells did not efficiently recognize infected macrophages. As we are unaware of any class I MHC Mtb epitopes that are presented on K^b or D^b by Mtb-infected cells, we chose to use Mtb-infected macrophages as a physiological stimulus. CD8 T cells from Mtb-infected WT or MHCII KO mice (Figure 2.7 A-C) or from Mtb-infected helped and helpless mice (i.e., the transfer model; Figure 2.7 D-F) were purified and cultured with uninfected or Mtb-infected macrophages. The CD8 T cells from WT mice produced more IFNγ, TNF, and IL-2 than CD8 T cells from MHCII KO mice (Figure 2.7 A-C). Similarly, CD8 T cells cotransferred with P25 cells produced more IFNγ and IL-2 than CD8 T cells transferred alone (Figure 2.7 D-E). TNF production was comparable in the adoptive transfer model (Figure 2.7 F). Thus, we showed that CD4 T cells provide help for CD8 T cell production of IFNγ in both of our helped/helpless CD8 T cell models using Mtb-infected macrophages as a physiological stimulus. This is consistent with previous studies that demonstrated a reduction of IFNγ-producing CD8 T cells in the absence of CD4 T cells [85, 87]. Additionally, we show that CD4 T cell help is critical for CD8 T cell production of TNF and IL-2. These functional changes parallel the effector vs. exhausted features that distinguish "helped" vs. "helpless" CD8 T cells in both models.

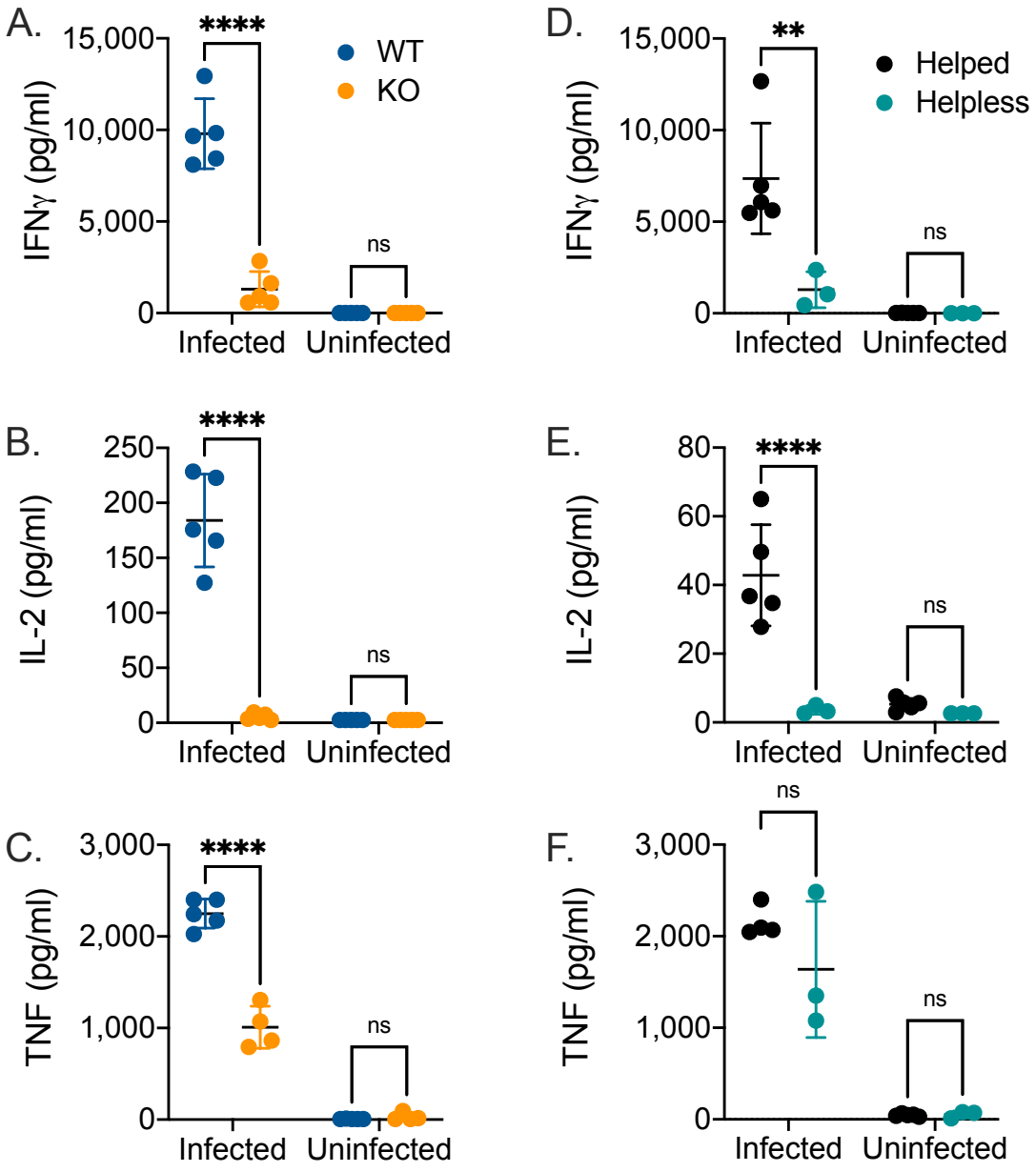

A.
IFNγ (pg/ml)
15,000
10,000
5,000
0
****
ns
WT
KO
Infected
Uninfected
D.
IFNγ (pg/ml)
15,000
10,000
5,000
0
**
ns
Helped
Helpless
Infected
Uninfected
B.
IL-2 (pg/ml)
250
200
150
100
50
0
****
ns
Infected
Uninfected
E.
IL-2 (pg/ml)
80
60
40
20
0
****
ns
Infected
Uninfected
C.
TNF (pg/ml)
3,000
2,000
1,000
0
****
ns
Infected
Uninfected
F.
TNF (pg/ml)
3,000
2,000
1,000
0
ns
ns
Infected
Uninfected

Figure 2. 7 CD4 T cell help enhances CD8 T cell cytokine productions upon recognition of infected macrophages.

Purified CD8 T cells from WT or MHCII KO mice 8wpi (A, B, C) or from helped or helpless mice (i.e., the adoptive transfer model) 7wpi (D, E, F) were cultured with Mtb-infected macrophages (Mϕ) at a CD8:Mϕ ratio = 1:1. IFNγ (A, D), IL-2 (B, E), and TNF (C, F) were determined 18-24 hours later. The data are representative of three experiments with 5 mice/group (A-C) or two independent experiments with 3-5 mice/group (D-F). Bars, mean ± SD. Statistical significance was analyzed by two-way ANOVA. p-values: **, $p<0.01$; ****, $p<0.0001$. ns, no significant differences.

“Helped” CD8 T cells more effectively restrict Mtb growth than “helpless” CD8 T cells

Whether helped CD8 T cells mediate better protection during Mtb infection is unknown. This question is difficult to address *in vivo* because it is difficult to separate the helper function of CD4 T cells from their effector function. Therefore, we quantified the ability of purified “helped” or “helpless” CD8 T cells to restrict intracellular bacterial growth *in vitro*. Using this approach, we found CD8 T cells from the lungs of WT mice inhibited Mtb growth effectively, even when the CD8: macrophage ratio was as low as 1:125 (Figure 2.8 A), although the potency varied between experiments. Bacterial control was MHCI-restricted since CD8 T cells were able to restrict bacterial growth in infected WT macrophages but not in infected K^bD^b KO macrophages (Figure 2.8 B). WT CD8 T cells inhibited Mtb growth significantly better than MHCII KO CD8 T cells (Figure 2.8 C). Interestingly, the colony forming unit (CFU) recovered after adding WT CD8 T cells was sometimes less than the input (i.e., day 1), suggesting that CD8 T cells are capable of promoting killing of Mtb under some conditions. These results were confirmed using CD8 T cells purified from Mtb-infected helped and helpless mice (i.e., the transfer model). Again, helped CD8 T cells inhibited bacterial growth significantly better than helpless CD8 T cells (Figure 2.8 D). The ability of CD8 T cells to control bacterial growth depended on when they were isolated from mice. As MHCII KO mice survive longer after Mtb infection than T cell deficient mice, CD8 T cells must be able to mediate some protection *in vivo*. We hypothesized that CD4 T cell help

is important in maintaining the function of CD8 T cells and preventing exhaustion. Therefore, we compared the ability of WT and MHCII KO CD8 T cells to restrict Mtb growth when isolated from mice at 5 vs. 8 wpi. MHCII KO CD8 T cells obtained after 5 wpi were able to inhibit intracellular bacterial growth while CD8 T cells isolated 8 wpi had lost their ability to restrict Mtb growth (Figure 2.8 E). WT CD8 T cells isolated at both timepoints similarly controlled bacterial growth. In order to compare experiments where Mtb grew differently, the growth from day 1 to endpoint was normalized and the percent inhibition by CD8 T cells was calculated. The 100% inhibition indicated no Mtb growth, while 0% inhibition indicated Mtb grew similarly as in macrophages without CD8 T cells (Figure 2.8 F). This correlated with greater inhibitory receptor expression on MHCII CD8 T cells 8 wpi (Figure 2.8 G). These data show that “helped” CD8 T cells restricted Mtb growth more efficiently than “helpless” CD8 T cells, and this effect is due to a loss of function of “helpless” CD8 T cells late during infection.

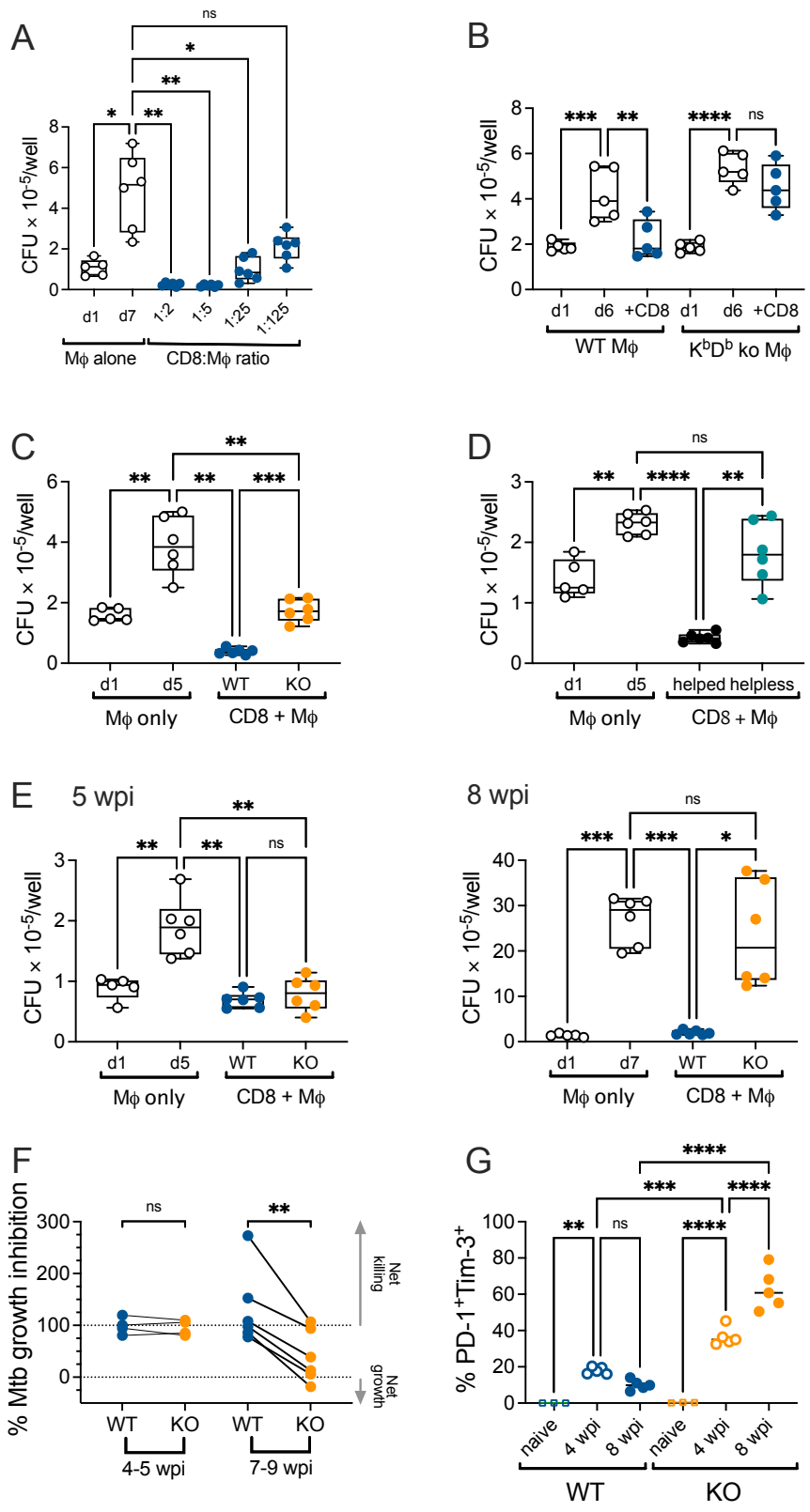

A
B
C
D
E
5 wpi
8 wpi
F
G
CFU × 10-5/well
Mϕ alone
CD8:Mϕ ratio
WT Mϕ
KbDb ko Mϕ
Mϕ only
CD8 + Mϕ
helped helpless
% Mtb growth inhibition
Net killing
Net growth
4-5 wpi
7-9 wpi
% PD-1+Tim-3+
naive
4 wpi
8 wpi
WT
KO

Figure 2. 8 "Helped" CD8 T cells are better at Mtb control than "helpless" CD8 T cells.

Purified CD8 T cells from WT or MHCII KO mice at 8 wpi (A-C, and E), or purified CD8 T cells from helped or helpless mice (i.e, the adoptive transfer model) at 7 wpi (D) were cultured with Mtb infected macrophages (Mϕ) at a CD8:Mϕ ratio = 1:2 unless otherwise indicated. Macrophages were lysed 4-6 days later and the colony forming unit (CFU) per well was determined. (A) Purified WT CD8 T cells were cultured with macrophages at different T cell to macrophage ratios. (B) Purified WT CD8 T cells were cultured with WT or K^bD^b macrophages. (C) Purified WT or MHCII KO CD8 T cells were cultured with macrophages. (D) Purified helped or helpless CD8 T cells were cultured with macrophages. (E) Purified WT or MHCII KO CD8 T cells from 5 or 8 wpi were cultured with macrophages. (F) Percent inhibition by CD8 T cells from early and late timepoints was calculated. The 100% inhibition indicated no Mtb growth since day 1, while 0% inhibition indicated Mtb grew similarly to conditions that had no T cells. (G) Frequencies of PD-1^+Tim-3^+ CD8 T cells from spleens of naïve and lungs of 4 and 8 wpi mice. Bar, mean. (A-E) The whiskers of the Box and whisker plots indicate the maximum and minimum. Representative of 3 (A, D), 2 (B, G),6 (C), or 4 (E) experiments. Statistical significance was analyzed by one-way ANOVA. p-values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

CD8 T cells inhibit intracellular Mtb growth through both IFNγ dependent and independent mechanisms

CD8 T cells can potentially inhibit intracellular Mtb growth through producing IFNγ or/and cytotoxic function. To address the requirement of IFNγ signaling in Mtb growth inhibition, we cultured CD8 T cells with infected IFNγR KO macrophages. We found CD8 T cells significantly restricted Mtb growth even in IFNγR KO macrophages, despite there was a trend for reduced ability of CD8 T cells (Figure 2.9 A, B). We then tested whether perforin dependent mechanism was involved in Mtb growth control. CD8 T cells deficient in perforin were cultured with infected macrophages with different CD8 T cell to macrophage ratios. WT and perforin KO CD8 T cells had comparable abilities to inhibit Mtb growth (Figure 2.9 C, D). Perforin KO CD8 T cells have been shown to have increased ability to produce IFNγ [98]. Thus, to exclude the possibility that more IFNγ made by perforin KO CD8 T cells contributed to the inhibition, we compared WT and perforin KO CD8 T cells' ability to restrict Mtb growth in IFNγR KO macrophages. We showed perforin KO CD8 T cells didn't have impaired ability to restrict Mtb growth even in IFNγR KO macrophages (Figure 2.9 D). These results suggest CD8 T cells use both IFNγ dependent and independent pathways to control intracellular Mtb.

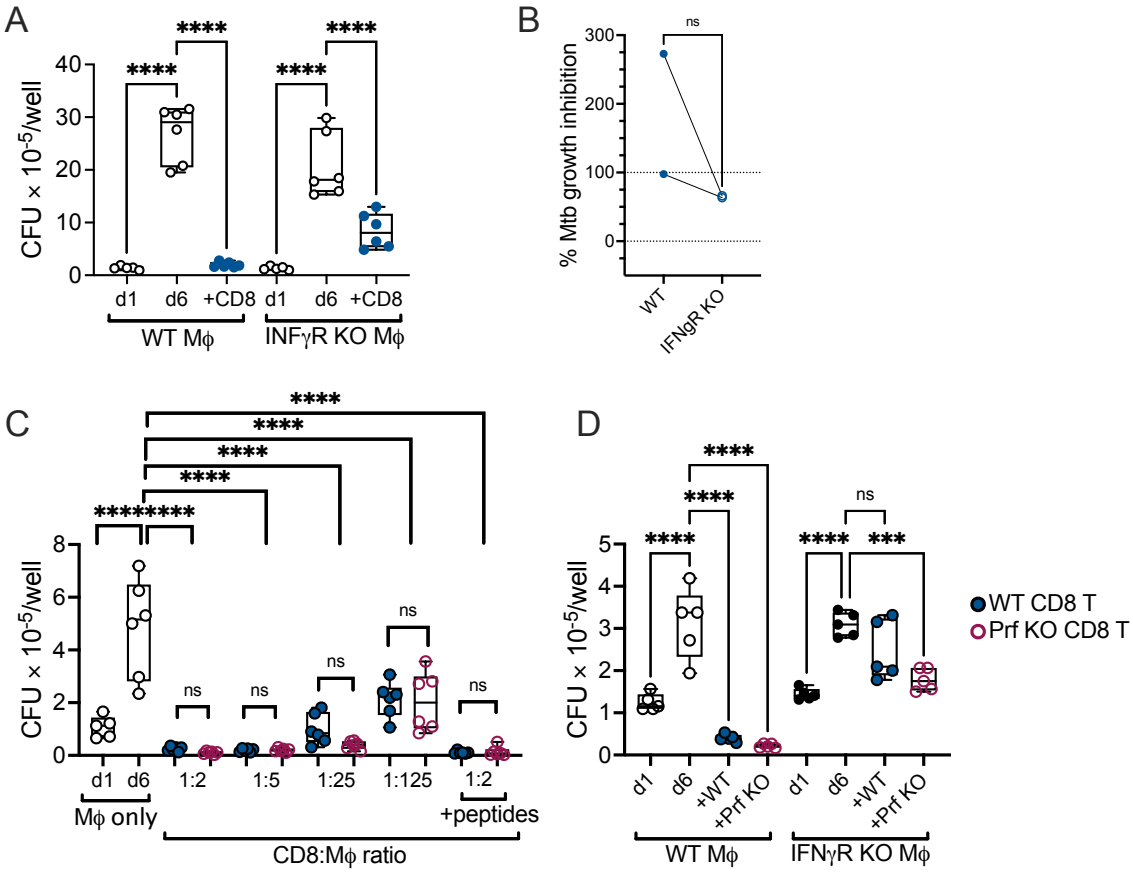

A
CFU x 10-5/well
d1
d6
+CD8
WT Mϕ
INFγR KO Mϕ
B
% Mtb growth inhibition
ns
WT
IFNgR KO
C
CFU x 10-5/well
Mϕ only
CD8:Mϕ ratio
1:2
1:5
1:25
1:125
+peptides
D
CFU x 10-5/well
+WT
+Prf KO
WT Mϕ
IFNγR KO Mϕ
WT CD8 T
Prf KO CD8 T

Figure 2. 9 CD8 T cells inhibit intracellular Mtb growth through both IFNγ dependent and independent mechanisms.

(A) Purified CD8 T cells at 8 wpi were cultured with infected WT or IFNγR KO macrophages (Mϕ) at a CD8:Mϕ ratio = 1:2. Macrophages were lysed 6 days later and the colony forming unit (CFU) per well was determined. (B) Percent inhibition by CD8 T cells in infected WT or IFNγR KO macrophages was calculated from two independent experiments. The 100% inhibition indicated no Mtb growth since day 1, while 0% inhibition indicated Mtb grew similarly to conditions that had no T cells. (C) CD8 T cells from WT or perforin KO mice at 8 wpi were cultured with Mtb infected macrophages at different CD8:Mϕ ratios. Macrophages were lysed 6 days later and the colony forming unit (CFU) per well was determined. (D) CD8 T cells from WT or perforin KO mice at 8 wpi were cultured with infected WT or IFNγR KO macrophages at CD8:Mϕ ratios= 1:2. Macrophages were lysed 6 days later and the colony forming unit (CFU) per well was determined. Statistical difference was analyzed by one-way ANOVA. p-values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Exhausted states accumulate on MHCII KO CD8 T cells as infection progresses

To better characterize how the exhausted features developed on MHCII KO CD8 T cells, lung parenchymal CD8 T cells from MHCII KO mice were analyzed at 3, 5, and 8 wpi by flow cytometry. UMAP projection for each timepoint was created and overlayed onto each other (Figure 2.10 A). We found significant overlaps in UMAP projections between 3 wpi and 5 wpi, while UMAP projections from 5 wpi vs. 8 wpi and 3 wpi vs. 8 wpi were more separated. Phenograph analysis identified 11 distinct clusters and the statistical differences among timepoints were analyzed (Figure 2.10 C, D). Indeed, we found minimal differences between 3 wpi to 5 wpi. In contrast, major differences in exhausted features were found between 5 wpi and 8 wpi. There was a rapid accumulation of terminally exhausted cells (cluster j and k) between 5 wpi and 8 wpi (Figure 2.10 B, D). Greater frequencies of CD8 T cells from 5 wpi were found in clusters appeared to be exhausted progenitors (cluster e and f) (Figure 2.10 B, D). Moreover, CD8 T cells from 5 wpi were also enriched in cluster g. This cluster was $PD1^{+}Tbet^{+}slamf7^{+}$, consisted with previous finding that exhausted cells transiently expressed effector signatures on the way to be terminally exhausted (Figure 2.10 B, D) [155]. We conclude exhausted features develop on MHCII KO CD8 T cells mostly between 5 and 8 wpi.

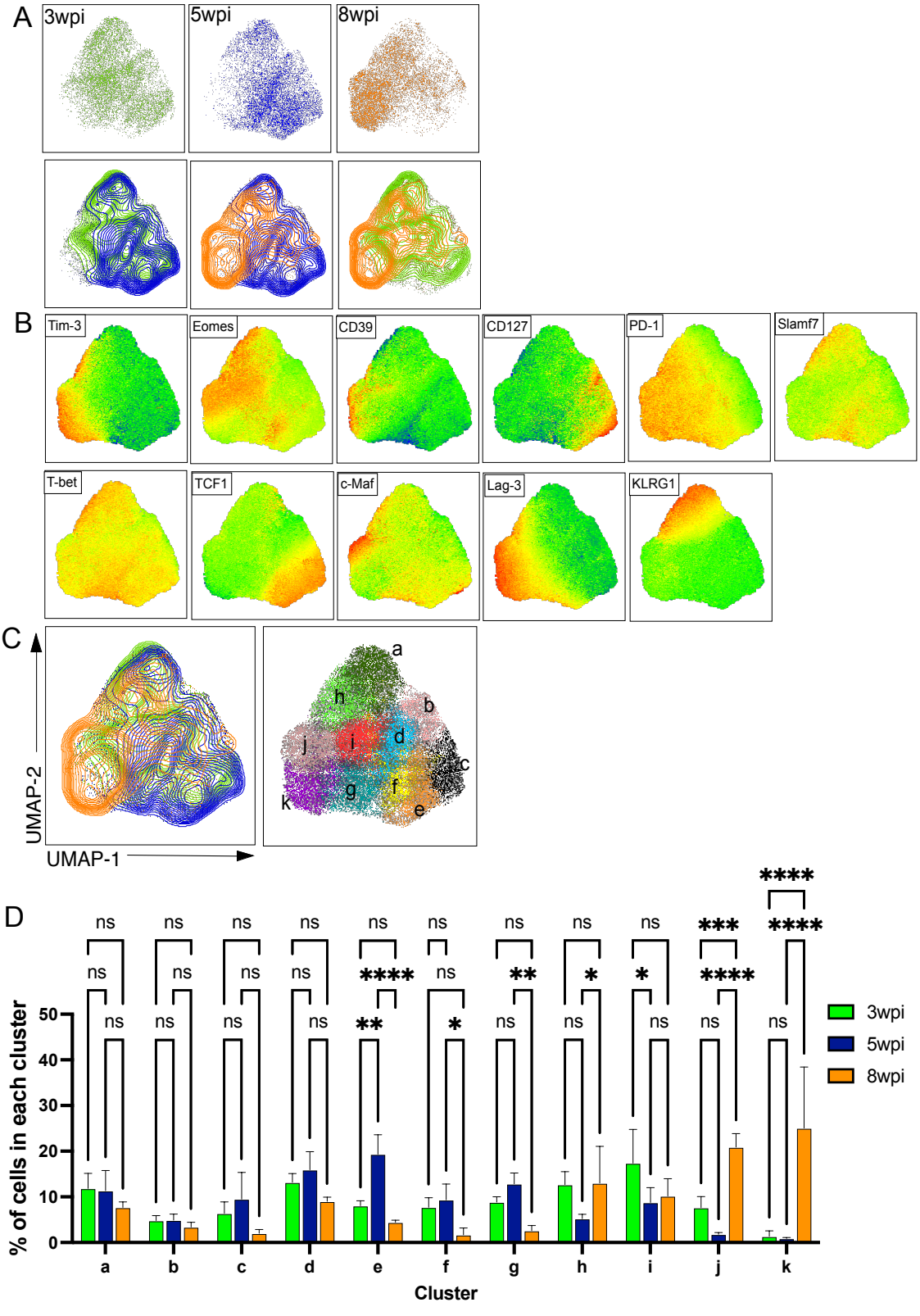

A
3wpi
5wpi
8wpi
B
Tim-3
Eomes
CD39
CD127
PD-1
Slamf7
T-bet
TCF1
c-Maf
Lag-3
KLRG1
C
UMAP-2
UMAP-1
a
b
c
d
e
f
g
h
i
j
k
D
% of cells in each cluster
Cluster
3wpi
5wpi
8wpi
ns
****
***
**
*

Figure 2. 10 Exhausted states accumulate on MHCII KO CD8 T cells as infection progresses.

MHCII KO mice were infected with aerosolized Mtb. Anti-CD45-AF647 was injected intravenously 3 minutes before euthanasia at 3, 5, and 8 wpi. Lung parenchymal CD8 T cells from each timepoint were used to create UMAP projection and analyzed with PhenoGraph. (A) Pairwise comparisons between timepoints were overlayed onto each other. (B) UMAP projections were overlayed with expression of each indicated marker. (C) 11 clusters were identified with PhenoGraph analysis. (D) The percentage of each cluster among timepoints were compared statistically. Bars, mean ± SD. Statistical significance was analyzed by two-way ANOVA (D). p values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Discussion

The relative importance of CD4 and CD8 T cells for immunity to Mtb continues to be debated. Tackling this question is confounded by several issues. CD4 and CD8 T cells differ in the way they are activated. CD8 T cells survey the cytosol, which is sampled by MHCI, while CD4 T cells survey antigens produced in endosomal vesicles or acquired by endocytosis and sampled by MHCII. Thus, assaying T cell responses by in vitro stimulation with exogenous antigen (e.g., PPD or Mtb lysate) biases the results towards CD4 T cells. Optimally stimulating CD8 T cells requires defined epitopes or delivering the antigen into the cytosol. Thus, many immunogenicity studies find vaccines elicit primarily CD4 T cells responses, but this could be prejudiced by the stimulation conditions. Consequently, many investigators focus on vaccine strategies that elicit CD4 T cell effector responses. We hypothesize the potential of CD8 T cells to mediate protection is underestimated because they require CD4 T cell help to optimally express effector functions that inhibit Mtb growth.

We used RNA-seq to detect differences between WT and MHCII KO CD8 T cells during Mtb infection in the mouse model. These “helped” vs. “helpless” features were confirmed by flow cytometry in two independent models. Among the helped CD8 T cells, there were higher frequencies of SLECs and MPECs, and cells that expressed NK receptors. This may explain why CD4 T cells enhanced CD8 T cell lysis of Mtb-infected macrophages [136]. In contrast, helpless CD8 T cells resembled exhausted cells. We measured cytokine productions by CD8 T

cells in response to Mtb-infected macrophages. Helped CD8 T cells secreted more IFNγ, TNF, and IL-2, while helpless CD8 T cells produced less cytokines. Previous studies found CD4 T cells enhance Mtb-specific CD8 T cell production of IFNγ [85, 87] , and our data suggest that maintenance of cytokine production depends on CD4 T cells preventing CD8 T cell exhaustion.

Since CD4 T cells mediate protection during Mtb infection, it's difficult to study CD4 T cell help to CD8 T cells without the confounder of direct protection by CD4 T cells. Because of this, none of the previous studies addressed whether CD4 T cell help is necessary for protection mediated by CD8 T cells. We developed a novel adoptive transfer model to identify characteristics of helped and helpless CD8 T cell responses by using P25 TCRtg CD4 T cells that provide help but only limited protection. The combination of polyclonal CD8 T cells plus P25 CD4 T cells led to a synergistic effect in the survival of Mtb-infected mice. Finally, lung CD8 T cells purified from both of our models and cultured with infected macrophages showed that helped CD8 T cells directly restricted Mtb growth more efficiently than helpless CD8 T cells. Without CD4 T cells in vivo, mice succumb prematurely to TB despite lung IFNγ levels remaining elevated [86]. Thus, it is uncertain whether greater CD8 T cells production of IFNγ in vivo would correlate with protection in the absence of CD4 T cells. These results highlight the requirement of CD4 T cells for protective CD8 T cell responses and indicate that compromised CD8 immunity likely contributes to the early death of mice lacking CD4 T cells.

An underappreciated role of CD4 T cells is to help CD8 T cells resist exhaustion, a state characterized by the expression of multiple co-inhibitory receptors and accompanied by the gradual loss of effector functions. Exhausted CD8 T cells express a unique transcriptional network that inhibits their differentiation into effector or memory cells [153, 162]. How CD8 T cell exhaustion contributes to TB pathogenesis is not completely clear; nevertheless, T cells with features of exhaustion are detected during TB both in mice and in people [163]. Why helpless CD8 T cells become exhausted during the early stages of infection is unknown. One hypothesis is antigen persistence during chronic infection leads to persistent TCR engagement and exhaustion, supported by the finding that the transcription factors downstream of TCR signaling, including IRF4, NFAT, Nr4a1, Nr4a2, and Nr4a3, enhance exhaustion [164-166]. Alternatively, suboptimal priming of CD8 T cells in the absence of CD4 help favor the formation of exhausted progenitors rather than effector cells [131, 167, 168]. Our results are better explained by the chronic antigen stimulation model. We found a difference between WT and MHCII KO mice in inhibitory receptor expression and lung CFU by 4 wpi. We speculate that the higher bacterial burden in MHCII KO mice leads to CD8 T cell exhaustion.

An area for further investigation is the molecular nature of help signals during TB. When CD4 T cells recognize antigen presenting cells (APC), CD40/CD40L interaction, combined with innate signaling by pattern recognition receptors, leads to DC activation, cytokine production and upregulation of co-

stimulatory molecules [134, 135]. Cytokines including IL-12, IL-15, and type I interferon, and co-stimulatory ligands CD70 and CD80/86, are the molecular basis of “help” that DCs relay to CD8 T cells [67]. In addition, CD4 T cells produce IL-2 and IL-21, which promote CD8 T cell survival and acquisition of effector function [169]. Thus, CD4 T cell help CD8 T cells both directly and indirectly. IL-2 treatment also restored T cell responses in a mouse model, in which Mtb proteins were repeatedly administered to study T cell dysfunction [170]. CD8 T cells require IL-21 to control chronic viral infection [125-127], and we showed previously that IL-21 receptor deficiency impairs CD8 T cell responses during TB and leads to increased Tim-3 and PD-1 expression [139]. Whether exhaustion of helpless CD8 T cells develops because of reduced IL-2 or IL-21 levels needs further investigation. An interesting clue is that at 5 wpi, both helped and helpless CD8 T cells restricted Mtb growth in vitro; however, the function of helpless CD8 T cells obtained later during infection seemed to decline. This may indicate that “help” is needed to maintain effector function or prevent T cell exhaustion.

Mtb-specific CD8 T cell responses are detected in infected people and animal models and are recognized as an integral part of immune response to Mtb. CD8 T cells were considered to play a minor role compared to CD4 T cells based on only moderate reduction in the survival of mice with defective CD8 T cell responses compared to normal mice. Here, using a variety of approaches including two independent in vivo models, we unequivocally show that CD4 T cells play an essential role in helping CD8 T cells develop into effector T cells that can restrict

Mtb growth. These data are consistent with a greater appreciation for the role of CD8 T cells in primary Mtb infection and Mtb-challenge after vaccination in the non-human primate model [77, 78]. We suggest that vaccine strategies that enhance synergy between CD4 and CD8 T cells would be more effective at eliciting protective immunity

CHAPTER III. THE MECHANISMS OF CD4 T CELL HELP DURING *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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Attributions:

Conceptualization, Y.L. and S.M.B.; Methodology, Y.L., and S.M.B.; Investigation, Y.L., S.B., and K.C.; Formal analysis, Y.L.; Writing, Y.L.; Supervision, S.M.B.; Funding Acquisition, S.M.B.

Abstract

The molecular mechanisms of CD4 T cell help were addressed. We previously identified helped CD8 T cells as cytotoxic effector cells, and that they mediated protection both *in vitro* and *in vivo*. In contrast, helpless CD8 T cells were at different states of exhaustion. Here, we studied the involvements of IL-21, IL-2, and CD40-CD40L signalings in shaping the helped vs. helpless CD8 T cell characteristics. Whether these pathways were required for CD8 T cell mediated protection were also investigated. We found IL-21 promotes the cytotoxic features of helped CD8 T cells. In the absence of IL-21 signaling, CD8 T cells have impaired ability to inhibit intracellular Mtb growth. On the other hand, treating helpless mice with anti-CD40 agonist antibody partially restored CD8 T cell exhaustion, and we observed a reduction in splenic bacterial burden in anti-CD40 treated mice. Lastly, we showed that adding exogenous IL-2 modestly improved helpless CD8 T cells' ability to restrict intracellular Mtb growth. These results suggest the complexity of the help signals CD4 T cells provided to CD8 T cells, in which signals directly from CD4 T cells and signals relayed by antigen presenting cells (APCs) are involved.

Introduction

A field remaining to be studied is the molecular mechanisms of CD4 T cell help in TB. CD4 T cells can directly help CD8 T cells by producing cytokines that support the survival, expansion, and effector functions of CD8 T cells, including IL-21 and IL-2. In addition, CD4 T cell help can be delivered to CD8 T cells through antigen presenting cells (APCs). Once receiving activating signaling through CD40, which engages with CD40L on activated CD4 T cells, APCs upregulate expression of MHCI and co-stimulatory receptors and production of pro-inflammatory cytokines. These stimulating signals then act on CD8 T cells, shaping their differentiation and functions. Many studies have shown several signaling pathways involved in this process, however, the requirement of these pathways in promoting the “helped” CD8 T cell responses during TB is unknown. In this chapter, I studied the roles of IL-21, IL-2, and CD40-CD40L as help signals to generate optimal CD8 T cell responses.

IL-21, IL-2, and CD40-CD40L signaling have well-established roles in CD4 T cell help. IL-21 is essential for CD4 T cells to sustain antiviral activity of CD8 T cell responses in chronic viral infection [125-127], and it maintains CD8 T cell function through induction of transcription factor BATF [171]. IL-2 was identified as an important mediator of CD4 T cell help for generating memory CD8 responses. IL-2 produced by CD4 T cells, or by CD8 T cells after activated by licensed DCs, inhibits TRAL expression and prevents apoptosis of memory CD8 T cells. CD40-

CD40L signaling was the first reported molecular mechanism of CD4 T cell help [134, 135]. Because of its role in activating APCs and providing stimulating signals to CD8 T cells, anti-CD40 agonist is included in vaccine strategies to substitute the need for CD4 T cells, and it has been used for cancer immunotherapy to generate anti-tumor responses or combined with inhibitory receptor blockade. Moreover, IL-21 and IL-2 has been suggested to play a role in antagonizing CD8 T cell exhaustion.

The contribution of these signaling pathways in protective immunity to Mtb has been studied. Deficiency in IL-21 signaling resulted in increased susceptibility. IL-21R KO mice have higher bacterial burden and succumbed early after infection, which has been attributed to the impaired T cell priming, expansion, and effector function in the absence of IL-21 signaling [139]. CD4 T cells producing IFNγ, TNFa, and IL-2 are associated with protection in TB, and recombinant IL-2 has been used as immunotherapy to pulmonary TB. CD40 KO mice also died prematurely after Mtb infection, although it's likely due to defective IL-12 production. Whether these signals contribute to immunity against Mtb due to their role as "help signals" is unknown.

We had identified "helped" vs. "helpless" CD8 T cell features previously. While helped CD8 T cells are effector cells with cytotoxic ability, helpless CD8 T cells are exhausted. We also developed a Mtb growth inhibition assay to quantify CD8 T cell ability to restrict Mtb *in vitro*. Here, we tested the requirement of help signals in shaping the cytotoxic vs. exhausted states on CD8 T cells. In addition,

whether providing/ blocking help signals alters CD8 T cells' ability to inhibit Mtb growth was studied. Finally, we determined whether reinvigoration of exhausted CD8 T cells improved Mtb control. We found IL-21 enhanced cytotoxicity of CD8 T cells, while anti-CD40 agonist partially restored exhausted CD8 T cell features after infection. Furthermore, Mtb growth inhibition by MHCII KO CD8 T cells was improved by IL-2, but not inhibitory receptor blockade.

Results

IL-21 signaling enhances NK receptor, but prevents inhibitory receptor expressions on CD8 T cells

To test whether IL-21 signaling is involved in CD4 T cell help to CD8 T cells during TB, we measured the "helped vs. helpless" features on $CD44^{+}CD62L^{-}$ CD8 T cells in lungs of WT and IL-21R KO mice after infection. A greater percentage of WT CD8 T cells expressed NK receptor NKG2A/C/E, and the frequencies of $NKG2A/C/E^{+}NKG2D^{+}$ or $2B4^{+}CD226^{+}$ CD8 T cells among total CD8 T cells were also greater in WT mice (Figure 3.1 A). Moreover, we showed higher expression of transcription factor T-bet in WT CD8 T cells (Figure 3.1 B). WT and IL-21R KO CD8 T cells had similar frequency in co-expression of inhibitory receptors Tim-3 and PD-1, which are usually an indicator of terminally exhausted cells. Nevertheless, when we performed UMAP and Phonograph analysis, greater frequencies of IL-21R KO CD8 T cells were found in cluster 7, which appeared to be terminally exhausted cells based on its expression of PD1, Tim-3, Lag3, CD39, and transcription factor c-Maf (Figure 3.1 C-E). These features indicate WT CD8 T cells have effector states with cytotoxic function, while greater frequencies of IL-21R KO CD8 T cells are exhausted. Although we identified helped vs. helpless features of WT and IL-21R KO CD8 T cells, the differences were much smaller compared to what we found in the WT/MHCII KO and the adoptive transfer model. This may be due to the analysis on total lung CD8 T cells rather than parenchymal

CD8 T cells. Since intravascular CD8 T cells didn't interact with infected cells, they expressed different features and may have diluted the phenotypes we observed here. Nevertheless, we showed IL-21 signaling is involved in shaping helped and helpless features of CD8 T cells.

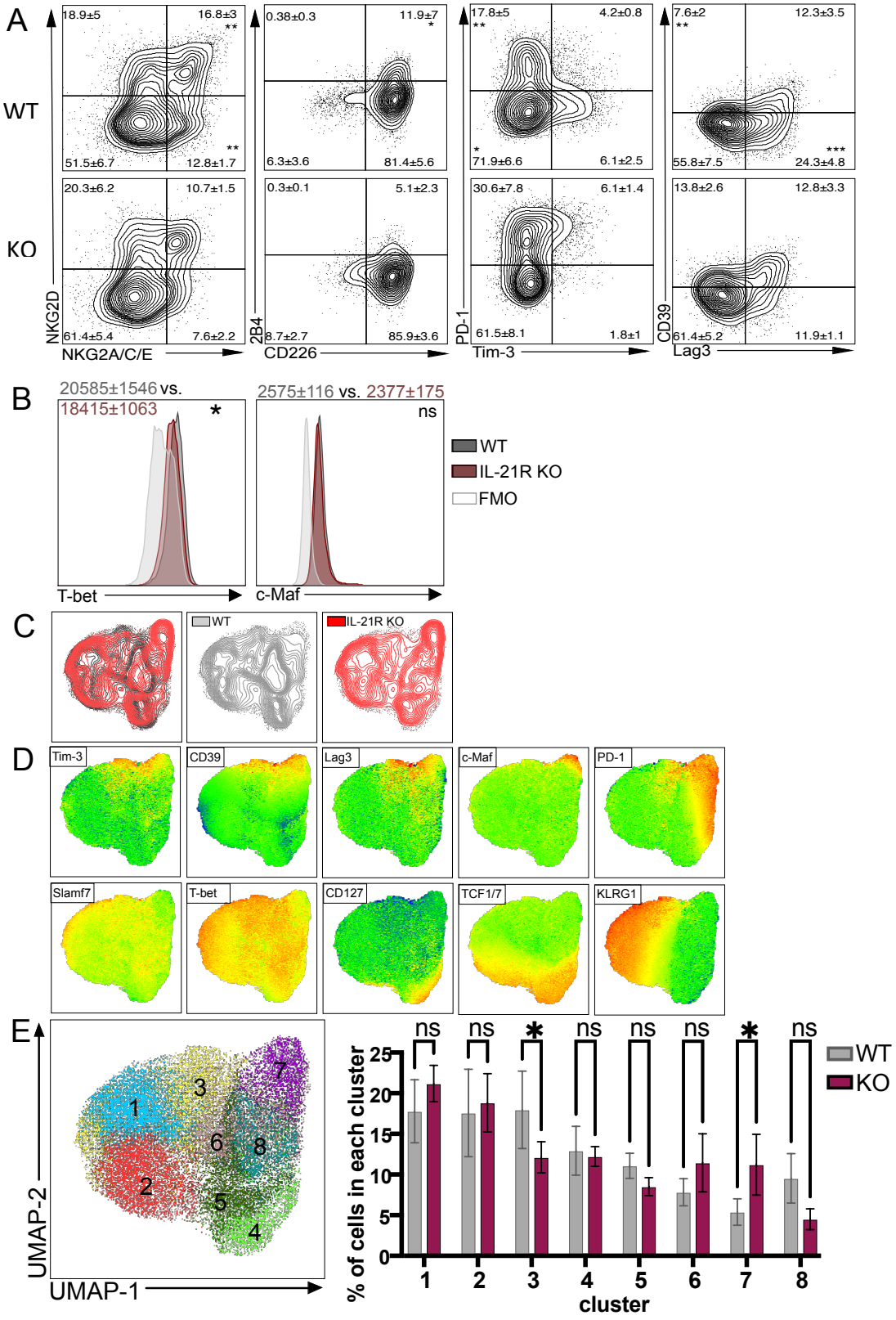
A
WT
KO
NKG2D
NKG2A/C/E
18.9±5
16.8±3
51.5±6.7
12.8±1.7
20.3±6.2
10.7±1.5
61.4±5.4
7.6±2.2
2B4
CD226
0.38±0.3
11.9±7
6.3±3.6
81.4±5.6
0.3±0.1
5.1±2.3
8.7±2.7
85.9±3.6
PD-1
Tim-3
17.8±5
4.2±0.8
71.9±6.6
6.1±2.5
30.6±7.8
6.1±1.4
61.5±8.1
1.8±1
CD39
Lag3
7.6±2
12.3±3.5
55.8±7.5
24.3±4.8
13.8±2.6
12.8±3.3
61.4±5.2
11.9±1.1
B
20585±1546 vs.
18415±1063
T-bet
2575±116 vs. 2377±175
ns
c-Maf
WT
IL-21R KO
FMO
C
WT
IL-21R KO
D
Tim-3
CD39
Lag3
c-Maf
PD-1
Slamf7
T-bet
CD127
TCF1/7
KLRG1
E
UMAP-2
UMAP-1
1
2
3
4
5
6
7
8
% of cells in each cluster
cluster
WT
KO
ns

Figure 3. 1 IL-21 signaling enhances NK receptor, but prevents inhibitory receptor expressions on CD8 T cells.

WT and IL-21R KO mice were infected with aerosolized Mtb for 8 weeks. (A) $CD44^{+}CD62L^{-}$ lung CD8 T cells were analyzed by flow cytometry to determine the frequencies of NK and inhibitory receptors. The mean % ± SD for each quadrant is indicated (n=5/group). The percentage of WT vs. KO cells in each quadrant was compared, and if statistically significant, is indicated in the WT quadrant. (B) The median fluorescence intensity (MFI) of each transcription factor, ± SD, are indicated. UMAP projections from the flow cytometric analysis of $CD44^{+}CD62L^{-}$ lung CD8 T cells were overlayed between WT or KO CD8 T cells (C), or with expression of the indicated markers (D). (E) Eight clusters were identified using PhenoGraph and the percentage of each cluster among WT or IL-21R KO CD8 T cells were analyzed statistically. Bars, mean ± SD. Experiment was performed with 5 mice/group. Statistical significance was analyzed by two-way ANOVA. p-values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; #, $p<0.0001$. ns, no significant differences.

IL-21R KO CD8 T cells have impaired ability to control Mtb growth

To determine whether IL-21R KO CD8 T cells lost the ability to restrict intracellular Mtb growth as we observed on helpless CD8 T cells from two other models, purified CD8 T cells from Mtb infected WT or IL-21R KO mice at 8wpi were cultured with Mtb infected macrophages. We showed IL-21R KO CD8 T cells had impaired ability to control Mtb growth at low T cell to macrophage ratio (i.e., T: macrophage = 1:5). We didn't observe any difference in ability of WT and IL-21R KO CD8 T cells to inhibit Mtb growth at high T cell to macrophage ratio, which could reflect WT CD8 T cells having better cytotoxic function that required cell contact to mediate Mtb control. In conclusion, defective IL-21 signaling on CD8 T cells hindered their restriction of Mtb growth *in vitro* (Figure 3.2).

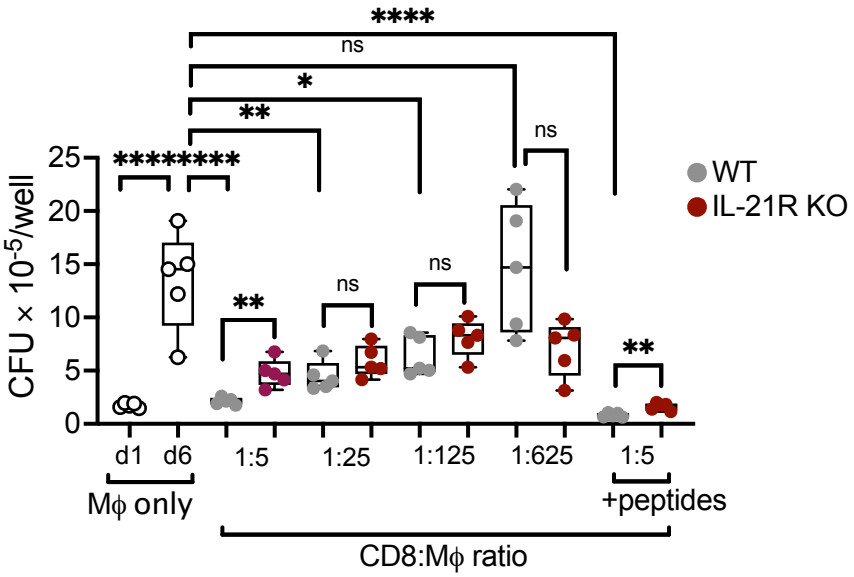

Figure 3. 2 IL-21R KO CD8 T cells have impaired ability to control Mtb growth. Purified CD8 T cells from WT or IL-21R KO mice at 8wpi were cultured with Mtb infected macrophages (Mϕ) at different CD8 T cell to Mϕ ratio. Macrophages were lysed 6 days later and colony forming unit (CFU) per well was determined. The whiskers of the box plots indicate the maximum and minimum. Statistical significance was analyzed by one-way ANOVA (compared to d6) and multiple t tests (between WT and IL-21R KO CD8 T cells). P values: *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$. ns, no significant differences.

Anti-CD40 treatment partially reverts the exhaustion feature on helpless CD8 T cells

We considered whether providing the "help signal" can restore the helpless state of MHCII KO CD8 T cells. Anti-CD40 was intraperitoneally injected into MHCII KO mice every week after Mtb infection, and at 7wpi, lung parenchymal CD8 T cells from these mice were analyzed and compared to MHCII KO mice that didn't receive the treatment. We found a decreased frequency of CD8 T cells that expressed both PD-1 and Tim-3, but an increased frequency of CD8 T cells that expressed neither receptors in anti-CD40 treated MHCII KO mice (Figure 3.3 A). This suggested that a lower frequency of terminally exhausted CD8 T cells was generated following anti-CD40 treatment. Indeed, when we performed UMAP and PhenoGraph analysis, there was a lower frequency of anti-CD40 treated MHCII KO CD8 T cells in cluster k, in which appeared to be terminally exhausted cells, characterized by the co-expression of PD1, Tim3, Lag3, CD39, and transcription factor c-Maf and Eomes (Figure 3.3 C-E). Anti-CD40 treatment also decreased the frequency of cells in cluster g, the cluster characterized by the expression of PD-1, Tim-3, and transcription factor T-bet and was phenotypically similar to exhausted cells in transition states. In contrast, anti-CD40 treatment increased the frequency of cells that were KLRG1^{+}Tbet^{+}PD1^{-}, which were likely effector cells (i.e., cluster a). However, there weren't as many effector clusters as we found in WT CD8 T cells. The frequencies of CD8 T cells that expressed NK receptors, including NKG2D, NKG2A/C/E, and 2B4, didn't increase in anti-CD40 treated

MHCII KO mice (Figure 3.3 A). In summary, anti-CD40 treatment partially restores the exhausted states of MHCII KO CD8 T cells, but doesn't reproduce the cytotoxic effector cells that we observed in helped CD8 T cells.

To see if the reverted exhaustion features correlate with control of bacterial burden, we compared bacterial burdens in lungs and spleens of anti-CD40 treated vs. non-treated MHCII KO mice. Although the bacterial burdens in lungs were comparable, we found a reduced splenic bacterial burden in anti-CD40 treated compared to the non-treated MHCII KO mice. The similar bacterial burdens in lungs perhaps reflect the modest restoration of exhaustion, and the absence of cytotoxic effector cells after anti-CD40 treatment (Figure 3.4).

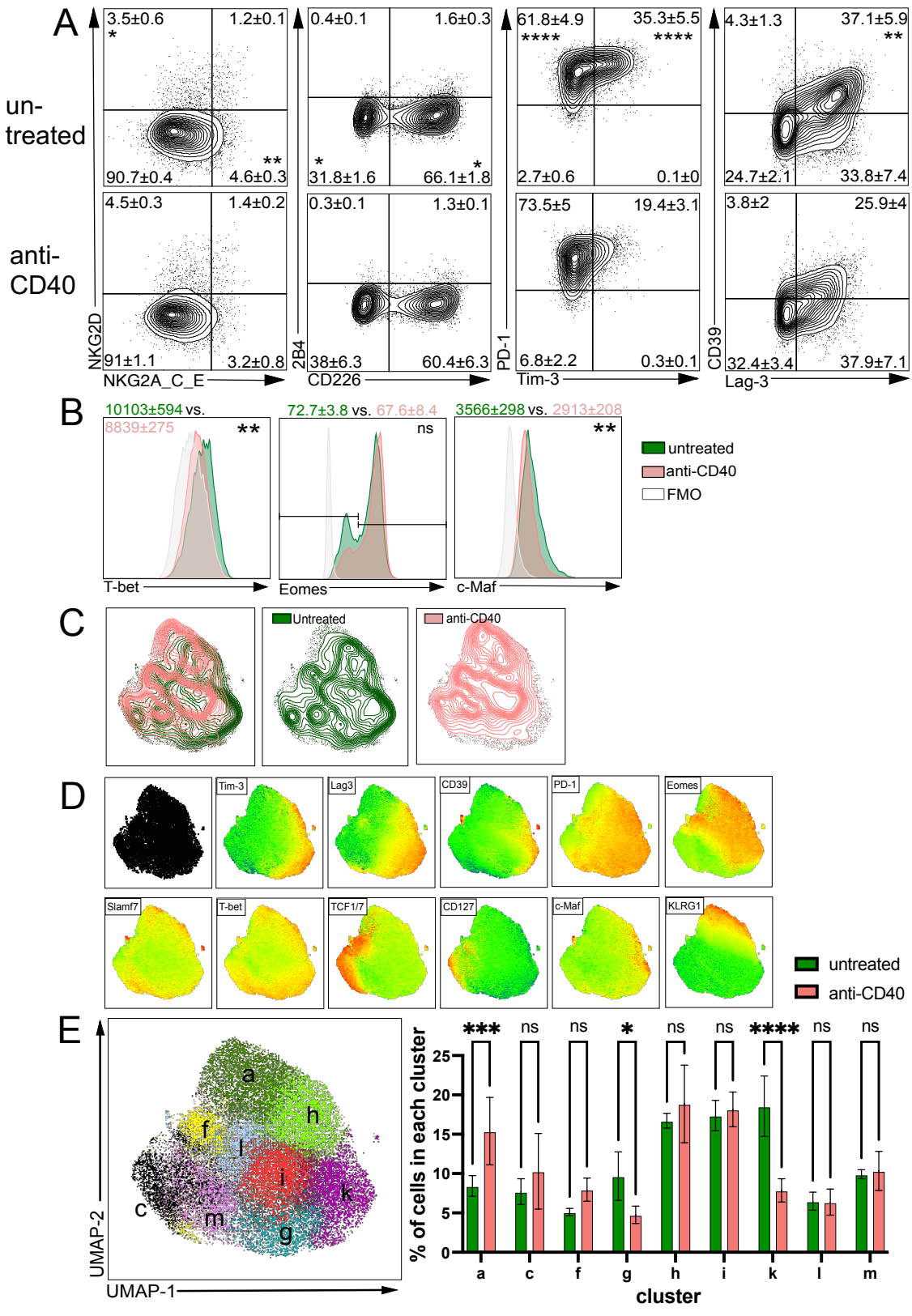
A
un-treated
anti-CD40
3.5±0.6
1.2±0.1
90.7±0.4
4.6±0.3
0.4±0.1
1.6±0.3
31.8±1.6
66.1±1.8
61.8±4.9
35.3±5.5
2.7±0.6
0.1±0
4.3±1.3
37.1±5.9
24.7±2.1
33.8±7.4
4.5±0.3
1.4±0.2
91±1.1
3.2±0.8
0.3±0.1
1.3±0.1
38±6.3
60.4±6.3
73.5±5
19.4±3.1
6.8±2.2
0.3±0.1
3.8±2
25.9±4
32.4±3.4
37.9±7.1
NKG2D
NKG2A_C_E
2B4
CD226
PD-1
Tim-3
CD39
Lag-3
B
10103±594 vs. 8839±275
72.7±3.8 vs. 67.6±8.4
3566±298 vs. 2913±208
T-bet
Eomes
c-Maf
untreated
anti-CD40
FMO
C
Untreated
anti-CD40
D
Tim-3
Lag3
CD39
PD-1
Eomes
Slamf7
T-bet
TCF1/7
CD127
c-Maf
KLRG1
untreated
anti-CD40
E
UMAP-2
UMAP-1
% of cells in each cluster
cluster
a
c
f
g
h
i
k
l
m

Figure 3. 3 Anti-CD40 treatment reverts the exhaustion feature on helpless CD8 T cells.

Anti-CD40 was intraperitoneally injected into aerosolized Mtb infected MHCII KO mice at day 8 post infection, and once every week until 7 wpi. (A) Anti-CD45-AF647 was injected intravenously 3 minutes before euthanasia of anti-CD40 treated or untreated mice, and lung parenchyma ($CD45iv^{-}CD44^{+}CD62L^{-}$) CD8 T cells were analyzed by flow cytometry to determine the frequencies of NK and inhibitory receptors. The mean % $\pm$ SD for each quadrant is indicated. The percentage of cells between anti-CD40 treated and untreated in each quadrant was compared, and indicated in the untreated quadrant if statistically significant. (B) The median fluorescence intensity (MFI) or the average percentage of cells expressing each transcription factor, ± SD, are indicated. UMAP projections from the flow cytometric analysis of lung parenchymal CD8 T cells were overlayed between anti-CD40 treated or untreated group (C), or with expression of the indicated markers (D). (E) Nine clusters that were identified using PhenoGraph, and clusters that were phenotypically similar to clusters found in Figure 2.10 were assigned with the same letters. The percentage of each cluster among anti-CD40 treated or untreated KO CD8 T cells were analyzed statistically. Bars, mean ± SD. Experiment was performed with 5 mice/group. Statistical significance was analyzed by two-way ANOVA. p-values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; #, $p<0.0001$. ns, no significant differences.

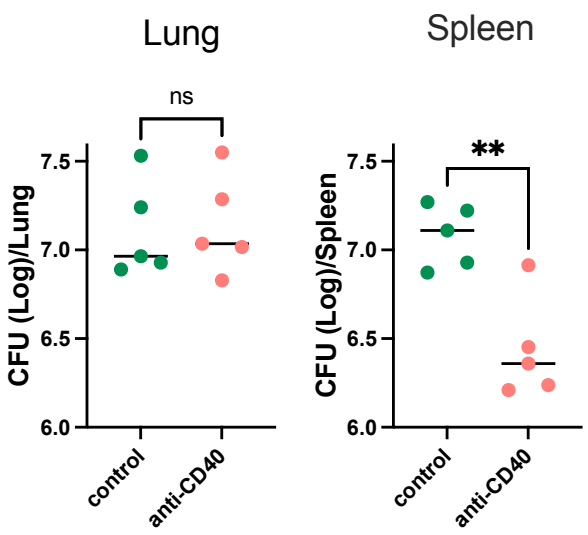

Figure 3. 4 Anti-CD40 treatment reduces bacterial burden in spleens of MHCII KO mice.

Anti-CD40 was intraperitoneally injected into aerosolized Mtb infected MHCII KO mice at day 8 post infection, and once every week until endpoint. At 7 wpi, anti-CD40 treated or untreated mice were euthanized and bacterial burden at lungs and spleens were determined. Experiment was performed with 5 mice/group. Bars, mean $\pm$ SD. Statistical significance was analyzed by unpaired t test. **, $p<0.01$. ns, no significant differences.

IL-2 slightly enhances the inhibition of Mtb growth by CD8 T cells

IL-2 is a known “help signal” that CD4 T cells deliver to improve CD8 T cell survival and function. Moreover, IL-2 treatment has been used to revert T cell exhaustion. We tested whether adding exogenous IL-2 when co-culturing CD8 T cells and macrophages improved CD8 T cells’ ability to inhibit Mtb growth. We found that adding IL-2 slightly enhanced the inhibition by both WT and MHCII KO CD8 T cells, although this effect was neither statistically significant nor dose-dependent (Figure 3.5 A-C). The modest improvement by WT CD8 T cells was likely due to the great inhibition they already mediated in the absence of IL-2. Despite the limited improvement of IL-2 treated compared to non-treated MHCII KO CD8 T cells, IL-2 treated MHCII KO CD8 T cells marginally inhibited Mtb growth compared to the condition without CD8 T cells (i.e., d6) (Figure 3.5 A,B). Furthermore, anti-IL-2 blocking antibody had minimal effect on control of Mtb growth by WT CD8 T cells, nor did it aggravate the loss of function on MHCII KO CD8 T cells (Figure 3.5 A). In summary, adding IL-2 *in vitro* has limited effects to restore the ability of MHCII CD8 T cells.

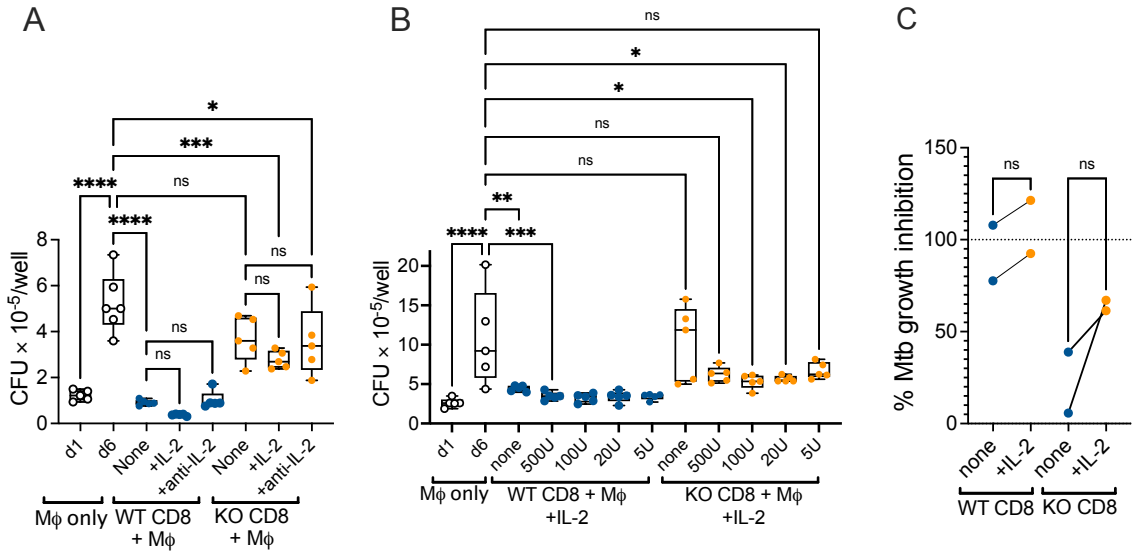

Figure 3. 5 IL-2 treatment marginally enhances CD8 T cells' ability to inhibit Mtb growth.

(A-B) Purified CD8 T cells from WT or MHCII KO mice at 8 wpi were cultured with Mtb infected macrophages (Mϕ) at a ratio CD8: Mϕ =1:3 in the presence of 100U/ml IL-2, unless indicated, or 25 ug/ml of anti-IL-2. Macrophages were lysed 6 days later and colony forming unit (CFU) per well was determined. (C) Percent inhibition by CD8 T cells with or without 100U/ml of IL-2 from (A) and (B) was calculated. The 100% inhibition indicated no Mtb growth since day 1, while 0% inhibition indicate Mtb grew similarly to conditions that have no CD8 T cells. The whiskers of the box plots indicate the maximum and minimum. Statistical significance was analyzed by one-way ANOVA (A-B) and Mixed-effects analysis (C). p values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Inhibitory receptors blockade does not improve MHCII KO CD8 T cells to control Mtb growth

Inhibitory receptor blockade has been used to revert T cell exhaustion. We tested whether in the presence of anti-inhibitory receptor blocking antibodies, MHCII KO CD8 T cells controlled intracellular Mtb growth. CD8 T cells from Mtb infected WT or MHCII KO mice at 8wpi were purified and cultured with infected macrophages in the presence of anti-PD1, anti-Tim-3, combination of anti-PD1 and anti-Tim-3, or isotype antibodies. Macrophages were lysed after 6 days and plated to assay intracellular Mtb burden. MHCII KO CD8 T cells marginally controlled Mtb growth when given anti-Tim3, but failed to inhibit Mtb growth in the presence of anti-PD1 or both anti-PD1 and anti-Tim-3 antibodies (Figure 3.6). Inhibitory receptor blockade didn't enhance the control by WT CD8 T cells as expected, since low frequencies of WT CD8 T cells expressed these inhibitory receptors. We conclude inhibitory receptor blockade doesn't restore the ability of MHCII KO CD8 T cells to restrict Mtb growth.

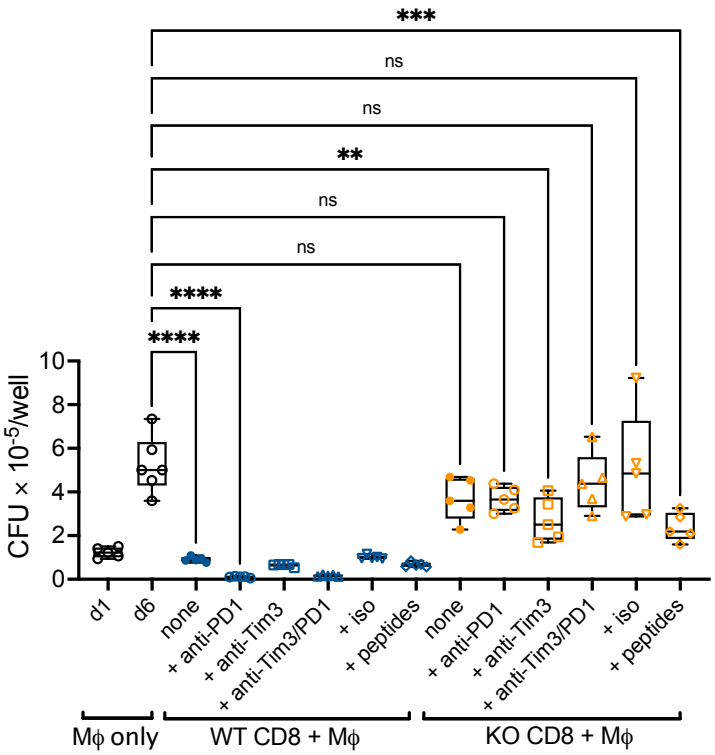

Figure 3. 6 Inhibitory receptors blockade does not restore MHCII KO CD8 T cells to control intracellular Mtb growth.

Purified CD8 T cells from WT or MHCII KO mice at 8wpi were cultured with Mtb infected macrophages at a ratio CD8 T: M =1:3 in the presence of 25 ug/ml of anti-PD-1, anti-Tim-3, combination of anti-PD-1 and anti-Tim-3, or isotype control. 10 μm of $TB10.4_{4\text{-}11}$ and $32A_{309\text{-}318}$ peptides were added to enhance CD8 T cell recognition in one of the conditions. Macrophages were lysed 6 days later and colony forming unit (CFU) per well was determined. The whiskers of the box plots indicate the maximum and minimum. Statistical significance was analyzed by one-way ANOVA. p values: **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Discussion

In this chapter, the molecular mechanisms of CD4 T cell help during TB were addressed. The signaling pathways involved in promoting cytotoxic function and preventing exhaustion of CD8 T cells, and their requirements of Mtb restriction *in vitro* or *in vivo* need to be investigated. IL-21, IL-2, and CD40-CD40L signaling were tested because of their well-established roles in CD4 T cell help, and their suggested contribution in immunity to Mtb. I found IL-21 and IL-2 signaling played roles in the inhibition of intracellular Mtb growth. The ability of CD8 T cells to control Mtb growth *in vitro* were decreased in the absence of IL-21 signaling, while adding exogenous IL-2 modestly improved Mtb growth restriction. In contrast, anti-CD40 treatment partially reverted exhausted CD8 T cells from lungs of Mtb-infected MHCII KO mice. Although anti-CD40 treatment had limited effect on diminishing Mtb burden in lungs of MHCII KO mice, we observed a significant reduction in splenic bacterial burden.

Our results highlighted the role of IL-21 signaling in cytotoxic CD8 T cells. CD8 T cells from infected IL-21R KO mice express a lower percentage of NK receptor NKG2A/C/E and 2B4, suggesting they had impaired cytolytic functions. Indeed, IL-21R KO CD8 T cells have decreased ability to inhibit Mtb growth when cultured with infected macrophages. These findings are consistent with a previous study that IL-21 was indispensable for generating cytotoxic CD8 T cells during chronic LCMV infections [129]. The effector functions CD8 T cells used to inhibit

intracellular Mtb growth is not fully uncovered. Although we showed perforin KO CD8 T cells' ability to control Mtb growth wasn't impaired, we didn't address Fas-FasL or TNF dependent cytotoxicity. Moreover, the deficiency of perforin may be compensated by other redundant pathways (i.e. Fas-FasL and/or TNF pathway). Thus, we suggested cytolysis pathways were involved in CD8 T cell mediated inhibition of intracellular Mtb growh.

Interestingly, I found that CD8 T cells from infected IL-21R KO mice showed minimal T cell exhaustion, which may seem to contrast with previous findings that T cells from IL-21R KO mice were more exhausted [139], but in this study, Booty and Barreira-Silva et al measured exhausted features at a later timepoint (i.e., 16wpi). However, IL-21R KO mice have higher bacterial burden in lungs and spleens even at early timepoints. Since high bacterial loads may lead to T cell exhaustion, the role of IL-21 in T cell exhaustion needs to be carefully characterized. Indeed, infected mice with BCG expressing IL-21 didn't diminish the formation of exhausted cells [172]. The involvement of IL-21 independent of antigen stimulation to inhibit T cell exhaustion needs to be addressed.

Whether introduction of "help signals" can substitute the need for CD4 T cells and promote effective CD8 T cell responses is of great interest. Anti-CD40 agonistic antibody has been used in cancer therapy because of its function to activate T cell responses against tumor associated antigens, and to directly target tumor cells that expressed CD40. Treating Mtb infected mice with anti-CD40 antibody partially improved the exhausted states on lung CD8 T cells from MHCII

KO mice, but this improvement didn't reduce Mtb burden in lungs. Nevertheless, we observed a significant decrease in splenic bacterial burden on anti-CD40 treated MHCII KO mice. CD8 T cells have been previously shown to provide better protection in spleens compared to lungs, but it remains unknown whether the reduction in splenic Mtb burden was mediated by CD8 T cells or through APCs in spleen that were activated by CD40-anti-CD40 antibody interaction. In addition, exogenous IL-2 was added to enhance the performance of helpless CD8 T cells *in vitro*. I showed IL-2 treatment modestly enhanced control of Mtb growth by MHCII KO CD8 T cells. Whether IL-2 treated MHCII KO CD8 T cells controlled Mtb growth because the reinvigoration of exhausted features or/and the improvement of effector functions was not addressed. The role of IL-2 in CD8 T cell mediated control of Mtb growth was further complicated by the fact that blocking IL-2 signaling had negligible effect. This result seemed to suggest IL-2 wasn't produced in the culture, however, we detected a significant amount of IL-2 produced by helped CD8 T cells when cultured with infected cells.

Finally, whether reinvigoration of exhausted CD8 T cells by inhibitory receptor blockade led to enhanced Mtb growth control *in vitro* was tested. We showed anti-PD1, anti-Tim-3, and the combination of these two antibodies have minimal effect on improving the functions of exhausted CD8 T cells. A previous study showed $TCF1^{+}CXCR5^{+}$ exhausted progenitor cells respond to inhibitory receptor blockade, and these precursor cells proliferated and differentiated into cells that have effector functions. Although purified total CD8 T cells were used in

the CFU assay, a cluster of $PD1^{int}TCF1^{+}Eomes^{-}$ progenitor cells were previously identified in MHCII KO CD8 T cells. How this cluster of cells responded to inhibitory receptor blockade needs further characterization. In addition, whether administration of anti-inhibitory receptor blocking antibodies *in vivo* results in a more significant change is another direction for future studies.

In summary, the signals CD4 T cells provide to help CD8 T cells are complicated. We showed mediators directly from T cells (i.e., IL-21 and IL-2) and through APCs (i.e., CD40-CD40L) are involved. However, deficiency in any of these signaling pathways has limited effect on CD8 T cell responses compared to the absence of CD4 T cells, suggesting CD4 T cells help CD8 T cells by multiple mechanisms. Moreover, our results suggest the possibility that individual signaling pathways may play a different role in shaping CD8 T cell responses. For example, IL-21 promotes cytotoxicity of CD8 T cells, while signaling through CD40 prevents exhaustion. Collectively, these help signals generate an optimal CD8 T cell response.

CHAPTER IV. UNEXPECTED ROLE OF CD8 T CELLS FROM MHCII KO MICE IN MEDIATING PROTECTION

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Attributions:

Conceptualization, Y.L. and S.M.B.; Methodology, Y.L., and S.M.B.; Investigation, Y.L. and S.B.; Formal analysis, Y.L.; Writing, Y.L.; Supervision, S.M.B.; Funding Acquisition, S.M.B.

Abstract

Mtb becomes “dormant” in response to low oxygen and the high immune pressure inside granulomas. 18b, a streptomycin auxotroph of Mtb, provides a model to study immune responses during Mtb dormancy. We leveraged the 18b property of manageable growth, and used it to study CD8 T cell responses in WT and MHCII KO mice with the same bacterial burden. In contrast to Erdman infection, we found lower 18b specific responses in MHCII KO mice after infection, suggesting CD4 T cell help is indispensable to generate CD8 T cell responses in 18b infection. Moreover, I observed an altered hierarchy of immunodominance, in which dominant TB10 specific CD8 T cells decreased, while subdominant 32A specific CD8 T cells increased.

To study protection mediate by CD8 T cells, purified CD8 T cells from 18b infected WT or MHCII KO mice were transferred into TCRα KO mice before challenge with Mtb. Unexpectedly, we found MHCII KO CD8 T cells mediated better protection in TCRα KO mice. Through a series of experiments, we showed the deficiency in MHCII molecule perhaps during development, but not deficiency in CD4 T cells, contributed to MHCII KO CD8 T cell mediated protection. This supports previous findings that MHCII molecules play role in regulating CD8 T cell behavior. I suggest this model provides insights into the mechanisms of CD8 T cell mediated protection.

Introduction

It is estimated that a quarter of the world population has latent TB, an asymptomatic phase of infection that can persist lifelong. At this stage, immune responses against Mtb are triggered and contain the infection through "walling off" the bacteria with granulomas. This results in a low oxygen and nutrient, but high immune pressure environment for Mtb. In response to the harsh environment, Mtb turns itself into a non-replicating and low metabolic "dormancy" state. To better understand this dormancy state, several animal models has been proposed, and 18b, a streptomycin auxotroph of Mtb, becomes a useful model among these strategies [21, 173, 174]. 18b grows in the presence of streptomycin, enters a non-replicating phase in a streptomycin free environment, and resumes growth when streptomycin is reinstated. Despite trying to be invisible to the immune system, 18b has shown to elicit T cell responses.

Because the growth of 18b is easily manipulated, we explored the possibility to use 18b to answer the remaining question we have for CD4 T cell help: Does the lack of CD4 T cell help or the increased bacterial burden in the absence of CD4 T cells contribute to CD8 T cell exhaustion? Despite our finding using two independent models that CD4 T cell help limits CD8 T cell exhaustion and that anti-CD40 agonist antibody partially reverts exhaustion, the question is not completely answered. We thought to address this question in a condition that helped and helpless CD8 T cells under same antigen stimulation, which can be

achieved through infecting WT and MHCII KO mice with 18b and maintaining similar bacterial burden in these mice with control of streptomycin. However, we found lower CD8 T cells responses in MHCII KO mice after 18b infection, making it not an ideal model to study exhaustion. Moreover, when we transferred purified CD8 T cells from 18b infected WT or MHCII KO mice into TCRα KO mice, we unexpectedly found MHCII KO CD8 T cells mediated better protection against the subsequent infection.

Through a series of experiments, we showed that deficiency in MHCII molecules, not CD4 T cells, contributed to MHCII KO CD8 T cell mediated protection. Although it's unexpected, the link between MHCII molecules and CD8 T cells has previously been shown. Several lines of evidence suggest MHCII molecules can regulate CD8 T cell proliferation and functions. Triggered by the recognition of self peptide-MHC complex, T cells undergo homeostatic proliferation in lymphopenic conditions. While CD4 T cells fail to proliferate in MHCII deficient lymphopenic conditions, CD8 T cells have substantial proliferation in MHCI deficient lymphopenic environment. This proliferation is abolished in both MHCI and MHCII deficient conditions, suggesting a role for MHCII in regulating CD8 T cells homeostatic proliferation [158]. Moreover, this regulation has been shown to occur through Lag3, a ligand of MHCII molecules. CD8 T cells from MHCII KO mice express more Lag3, and Lag3 blockade by antibody further enhances CD8 T cell expansion [158]. Lag3 deficient CD8 T cells are reportedly having greater ability to infiltrate into tumor [175], which are associated with tumor control. In this

chapter, I characterized CD8 T cell responses after 18b infection in WT and MHCII KO mice, and reported a role of MHCII molecules in regulating CD8 T cell functions, which contributed to protective immunity in TB.

Results

Comparable 18b burdens in WT and MHCII KO mice

We sought to dissociate the role of CD4 T help in preventing CD8 T cell exhaustion from the role that CD4 T cells play in maintaining low bacterial burden, leading to less antigen stimulation of CD8 T cells. To study CD8 T cell responses from WT and MHCII KO mice with same bacterial burden, we intravenously (iv) infected mice with 18b, a streptomycin auxotroph of Mtb. Streptomycin was given intraperitoneally (ip) to support 18b growth for the first week after infection. Consistent with previous studies, 18b grew in the presence of streptomycin, and persisted in mice for a long time without streptomycin (Figure 4.1 A). Importantly, the bacterial burdens were similar in lungs and spleens between WT and MHC II KO mice (Figure 4.1 B).

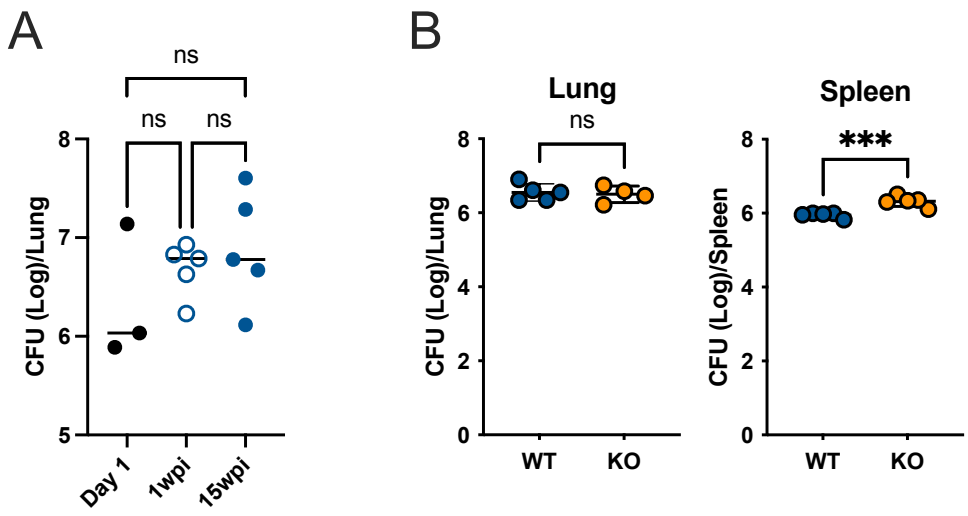


Figure 4. 1 Bacterial burdens after 18b infection.

WT (A) or WT and MHCII KO (B) mice were intravenously infected with 18b. Streptomycin was intraperitoneally given to infected mice every day for a week. Bacterial burdens in lung (A) or lung and spleen (B) were determined at indicated timepoints (A) or 4 weeks post infection (wpi) (B). Bar, median (A) and mean±SD (B). Statistical significance was analyzed by one-way ANOVA (A) or unpaired t test (B). p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Greater 18b specific CD8 T cell responses are generated in the presence of CD4 T cells

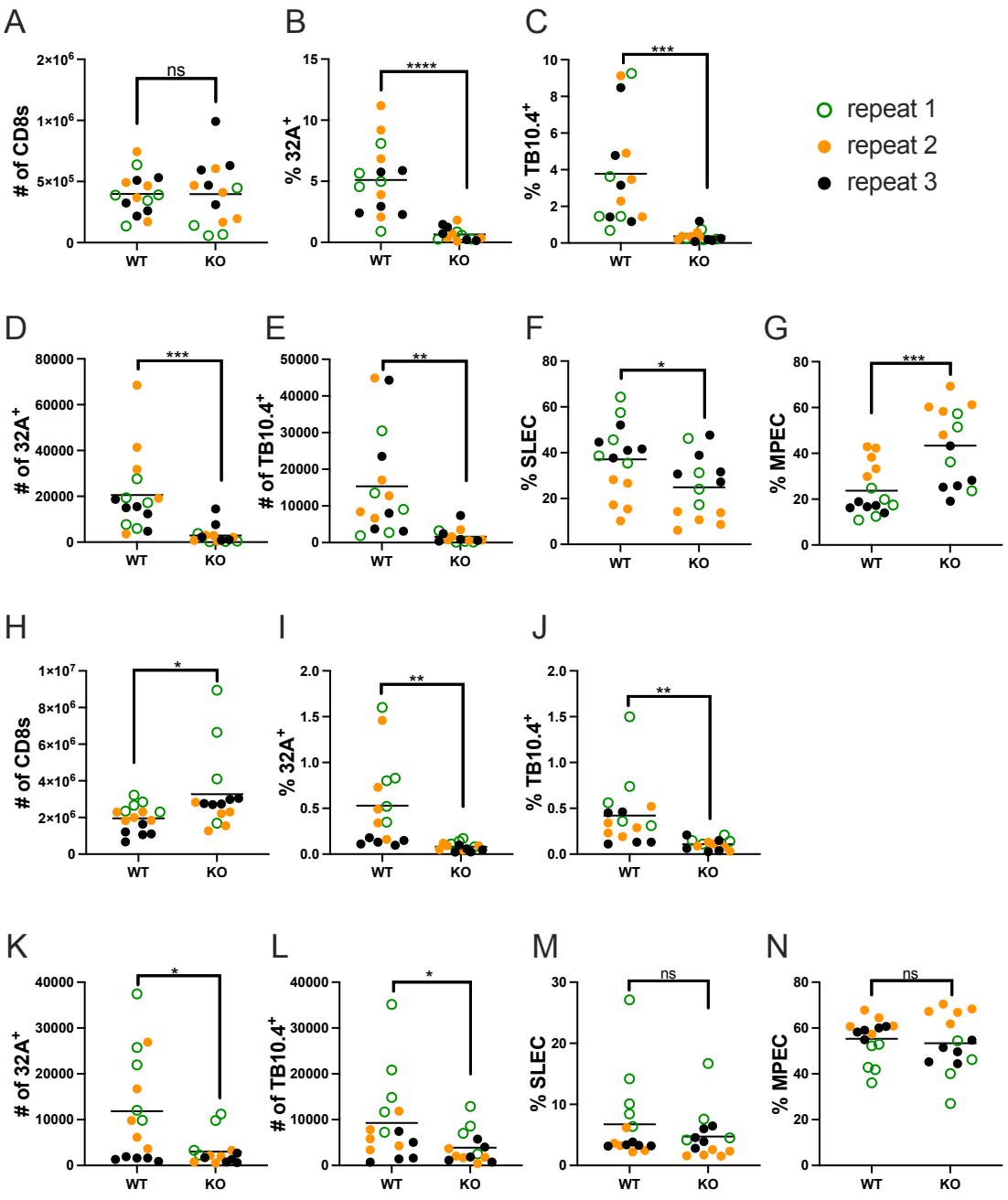
To measure 18b specific CD8 T cell responses, we assayed TB10.4 and 32A specific CD8 T cells from lungs and spleens of WT or MHCII KO mice at 4 wpi. We detected higher frequencies of both TB10.4 and 32A specific CD8 T cells in lung of WT mice (Figure 4.2 B,C). Despite smaller differences, perhaps reflecting the variation in individual mice, we observed more TB10.4 and 32A specific CD8 T cells in lung of WT mice (Figure 4.2 D,E). Interestingly, we found an altered hierarchy in immunodominant CD8 T cell responses in 18b infection compared to Erdman infection. The frequency of dominant TB10.4 specific CD8 T cells, which could account for 30-40% of total CD8 T cells in the lungs after Erdman infection, deceased in 18b infection. This was comparable to the frequency of subdominant 32A specific CD8 T cells (Figure 4.2 B,C,I,J) .

We previously found TB10.4 specific CD8 T cells didn't recognize infected macrophages [102]. To measure the portion of CD8 T cells elicited by 18b infection that recognized infected macrophages, we used Mtb-infected macrophage ELISPOT (MIME) assay that was developed in our lab [107]. In this assay, purified CD8 T cells were cultured with H37Rv-infected macrophages, and the recognition of infected macrophages was measured by IFNγ production. The proportion of CD8 T cells recognizing infected macrophages was calculated by the number of spots developed in the ELISPOT assay versus the number of CD8 T cells added. Consistent with a previous report, the frequencies of CD8 T cells recognizing

infected macrophages were low, but there were greater frequencies of CD8 T cells in WT mice that recognized infected macrophages (Figure 4.3 A).

The frequencies of TB10.4 and 32A specific CD8 T cell responses were even lower in spleens of 18b infected mice. Nevertheless, there was a greater frequency, and a modest increase in the number, of 32A specific CD8 T cells in WT mice (Figure 4.2 I,K). The frequency and number of TB10.4 specific CD8 T cells were similar in WT and MHCII KO mice in most of experiments, but a significantly greater frequency and number were observed in WT mice when all the data were combined and analyzed (Figure 4.2 J,L) Furthermore, we detected very few spots with MIME assay when purified splenic CD8 T cells were cultured with infected macrophages, indicating a very low proportion of CD8 T cells from spleens of 18b infected mice recognized infected cells (Figure 4.3 B).

We analyzed the differentiation of CD8 T cells by determining the frequencies of short-lived effector cells (SLEC) and memory precursor cells (MPEC). The frequencies of SLEC were modestly increased in lung CD8 T cells from WT mice (Figure 4.2 F). In contrast, there was much greater frequency of MPEC in MHCII KO mice, although we can't rule out the presence of naïve CD8 T cells base on this flow cytometry gating (Figure 4.2 G). The frequencies of SLEC and MPEC were similar in spleen CD8 T cells from WT and MHCII KO mice (Figure 4.2 M,N). Collectively, greater 18b specific CD8 T cell responses were generated in both lungs and spleens of WT mice.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WT
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repeat 1
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Figure 4. 2 Greater 18b specific responses are generated in WT mice after infection.

WT and MHCII KO mice were iv infected with 18b, and given streptomycin for first week after infection. At 4 wpi, CD8 T cells from lung (A-G) or spleen (H-N) were analyzed by flow cytometry to determine the number of CD8 T cells (A, H) and the frequencies (B,C,I, J) and absolute number (D,E,K, L) of TB10.4 and 32A specific CD8 T cells. The frequencies of $KLRG1^{+}CD127^{-}$(SLEC) or $KLRG1^{-}CD127^{+}$(MPEC) among CD8 T cells were determined (F, G, M, N). Colors were used to distinguish individual experiment. Bar, grand mean. Statistical difference was analyzed by unpaired t test for pooled data. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

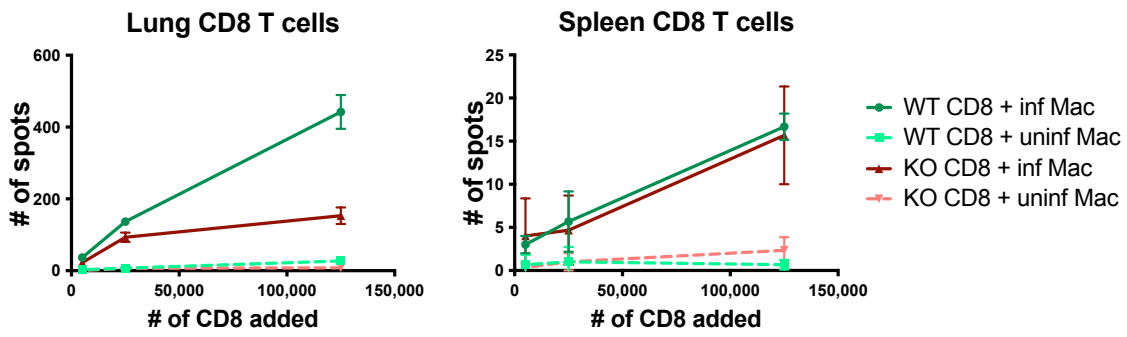

Figure 4. 3 The frequencies of CD8 T cells from 18b infected WT and MHCII KO mice that recognized infected macrophages.

WT and MHCII KO mice were iv infected with 18b, and given streptomycin for first week after infection. At 4wpi, CD8 T cells from lung or spleen were purified and cultured with H37Rv infected or uninfected TG-PMs. The frequencies of CD8 T cells secreted IFNγ were determined by IFNγ ELISPOT. Bar, mean±SD.

18b immunized MHCII KO CD8 T cells mediate better protection in immunodeficient mice

To study the ability of CD8 T cells to mediate protection *in vivo*, purified CD8 T cells from 18b infected mice can be transferred into TCRα KO mice before challenging the recipients with Mtb. Since less than a million of CD8 T cells per mouse can be purified from lungs of 18b infected mice, we transferred splenic CD8 T cells from 18b infected WT or MHCII KO mice into TCRα KO mice, and the recipients of WT or MHCII KO CD8 T cells and mice received no cells were then challenged with aerosolized Mtb. We found neither WT nor MHCII KO CD8 T cells mediated protection in lungs of recipients compared to mice that didn't receive any CD8 T cells, while MHCII KO CD8 T cells unexpectedly reduced bacterial burden in spleens of recipients (Figure 4.4).

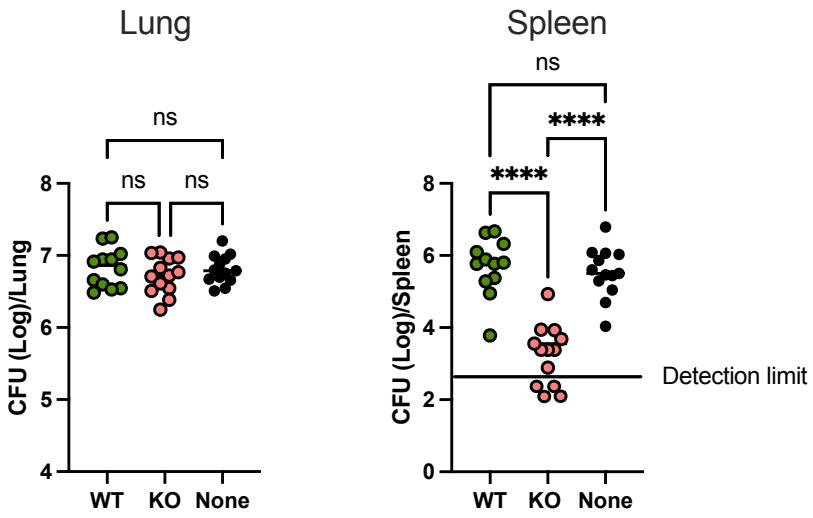

Figure 4. 4 CD8 T cells from 18b infected MHCII KO mice confer protection in spleens of TCRα KO mice.

CD8 T cells were purified from spleen of 18b infected WT or MHCII KO mice at 4 wpi, and adoptive transferred into TCRα KO mice. TCRα KO mice received WT or MHCII KO CD8 T cells, and TCRα KO mice didn't received CD8 T cells were then infected with aerosolized Mtb. Mtb burden in lungs and spleens of recipient mice were determined at 3 wpi. Data were combined from three independent experiments. Bar, median. Statistical difference was analyzed by one-way ANOVA. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Very few TB10.4 and 32A specific CD8 T cells in the recipient mice

To address potential mechanisms contributing to the protection mediated by MHCII KO CD8 T cells, we characterized CD8 T cell responses after challenge in lungs and spleens of recipients of WT or MHCII KO CD8 T cells. We found MHCII KO CD8 T cells tend to proliferate more in the recipients compared to WT CD8 T cells, although this didn't occur in all the experiments (Figure 4.5 A,B). Furthermore, not all the recipients generated CD8 T cells specific to TB10.4 or 32A, and even in the mice that generated TB10.4 or 32A specific CD8 T cells, the frequencies of these cells were very low (Figure 4.5 C-F). This may be contributed by the limited number of TB10.4 and 32A specific precursor cells that were transferred into TCRα KO mice. Nevertheless, it was less likely that TB10.4 and 32A specific CD8 T cells contributed to the protection since the low frequencies of these cells found in both donors and recipients.

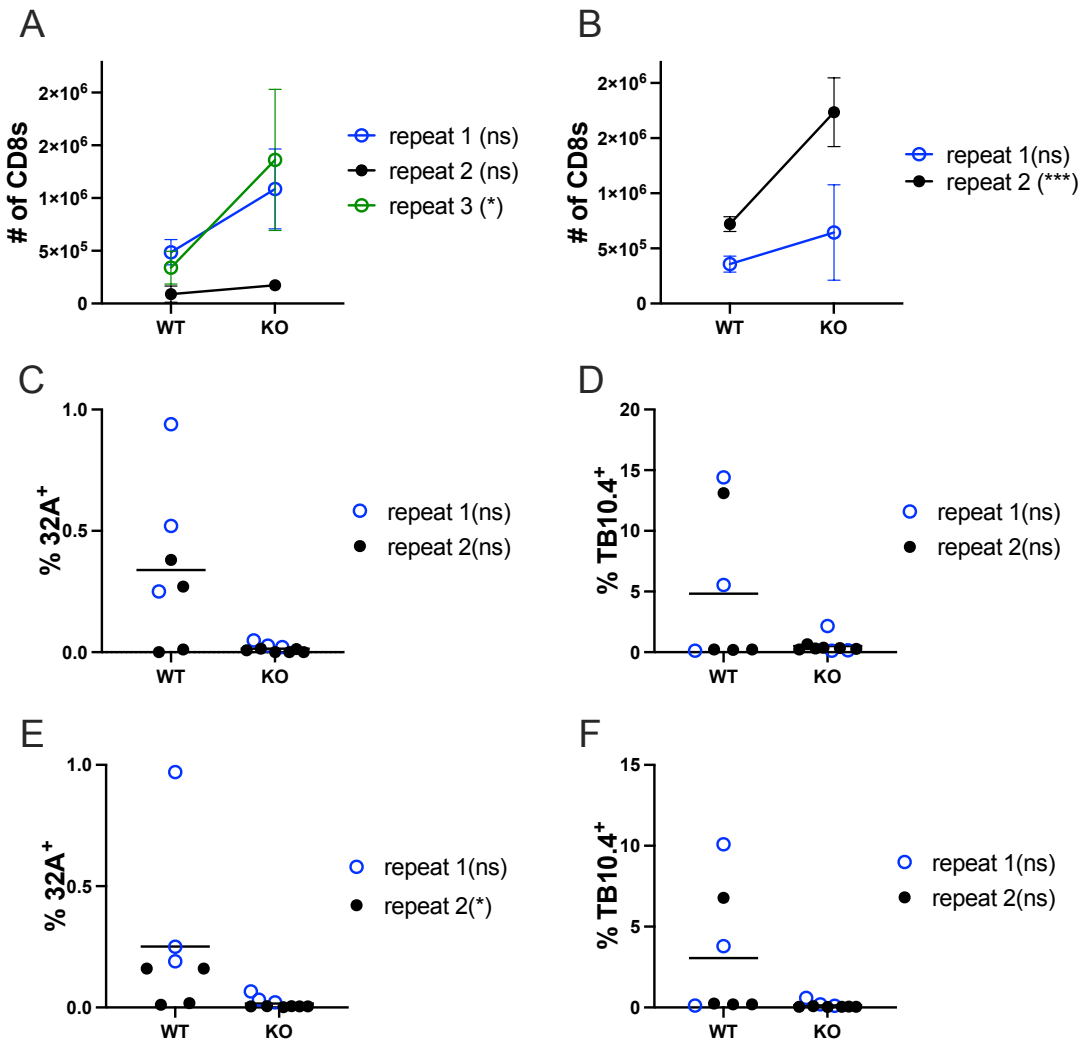
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Figure 4. 5 Very few TB10.4 and 32A specific CD8 T cells are detected in recipient mice.

Splenic CD8 T cells were purified from 18b infected WT or MHCII KO mice at 4 wpi, and adoptively transferred into TCRα KO mice. TCRα KO mice received WT or MHCII KO CD8 T cells were then infected with aerosolized Mtb, and CD8 T cells from lung (A,C,D) and spleens (B,E,F) of TCRα KO recipient mice were analyzed by flow cytometry at 3 wpi to determine the number of CD8 T cells (A,B) and frequencies of TB10.4 and 32A specific CD8 T cells (C-F). Colors are used to distinguish individual experiment. Bar, mean$\pm$SD (A,B) and grand mean (C-F). Statistical difference was analyzed for individual experiment with unpaired t test, and the results are labeled next to the legends. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Virtual memory (VM) cells less likely contribute to MHCII KO CD8 T cell mediated protection

Virtual memory cells (VMs) are antigen inexperienced, memory-like CD8 T cells that can be found in naïve mice. These cells are thought to arise from homeostatic proliferation, although never having experienced foreign antigens, they quickly respond and expand during infection [176-178]. More importantly, they can produce IFNγ and mediate bystander killing driven by cytokines [176]. We tested whether VMs mediated the protection we observed from recipients of MHCII KO CD8 T cells. VMs are $CD44^{hi}CD49d^{lo}$ and can be distinguished from naïve and true memory cells by the expression of these two markers (Figure 4.6 A). However, we didn't observe more VMs in naive MHCII KO mice, and these VMs didn't expand after Mtb infection or transferred into TCRα KO mice (Figure 4.6 B-D). Thus, VMs are less likely contribute to protection in recipients of MHCII KO CD8 T cells.

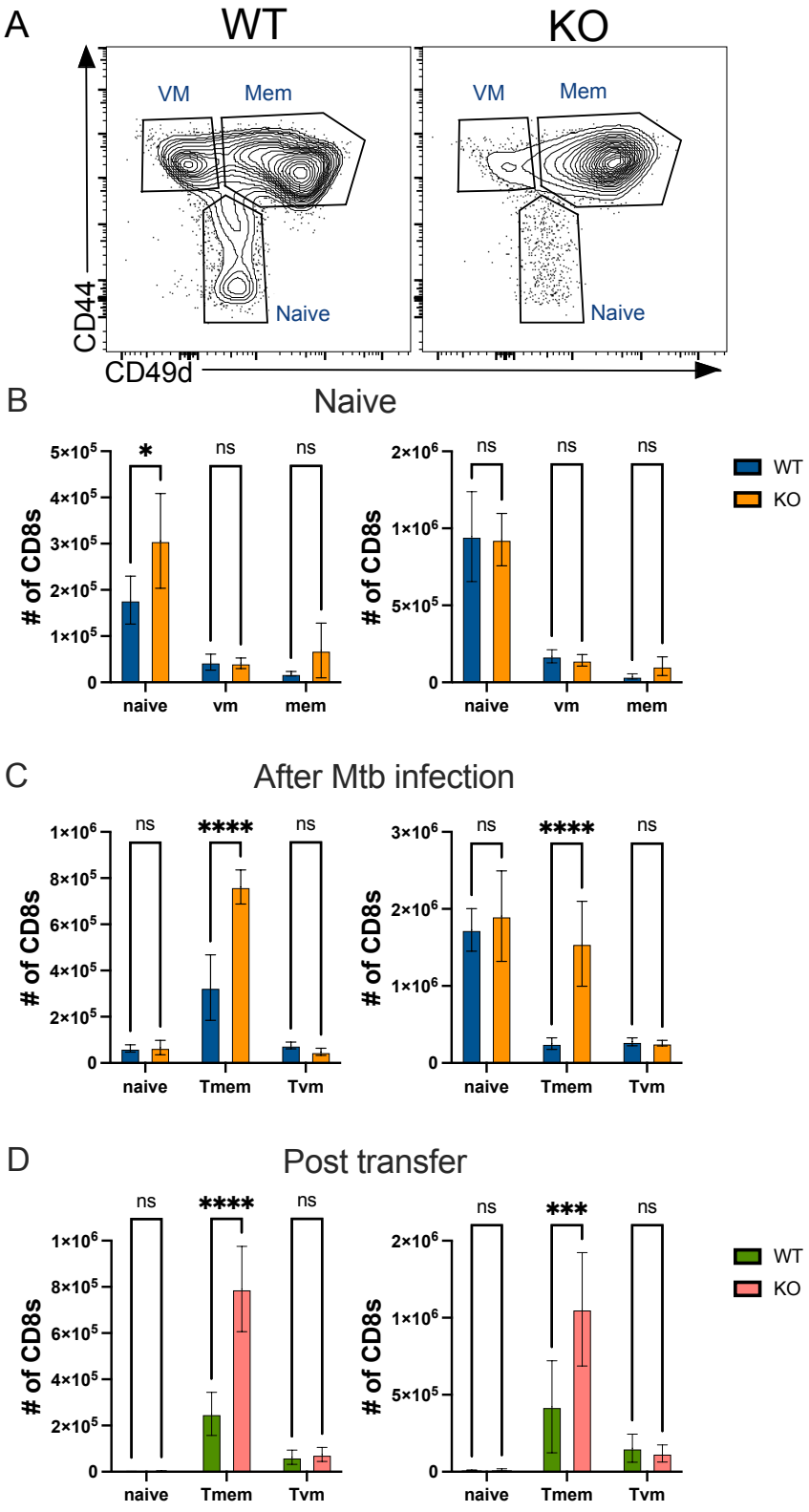
A
WT
KO
VM
Mem
Naive
CD44
CD49d
B
Naive
# of CD8s
naive
vm
mem
WT
KO
C
After Mtb infection
Tmem
Tvm
D
Post transfer

Figure 4. 6 MHCII KO mice have less virtual memory (VM) cells.

Lung naïve ($CD44^{lo}CD49d^{lo}$), VM ($CD44^{+}CD49d^{-}$), and memory ($CD44^{+}CD49d^{+}$) CD8 T cells were characterized using flow cytometry to determine cell numbers. (A) Representative flow plots showing gating strategy. Number of naïve, VM, and memory CD8 T cells in naïve WT and MHCII KO mice (B), Mtb infected WT and MHCII KO mice at 4wpi (C), and in TCRα KO mice received purified total WT or MHCII KO CD8 T cells at 3 weeks post transfer (D). Bars, Bar, mean ± SD. Statistical differences was analyzed by one-way ANOVA. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

CD8 T cells from 18b immunized, CD4 T cell depleted mice do not confer protection in immunodeficient mice

We considered whether the ability of MHCII KO CD8 T cells to protect the recipients arose from the absence of CD4 T cells, or it arose from the developmental or intrinsic differences in MHCII KO CD8 T cells. Although it's hard to associate CD4 T cell "help" to an impaired ability of CD8 T cells, one possible hypothesis could be that CD4 T cells promote the differentiation of SLEC cells, which could be less proliferative and thus mediate poor protection in recipients. To test the requirement of CD4 T cells, purified WT CD8 T cells from CD4 T cell depleted mice were used. Anti-CD4 was used to deplete CD4 T cells from C57BL/6J mice before infecting with 18b, and was continuously injected every five days for first three weeks after infection. Purified splenic CD8 T cells from CD4 T cell depleted or intact mice were transferred into TCRα KO mice before these recipients were challenged with Mtb. We didn't observe protection in lungs or spleens of recipients by CD8 T cells, regardless they were from intact or CD4 T cell depleted mice. This result shows an intrinsic feature of MHCII KO CD8 T cells contributed to the protection (Figure 4.7).

We also tested whether less TB10.4 and 32A specific CD8 T cells found in MHCII KO CD8 T cells after 18b infection resulted from the absence of CD4 T cells. The frequencies and numbers of TB10.4 and 32A specific CD8 T cells from lungs and spleens of 18b infected intact or CD4 T cell depleted mice were determined by flow cytometry. Although it's not statistically significant, we observed a trend for

decreased frequencies and numbers of TB10.4 and 32A specific CD8 T cells in lungs in the absent of CD4 T cells (Figure 4.8 A-D). We barely detected any TB10.4 specific CD8 T cells in spleens of neither mice, but we found more 32A specific CD8 T cells in spleen of intact mice. These results suggested CD4 T cells, at least in part, contributed to generate 18b specific CD8 T cell responses.

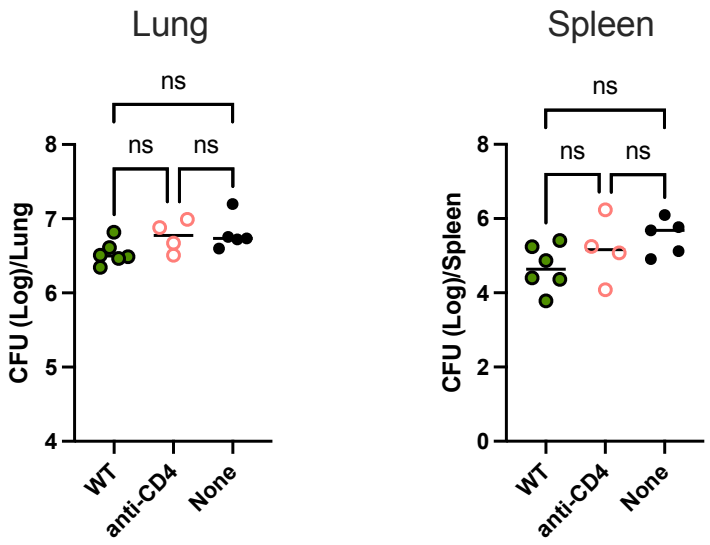

Figure 4. 7 Purified CD8 T cells from 18b infected, CD4 T cell depleted mice don't protect TCRα KO mice.

CD4 T cells were depleted from C57BL/6J mice by anti-CD4 antibody before 18b infection, and anti-CD4 was consciously given every five days for the first three weeks of infection. Purified CD8 T cells from CD4 T cell depleted or intact mice were transferred into TCRα mice, and the recipients were challenged with aerosolized Mtb. Bacterial burden in lungs and spleens of recipients were determined at 3 weeks post challenge. Bars, median. Statistical difference was analyzed by one-way ANOVA. ns, no significant differences.

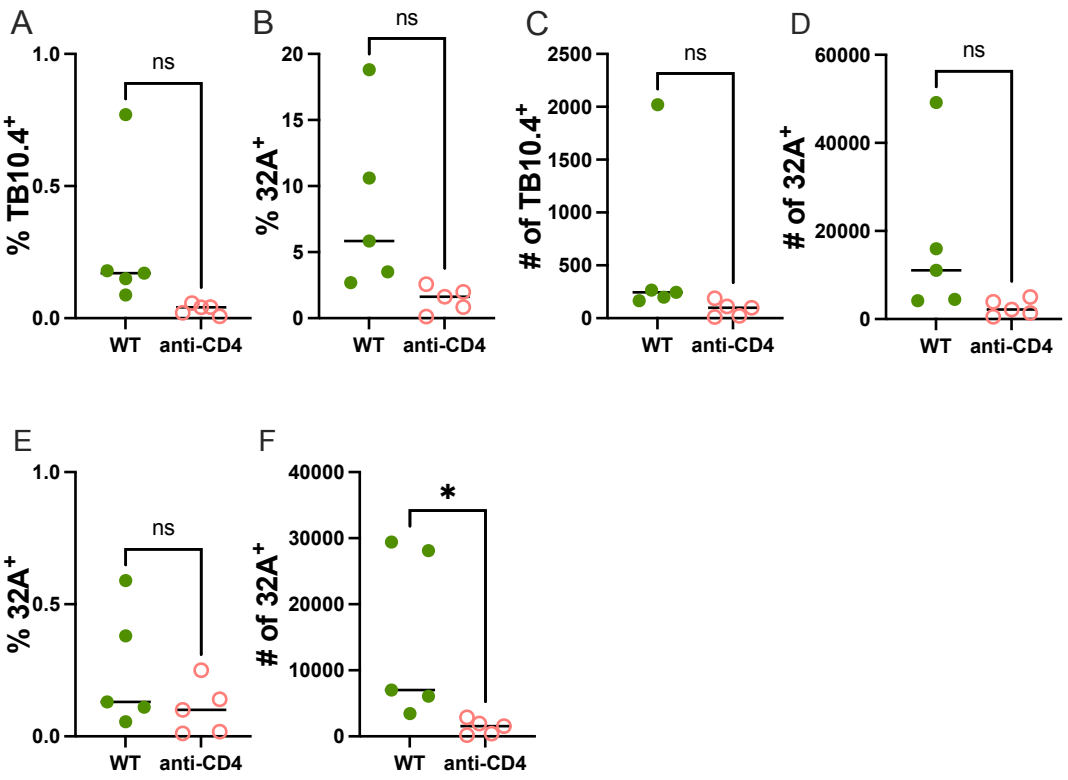

Figure 4. 8 TB10.4 and 32A specific CD8 T cells in intact or CD4 T cell depleted mice after 18b infection.

Anti-CD4 antibody was used to deplete CD4 T cells from C57BL/6J mice before, and consciously given for the first three weeks after 18b infection. The frequencies (A,B,E) and number (C,D,F) of TB10.4 or 32A specific CD8 T cells from lungs (A-D) and spleens (E,F) of intact or CD4 T cell depleted mice were determined by flow cytometry. Bars, median. Statistical difference was analyzed by unpaired t test. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

MHC II KO CD8 T cells express more Lag3 and are higher proliferative in immunodeficient mice

Previous study suggested that MHCII KO CD8 T cells were hyperreactive under lymphopenic conditions, which was due to the lack of inhibition signals delivered through Lag3-MHCII interaction. This study showed greater expression of Lag3 on MHCII KO CD8 T cells, and administration of anti-Lag3 blockade further enhanced the expansion [158]. To validate the hyperproliferative feature of MHCII KO CD8 T cells, we transferred proliferation-dye-stained WT and MHCII KO CD8 T cells at a 1:1 ratio into TCRα KO mice and assayed their proliferation based on the dilution of dye after 4 days. We found MHCII KO CD8 T cells proliferated more compared to WT CD8 T cells (Figure 4.9 A). We then measured Lag3 expression on CD8 T cells using flow cytometry, and confirmed MHCII KO CD8 T cells had greater expression of Lag3 in basal level (i.e., naïve cells), after Mtb infection, and after transfer into TCRα KO mice (Figure 4.9 B-D).

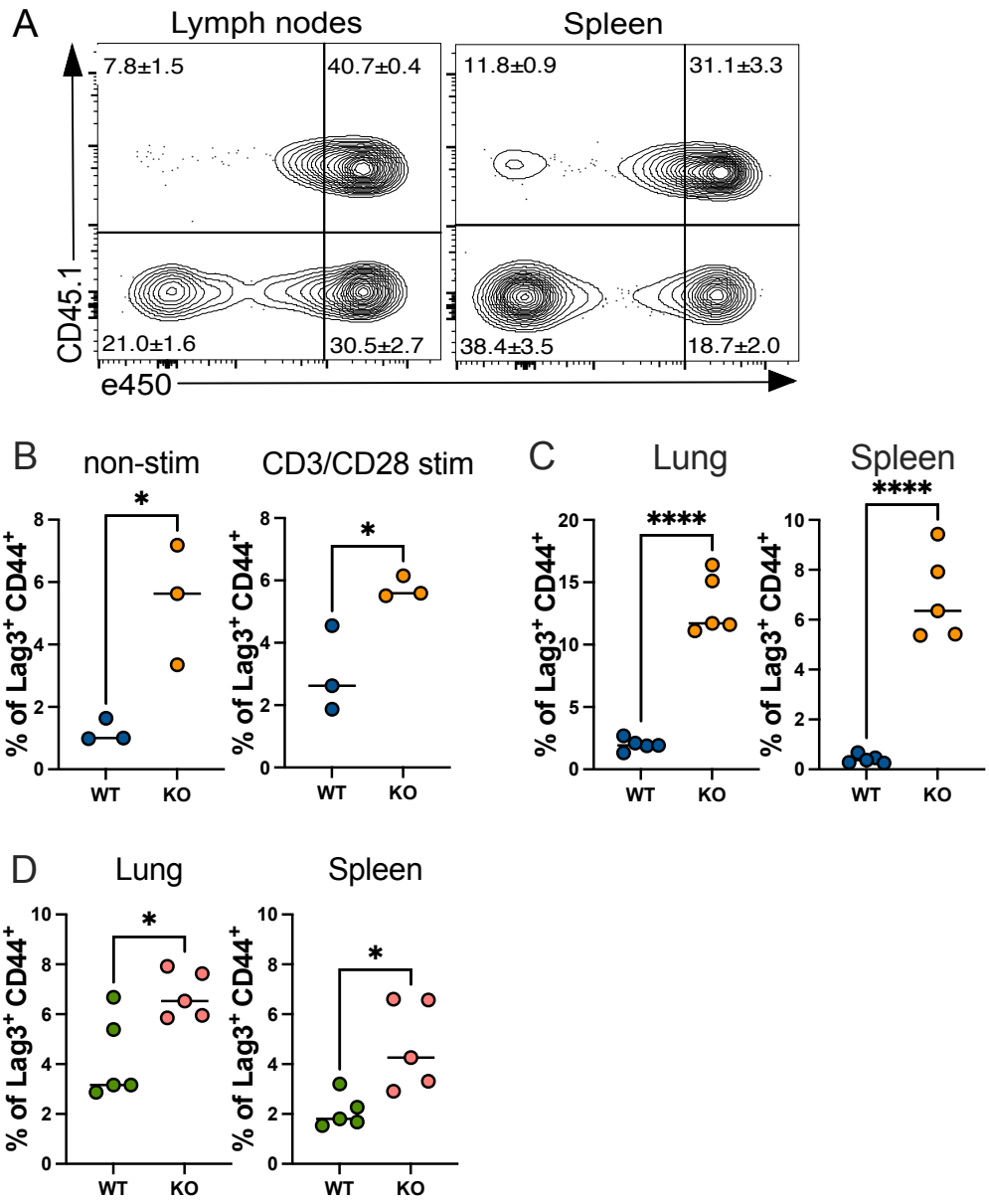
A
Lymph nodes
Spleen
7.8±1.5
40.7±0.4
11.8±0.9
31.1±3.3
21.0±1.6
30.5±2.7
38.4±3.5
18.7±2.0
CD45.1
e450
B
non-stim
CD3/CD28 stim
% of Lag3+ CD44+
WT
KO
C
Lung
Spleen
****
D
Lung
Spleen
*

Figure 4. 9 MHCII KO CD8 T cells express more Lag-3 and are hyperproliferative in TCRα KO mice.

(A) 10^6 of naïve WT (CD45.1) and MHCII KO CD8 T cells (CD45.2) were labeled with proliferation dye e450 and transferred into TCRα KO mice. TCRα KO were euthanized at 4 days post transfer, and CD8 T cells from lymph nodes and spleens were analyzed by flow cytometry. The mean % $\pm$SD for each quadrant is indicated (n=3/group). (B-D) The frequencies of CD8 T cells expressed $CD44^+Lag3^+$ were analyzed by flow cytometry from spleens of naïve WT and MHCII KO mice (B), lungs and spleens of Mtb infected WT and MHCII KO mice at 4wpi (C), and lungs and spleens of TCRα KO mice received purified WT or MHCII KO CD8 T cells at 3 weeks post transfer (D). Bars, median. Statistical difference was analyzed by unpaired t test. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Anti-Lag3 blockade does not enhance the protection by MHCII KO CD8 T cells in immunodeficient mice

We considered whether the hyperreactive nature of MHCII KO CD8 T cells correlated to the protection they mediated in the recipients. To answer this question, anti-Lag3 blockade was given to recipients of MHCII KO CD8 T cells, and we tested whether MHCII KO CD8 T cells would expand and bacterial burden in recipients can be further reduced. Purified splenic, 18b immunized MHCII KO CD8 T cells were transferred into TCRα KO mice before the recipients were challenged with aerosolized Mtb. Anti-Lag3 blockade was given to the recipients on the day of cell transfer, and every three days for three weeks. The recipients were euthanized, and the bacterial burden and CD8 T cell number in lungs and spleens were determined. There was comparably low bacterial burden in spleens between anti-Lag3 treated and control group (Figure 4.10 A), which may due to the low starting dose (i.e, day 1) of this aerosol infection and led to an inconclusive result. Nevertheless, the number of CD8 T cells in recipients didn't increase in response to anti-Lag3 blockade. Thus, we concluded anti-Lag3 blockade had minimal effect in promoting the proliferation or protection of MHCII KO CD8 T cells (Figure 4.10 B).

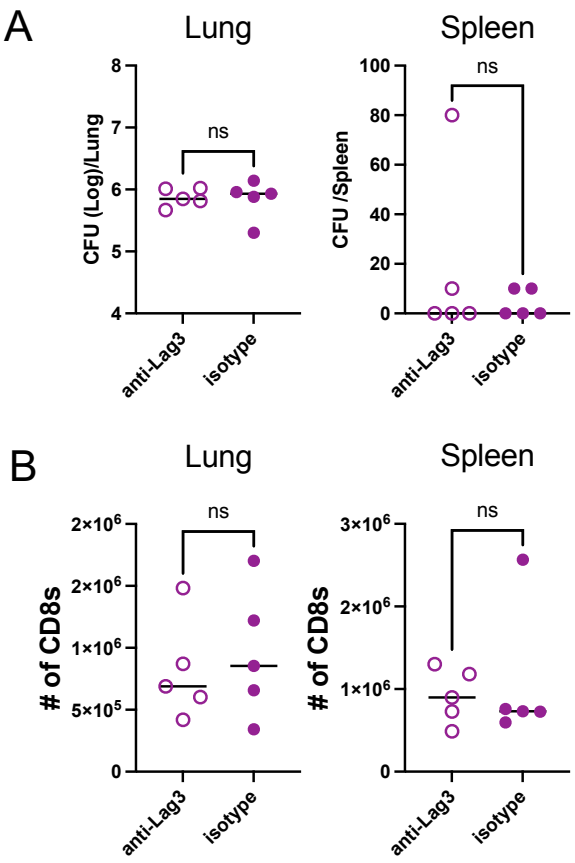

Figure 4. 10 Anti-Lag3 blockade does not enhance the protection by MHCII KO CD8 T cells in immunodeficient mice.

Purified CD8 T cells from 18b infected MHCII KO mice were transferred into TCRα KO mice before the recipients were challenge with aerosolized Mtb. Anti-Lag3 blocking antibody was given to the recipients every three days until euthanasia at 3 wpi. Bacterial burdens (A) and the number of CD8 T cells (B) in lungs and spleens of recipients were determined. Bars, median. Statistical difference was analyzed by unpaired t test. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Mtb infected MHCII KO CD8 T cells do not confer protection in immunodeficient mice

We hypothesized that 18b immunization was necessary for the protection in recipients medicated by MHCII KO CD8 T cells. To test whether CD8 T cells from Mtb infected MHCII KO mice transfer protection, purified CD8 T cells from spleens of aerosolized Mtb infected WT or MHCII KO mice were transferred into TCRα KO mice, and these recipients were then challenged with aerosolized Mtb. The recipients of MHCII KO CD8 T cells outlived the mice that received WT CD8 T cells in first experiment, but this result was not repeatable (Figure 4.11A). Conflicting results of bacterial burdens were also observed. Although MHCII KO CD8 T cells showed modest protection in lungs of recipients in first experiment, neither WT nor MHCII KO CD8 T cells mediated protection in second experiment (Figure 4.11 B). In summary, we concluded that CD8 T cells from Mtb infected MHCII KO mice didn't transfer protection in immunodeficient mice.

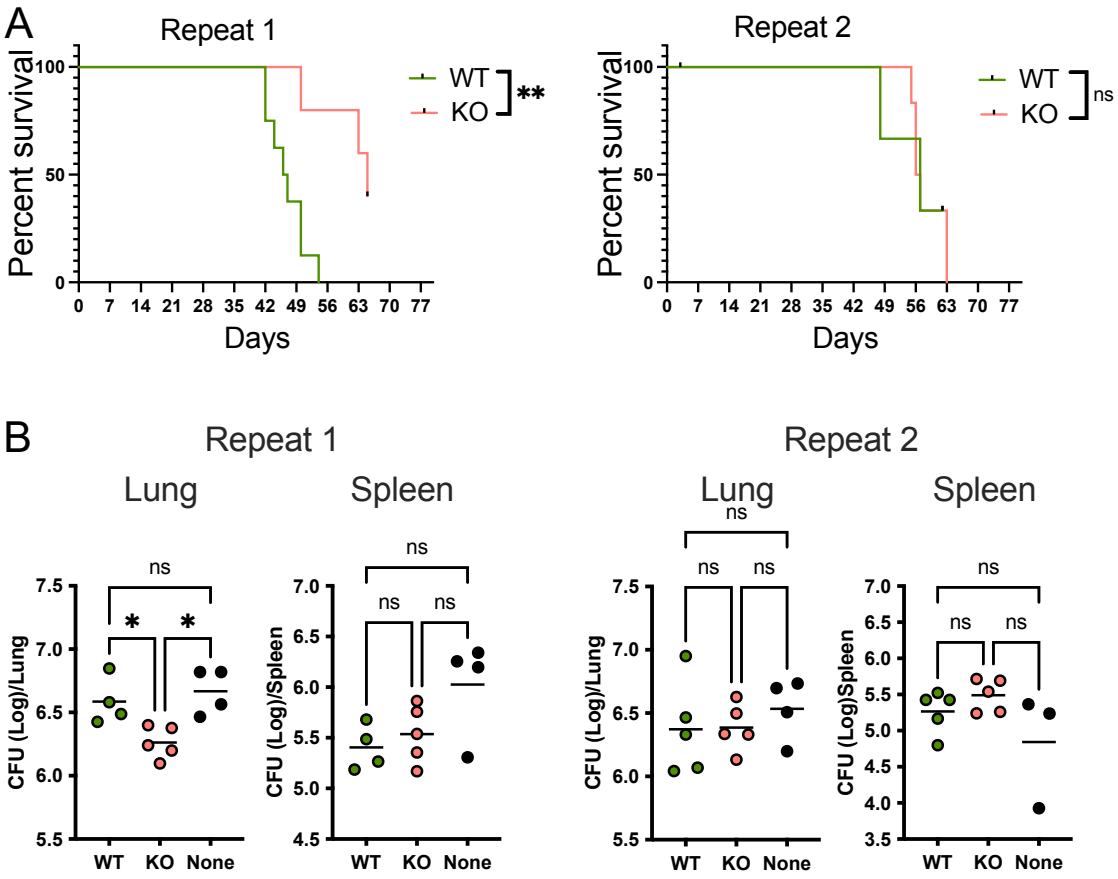
A
Repeat 1
Percent survival
100
50
0
0 7 14 21 28 35 42 49 56 63 70 77
Days
WT
KO
**
Repeat 2
Percent survival
100
50
0
0 7 14 21 28 35 42 49 56 63 70 77
Days
WT
KO
ns
B
Repeat 1
Lung
Spleen
Repeat 2
Lung
Spleen
CFU (Log)/Lung
CFU (Log)/Spleen
ns
*
WT KO None

Figure 4. 11 Mtb infected MHCII KO CD8 T cells don't confer protection in immunodeficient mice.

Purified CD8 T cells from aerosolized Mtb infected WT or MHCII KO CD8 T cells were transferred into TCRα KO mice. (A) The survival of recipients after challenged with aerosolized Mtb. Two independent experiments are shown. The statistical difference between the recipients of WT or MHCII KO CD8 T cells was determined by the log-rank test. (B) Bacterial burden in lungs and spleens of recipients and TCRα KO mice receiving no cells were determined at 3 wpi. Two independent experiments are shown. Statistical difference was determined by one-way ANOVA. p-values: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. ns, no significant differences.

Discussion

In this chapter, we reported an unexpected protective role of 18b immunized MHCII KO CD8 T cells against Mtb, which was likely because of its development in the absence of MHCII molecules and becoming hyperproliferative. Interestingly, fewer immunodominant antigens, including TB10.4 and 32A, specific CD8 T cells were found in MHCII KO mice. This contrasted to our previous finding that in Erdman infections, CD4 T cells were dispensable to generate Mtb specific CD8 T cell responses. CD4 T cells can help CD8 T cells through activating APCs, a process called “licensing”. The licensed APCs upregulate MHCI and co-stimulatory receptors and produce pro-inflammatory cytokines. However, APCs can also be activated by innate signaling through TLRs. Thus, infections that induced strong type I IFN response on APCs, for example, LCMV infection, may bypass the need for CD4 T cell help and directly activate CD8 T cells. It’s likely that in a more virulent Erdman infection, the need for CD4 T cell help was bypassed, whereas it’s indispensable to generate antigen specific CD8 T cell responses in 18b infection. This reduced TB10.4 and 32A specific responses were also observed in CD4 depleted mice after 18b infection.

We also observed an altered hierarchy of immunodominance in CD8 T cell responses. There was a decrease in frequencies of CD8 T cells specific for immunodominant epitopes TB10.4, while an increase in CD8 T cells specific for subdominant epitopes 32A. Why TB10.4 response is immunodominant among Mtb

specific CD8 T cells is unknown. Nevertheless, immunodominance is a phenomenon that often described in viral infection, and it's in part contributed by the number, type, and ability of APCs to present certain epitopes over others [179-183]. Moreover, dominant CD8 responses can actively suppressed subdominant responses [180-182]. The mechanisms for swapping positions in the hierarchy is also unclear, although it's be shown peripheral routes of infection (i.e., id and sc) sharpen the immunodominance, while systemic infection (i.e., iv and ip) generate broader repertoire [184]. We previously showed TB10.4 could be a decoy antigen that Mtb actively uses to evade CD8 T cells [102, 106]. Therefore, whether iv infection with 18b elicits broader immune responses (i.e., less immunodominant) that can confer better protection needs further investigation.

To address the mechanisms of protection mediated by 18b immunized MHCII KO CD8 T cells, we first addressed whether 18b elicited more CD8 T cells recognizing infected macrophages in MHCII KO mice. Since immunodominant antigens are likely decoy antigens, we hypothesize CD8 T cells that recognize non-dominant antigens presented on infected cells are protective [102, 106]. We performed MIME assay [107], in which the frequencies of CD8 T cells that secreted IFNγ when cultured with infected macrophages were assayed. We didn't find a greater frequency of MHCII KO CD8 T cells recognized infected cells. We considered whether the absence of CD4 T cells or the deficiency of MHCII contributed to MHCII KO CD8 T cell mediated protection. We depleted CD4 T cells in 18b infected mice and transferred purified CD8 T cells into TCRα KO mice, but

WT CD8 T cells from CD4 T-depleted mice didn't protect TCRα KO mice upon Mtb challenge. Thus, an intrinsic or development defect, which resulted from lack of MHCII, contributed to the protection by MHCII KO CD8 T cells. We then tested the possibility that MHCII KO mice have more VM cells, which can lead to protection. In contrast, we found naive MHCII KO mice have less VM cells, and this population didn't expand after Mtb infection. Finally, we studied the Lag3-MHCII regulation on CD8 T cells. We suspected the hyperproliferative nature in the absence of MHCII made CD8 T cells more protective. We determined whether the expansion of MHCII KO CD8 T cells when engaging anti-Lag3 antibody can reduced bacterial burden. We injected anti-Lag3 blocking antibody into TCRα KO mice that received MHCII KO CD8 T cells, but found no further reduction in bacterial burdens after treatment. However, the bacterial burdens in both anti-Lag3 treated and untreated mice were really low.

A spectrum of T cells with different proliferation capacities can be detected after Mtb infection and vaccination [46, 185-188]. The less differentiated cells, characterized by the expression of PD-1 and/or CXCR3, are thought to have better proliferation and cell renewal ability compared to the terminally differentiated CX3CR1, KLRG1, and/or Tim3 expressing cells [46, 185]. The less differentiated states are associated with better protection in TB partially because of their greater ability to produce IL-2 and/or multiple cytokines (i.e., polyfunctionality) [186]. In contrast, terminally differentiated cells expressing predominately IFNγ are associated with poor protection [186, 189, 190]. After vaccination, eliciting T cells

with less differentiated, proliferative central memory phenotypes are associated with vaccine induced protection [188, 191]. Interestingly, we found lower frequencies of SLEC in MHCII KO mice after 18b infection, but detected greater frequencies of SLEC in mice receiving MHCII KO CD8 T cells compared to mice that received WT CD8 T cells. This suggested MHCII KO CD8 T cells were able to differentiate into terminally effector cells after being transferred into TCRα KO mice. A better characterization of MHCII KO CD8 T cells in the recipients are needed to fully uncover the mechanisms of protection.

CD8 T cells mediate modest protection after Mtb infection even in immunodeficient mice. Here, we provided evidence that CD8 T cells can confer a two-log-reduction protection in spleens of recipients. Although we didn't find protection in lungs after infection, this preferential control in spleens by CD8 T cells have been shown previously. We attributed this protection to at least partially the intrinsic nature of MHCII KO CD8 T cells. The lack of Lag3- MHCII interaction alters the activation/proliferation threshold of MHCII KO CD8 T cells, leading to greater proliferation and differentiation in lymphopenic recipients. However, a prior 18b infection, which generated a distinct immune response than virulent Mtb, may also contribute to the protection. Moreover, the iv route of immunization was recently demonstrated to induce strong local and systemic immune responses after vaccination, leading to robust protection against Mtb in NHP [76]. Thus, a better characterization of immune responses induced by iv infection of 18b is needed. I

suggest that this model provides a unique opportunity to study CD8 T cell mediated protection against Mtb.

CHAPTER V: DISCUSSION

"Unity is strength." This proverb states the foundation of human society, and it's also the strategy our immune system uses when facing intruding pathogens. CD4 T cells are called T helper cells because of their role in regulating other immune cells. Many studies have shown an effective CD8 T cell response during viral infection and cancer depends on CD4 T cells. These signals that CD4 T cells provide to CD8 T cells are referred to CD4 T cell help. In Mtb infection, the relative importance of CD8 T cells in protective immunity has been debated. This contention arose from the observation that CD8 T cells play minimal role in experimental mouse model. We argue that in these studies, the contribution of CD8 T cells was evaluated as an individual cell type, which could be underestimated in the absence of CD4 T cell help. In this dissertation, I characterize "helped" vs. "helpless" CD8 T cell responses during Mtb infection and address how CD4 T cells provide help to CD8 T cells. I show CD4 T cell help has a significant impact on primary CD8 T cell responses, and CD8 T cells depend on CD4 T cells to mediate protection during TB. In the following sections, I discuss the impact of CD4 T cell help in CD8 T cell effector function and exhaustion, separately. Lastly, I reassess CD8 T cells' role in protective immunity to Mtb in the light of our findings and newly emerging results.

CD4 T cell help enhances CD8 T cell effector function

In chapter II, we used multiple unbiased approaches to study the role of CD4 T cell help in shaping CD8 T cell responses. We performed RNA-seq analysis on total lung CD8 T cells from Mtb infected WT and MHCII KO mice, validated the transcriptional signatures on lung parenchymal CD8 T cells using flow cytometry, and conducted cluster analysis on single cell data acquired by flow cytometry. We found “helped” CD8 T cells were clusters of effector cells with cytotoxic features, and contained a cluster that appeared to be memory precursors. In contrast, “helpless” CD8 T cells were in different states of exhaustion. The helped vs. helpless CD8 T cell features were confirmed with an adoptive transfer model in which CD8 T cells were transferred with or without Ag85b specific CD4 T cells into TCRα KO mice. Similar phenotypic differences between CD8 T cells that received help or not were found in this model. Thus, we identified shared characteristics on helped vs. helpless CD8 T cells from two independent models.

The effector features on helped CD8 T cells correlated with their greater functions. Helped CD8 T cells produced more IFNγ, TNF, and IL-2 when cultured with infected macrophages. The impaired ability of CD8 T cells to produce IFNγ during TB in the absence of CD4 T cell has been shown by two groups using different approaches [85, 87]. Bold et al. performed direct intracellular staining without re-stimulation to assess IFNγ production *in vivo*. They found the frequencies of IFN^+ CD8 T cells correlated with the frequencies of IFN^+ CD4 T cells [85]. In an adoptive transfer model in which CD4 T cells were depleted or non-

depleted from splenocytes before transferred into RAG KO mice, Green et al. showed the number of Mtb specific CD8 T cells and IFNγ production by CD8 T cells decreased without CD4 T cells [87]. We extended these findings showing CD4 T cells regulated IFNγ production by CD8 T cells with an overall impact on the transcriptome. Indeed, pathways associated with IFNγ production and regulation were enriched on WT CD8 T cells. The requirement of CD4 T cells for TNF and IL-2 production by CD8 T cells hasn't previously been reported. TNF synergizes with IFNγ to activate infected macrophages, promoting their microbicidal activity. IL-2 promotes CD8 T cell survival and expansion. These findings emphasized the essential role CD4 T cell played in regulating CD8 T cell effector functions.

An unexpected finding was that CD4 T cells enhanced NK receptor expression on CD8 T cells. Cytotoxic CD8 T cells with various surface receptors typically expressed by NK cells were described in leprosy and TB [75]. Balin and colleagues found that NK receptor expression on CD8 T cells was associated with their ability to produce cytotoxic granule proteins. CD8 T cells that expressed the greatest variety of NK receptors were the ones that secreted all perforin, granzyme, and granulysin [75]. A previous study addressed the requirement of CD4 T cells for CD8 T cell cytotoxicity by comparing the ability to of CD8 T cells from WT or CD4 KO mice to lyse target cells [136], but the result was complicated by later findings that CD4 KO mice contained MHCII restricted CD4 T cells. Nevertheless,

we found helped CD8 T cells from two independent models expressed more NK receptors, suggesting greater cytolysis ability of helped CD8 T cells.

How CD4 T cells provided help shaping CD8 T cell responses during TB was addressed in Chapter III. We focused on IL-21, IL-2, and CD40-CD40L signaling pathways because these pathways have well-established roles as “help signals”, and their requirements in protective immunity in TB were implied. With “helped” and “helpless” features we defined previously, we determined whether manipulating pathways mentioned above alters CD8 T cell characteristics. I found IL-21 was essential for cytotoxic features of CD8 T cells, and CD8 T cells deficient in IL-21 signaling had diminished ability to restrict intracellular Mtb growth. In contrast, anti-CD40 agonist antibody didn’t promote cytotoxic signatures on CD8 T cells, although it partially reverted CD8 T cell exhaustion. These results indicated signals directly from T cells (i.e., IL-21) and signals relayed by DCs (i.e., CD40-CD40L) were both involved in shaping CD8 T cell responses. However, one help signal failed to reproduce the full impact of CD4 T cells, suggesting CD4 T cells helped CD8 T cells using multiple pathways.

An unanswered question is when and how long CD4 T cell help is needed. The requirement of IL-21 to sustain CD8 T cell response in chronic viral infection suggests the need for a persistent CD4 T cell help [125-127]. We found helpless CD8 T cells purified from mice at 5 wpi inhibited Mtb growth, but they lost ability to restrict Mtb as infection progressed, consistent with the rapid accumulation of terminally exhausted cells between 5 to 8 wpi. However, we can’t rule out

suboptimal priming in the absence of CD4 T cell help makes CD8 T cells prone to lose functions. It's known that CD4 T cell help during priming, but not after, is important for generating functional memory responses [109, 110], which is regulated through imprinting epigenetic states on CD8 T cells [112, 192]. Thus, the signals CD8 T cells received during priming has a long-term effect on their differentiation. The fact that progenitor exhaustion cells form under conditions when DCs express less MHCI and co-stimulatory molecules reinforces this idea [131]. To address this question, CD4 T cell depletion can be performed after CD8 T cells are primed, and whether CD8 T cells express helped features without persistent signals from CD4 T cells should be assessed.

CD4 T cell help prevents CD8 T cell exhaustion

A previous underappreciated role of CD4 T cell help is to prevent CD8 T cell exhaustion. Through a sequence of analyses including RNA-seq, flow cytometry, and clustering using UMAP and Phenograph, we identified 4 clusters of cells in different exhausted states from MHCII KO CD8 T cells. Furthermore, the exhausted features on MHCII KO CD8 T cells associated with their loss control of Mtb *in vitro*. T cell exhaustion describes a state in which T cells gradually lose proliferation ability and effector functions and upregulate inhibitory receptors on cell surface. Exhausted T cells are frequently reported in chronic viral infections and tumors. In TB, T cell exhaustion is also observed, although its contribution to pathogenesis remains inconclusive. T cells from peripheral blood of active TB

patients have greater inhibitory receptor PD-1 expression compared to healthy donors, and anti-PD1 blockade enhances their proliferation and effector functions [193-195]. The T cell dysfunction and apoptosis correlated with Mtb loads [186, 190]. In experimental mice, the expression of PD-1 and Tim-3 on T cells marks the exhausted state, correlating with impaired functions [163]. In NHP models, despite the expression of inhibitory receptor PD-1, LAG-3, and CTLA-4 is detected on T cells in lung tissue [196, 197], it appears that T cells in granulomas are not exhausted [198]. Tolerogenic rather than exhausted T cells are found in granulomas. Nevertheless, our results validated the exhausted features on CD8 T cells in lungs of infected mice.

How helpless CD8 T cells become exhausted is still unanswered. There are several hypotheses explaining the formation of exhausted T cells. The most accepted hypothesis is the antigen persistence in chronic infection leads to constant TCR engagement. The term “exhaustion” nicely describes this hypothesis. It is supported by studies showing transcription factors in the proximal downstream of TCR signaling, including IRF4, NFAT, and Nr4a, enhance exhaustion [164-166]. Another hypothesis is the suboptimal priming leads to T cell exhaustion [167]. This hypothesis arose from the observation that exhausted cells can be detected from early stage of chronic infection. It’s also explained by recent studies showing that terminally exhausted cells derived from a population of $TCF1^+$ “progenitor” cells. The fact that not all the cells, but a specific progenitor population develops exhaustion, suggesting a population that isn’t properly primed eventually becomes

exhausted. Priming with DCs that have decreased MHCI and co-stimulatory molecule expression enhances the formation of TCF1$^+$ progenitor cells also supports this hypothesis [131]. Finally, the lack of appropriate cytokines, for example IL-2 and IL-21, is associated with T cell exhaustion.

The above hypotheses are not mutually exclusive, and all the circumstances could happen in the absence of CD4 T cells, leading to CD8 T cell exhaustion. In many infections, including TB, bacterial burdens increase in the absence of CD4 T cells. In addition, CD8 T cells are activated with “licensed” DCs that received help signals from CD4 T cells. Thus, CD8 T cells are less likely being properly primed in the absence of CD4 T cells. We found that the terminally differentiated effector cells decrease, while TCF1$^+$ exhausted progenitor cells form without CD4 T cells, suggesting the “helpless priming” favors the formation of exhausted progenitor cells. Lastly, CD4 T cells are the main producers of IL-2 and IL-21. In our study, Mtb burdens in lungs and spleens were higher in MHCII KO mice even as early as 4 wpi, and this correlated with MHCII KO CD8 T cells expressed more PD-1 and Tim-3 at this stage. The frequencies of MHCII KO CD8 T cells expressing both PD-1 and Tim-3 increased as the infection progressed. This may indicate that antigen persistence was the cause of CD8 T cell exhaustion. However, it is possible that suboptimal priming or diminished IL-2 and/or IL-21 production in MHCII KO mice also contributed to this.

To further differentiate whether the increased bacterial burden or the lack of help signals in the absence of CD4 T cells leads to CD8 T cell exhaustion,

helped and helpless T cells needs to be compared in an environment where they receive similar antigen stimulation. We thought to leverage Mtb strain 18b, a streptomycin auxotroph, to address this question. Since 18b depends on streptomycin to grow, we gave streptomycin to 18b infected WT and MHCII KO mice, and found similar bacterial burdens between these mice after streptomycin withdrawal. However,18b infection elicited weaker Mtb specific CD8 T cell responses in MHCII KO mice, which complicated the comparison of exhaustion between WT and MHCII KO CD8 T cells. A future plan is to generate T cell chimeric mice with Ag85b specific CD4 T cells, WT CD8 T cells, and IL-21R KO CD8 T cells. In this model, exhausted features developed on helped and helpless CD8 T cells can be addressed independent of antigen stimulation.

In conclusion, CD4 T cells have an indispensable role to prevent CD8 T cell exhaustion through several mechanisms. These results suggest exhaustion may cause CD8 T cells to lose effector functions, and introducing mediators that revert exhaustion may improve their function. Although we found IL-2 or inhibitory receptor blockade had minimal role to restore CD8 T cells' ability to restrict intracellular Mtb growth *in vitro*, these mediators could potentially improve CD8 T cell functions *in vivo*.

Helped CD8 T cells are protective

Whether "helped" CD8 T cells mediate protection needs to be addressed. The relative importance of CD4 and CD8 T cells during TB is contested and conflict

observations on human and experimental NHP and mice have been reported. Mice deficient in CD4 T cells are extremely susceptible to Mtb infection, while mice deficient in CD8 T cells survive relatively long. This seems to be supported by the "experiment of nature" wherein AIDS patients have higher risk to develop active TB diseases. Although there is no equivalent evidence to prove the requirement for CD8 T cells, a lack of human mutations or diseases that compromised CD8 T cell responses may never have occurred in a TB-prevalent area.

We argue lack of CD4 T cell help contributes to the ineffectiveness of CD8 T cells. In experimental mouse models, CD8 T cells' role is evaluated in experiments using antibody depletion, adoptive transfer, and genetic KO. However, these studies frequently assess CD8 T cells' contribution in conditions where their function could be compromised (i.e., helpless). Whether helped CD8 T cells are more protective is a challenging question to answer, because the direct protection from CD4 T cells could be a cofounder whenever the "help" is introduced. Therefore, this question hasn't been adequately addressed in previous studies. In Chapter II, we used two different approaches to address this question. Using an adoptive transfer model in which polyclonal CD8 T cells were transferred with or without Ag85b specific CD4 T cells into TCRα KO mice, we proved CD4 and CD8 T cells synergistically enhanced survival of recipient mice. This synergy was established by calculating a survival curve in a scenario where CD4 and CD8 T cells work independently, and we showed mice received both CD4 and CD8 T cells actually survived longer than the hypothesized scenario. In addition, purified

helped or helpless CD8 T cells were cultured with infected macrophages to quantify their abilities to inhibit Mtb growth. Helped, but not helpless, CD8 T cells restricted Mtb growth. Collectively, these results showed helped CD8 T cells mediate better protection. Therefore, the susceptibility of mice deficient in CD4 T cells is likely exacerbated by compromised CD8 T cell responses.

Whether CD4 T cells promote effective CD8 T cells in NHP model is unknown. Few studies employed CD4 T cell depletion in NHP and even fewer evaluated CD8 T cell responses. Nevertheless, Lin and colleagues showed reduced $CXCR3^+$ CD8 T cells in CD4 T cell depleted cynomolgus macaques [199]. Although the functions of $CXCR3^+$ CD8 T cells were not fully elucidated, the expression of CXCR3 was associated with polyfunctionality of CD8 T cells. Interestingly, they found not all the CD4 depleted macaques reactivated from an established latent infection. This was consistent with another study reported by Foreman and colleagues, in which they showed a group of latently infected rhesus macaques didn't develop reactivation after SIV infection, despite CD4 T cell numbers severely decreased in all the macaques [200]. While Lin et al. attributed these resistant macaques to incomplete deletion of CD4 T cells, Foreman's group found CD8 T cells from these macaques had increased granzyme B production. Whether CD4 T cell help is essential in NHP needs further investigation.

Nevertheless, CD8 T cells seem to play a more notable role in NHP compared to mice. This may due to the fact that NHP, like humans, express granulysin and group 1 CD1, whereas mice don't have orthologs of these genes.

Granulysin is a protein that has microbicidal ability, while group 1 CD1 can present Mtb lipid antigens to CD1 restricted CD8 T cells. Both molecules directly associate with CD8 T cell function, thus supporting the idea that NHP (and human) CD8 T cells are more effective. The role of CD8 T cells in NHP was best demonstrated in the study by Chan and colleagues, in which they depleted CD8 T cells from BCG immunized rhesus macaques using antibody, and found BCG induced protection was totally abolished in sequential challenge [78]. It's unclear whether CD8 T cells played role in BCG induced protection, or they conferred protection in challenge without BCG, but this experiment demonstrated an indispensable role of CD8 T cells.

Several vaccine candidates that conferred significant protection were also reported to induce significant CD8 T cell responses. Darrah and colleagues showed intravenous administer of BCG (iv BCG) remarkably protected rhesus macaques, with 6 out of 9 macaques had no sign of infection [76]. Kaushal and colleagues reported aerosolized mutant MtbΔsigH protected rhesus macaques [79]. In both studies, CD8 T cell responses were detected in BAL and blood after vaccination. Although the underlying mechanisms whereby these vaccines elicit CD8 T cell responses, and whether strong CD8 T cell responses contribute to vaccine induced protection needs further investigation, these results imply the importance of CD8 T cells. Interestingly, the safety of iv BCG and aerosolized MtbΔsigH to use in AIDS patients has been addressed. Despite being proved to be safe, the efficacies of these vaccines in AIDS patients have not been reported.

Whether CD8 T cells contribute to vaccine induced protection in AIDS patients is an important question to address.

In the light of emerging results, I suggest CD8 T cell responses should be considered and assessed in designing TB vaccines. Both our findings in mice and recent studies in NHP emphasize the role of CD8 T cells, which was previously underestimated. Despite some vaccines eliciting CD8 T cell responses as discussed above, current vaccine strategies focus on generating a durable CD4 T cell response. We suggest vaccines eliciting both CD4 and CD8 T cell responses are more likely to be successful than vaccines only generating CD4 T cell responses. The focus on CD4 T cell responses and their ability to produce cytokines may in part reflect the difficulty to elicit CD8 T cell responses through vaccination. While CD4 T cells recognize antigens that entered endocytic pathways and presented on MHCII molecules, CD8 T cells recognize peptides presented on MHCI, which are sampled from cytoplasm. Therefore, most of vaccine strategies including attenuated live bacteria, inactivated bacteria, and subunit protein elicit predominantly CD4 T cell responses. Nevertheless, the use of replication-deficient viral vectors encoding Mtb antigens provide a tool to generate CD8 T cell responses. Indeed, Hansen and colleagues reported a successful viral vector vaccine that was tested in NHP [77]. In this study, rhesus macaques were vaccinated with rhesus CMV vectors encoding 9 different Mtb antigens, and this vaccine conferred protection when used as booster vaccine with BCG or used as prime and booster vaccine without BCG. Strong Mtb specific

memory CD8 T responses were detected in vaccinated macaques. Although the correlation of CD8 T cells and protection wasn't established this study, it demonstrated that a vaccine strategy eliciting CD8 T cell responses can confer protection.

To sum up, newly emerging data suggest the need to reassess the role of CD8 T cells in Mtb infection. We found helped CD8 T cells mediated protection in infected mice, and recent findings in NHP also highlighted the essential role of CD8 T cells. We infer that vaccines eliciting both CD4 and CD8 T cell responses are more likely to be successful, and that strategies generating both CD4 and CD8 T cell responses should be carefully considered in future development.

CD8 T cell function

Despite emerging data suggesting that CD8 play an indispensable role during TB, the molecular mechanisms of CD8 T cell mediated protection are not fully elucidated. In this section, I discuss the potential pathways CD8 T cells may use to confer protection. CD8 T cells can secrete protective cytokines including IFNγ, TNF, and IL-2, and they have cytotoxic ability. To determine the effector functions used by CD8 T cells to inhibit intracellular Mtb growth, CD8 T cells deficient in perforin and/or macrophages deficient in IFNγR were co-cultured and Mtb growth in macrophages was determined. Despite having lower efficacy, CD8 T cells can significantly inhibit Mtb growth in IFNγR KO macrophages. This indicates CD8 T cells control Mtb replication through IFNγ dependent and

independent mechanisms. In contrast, perforin KO CD8 T cells were fully able to control intracellular Mtb growth. A previous study reported increased IFNγ production in perforin KO CD8 T cells [98]. Thus, to exclude the possibility that enhanced IFNγ by perforin KO CD8 T cells contributes to the inhibition, the ability of WT vs. perforin KO CD8 T cells to control Mtb growth in IFNγR KO macrophages was tested. Both WT and perforin KO CD8 T cells inhibited Mtb growth with comparable ability. CD8 T cells can use several pathways for cytolysis, including exocytosis of cytotoxic granule proteins (i.e., perforin dependent), production of TNFα, and Fas-FasL engagement. Cell lines derived from infected animals or human have been used to study the cytotoxic mechanisms CD8 T cell used to kill target cells. While some cell lines exclusively use either perforin or Fas-FasL dependent pathway, others use multiple mechanisms [53, 98, 99]. It's likely these cytolytic mechanisms are redundant. Thus, deficiency in perforin may be compensated by other cytotoxicity pathways.

Another unanswered question is whether CD8 T cells have any unique role in protective immunity against Mtb. Although CD8 T cells can secrete many of the same cytokines as CD4 T cells, CD4 T cells are the main producers in most cases. Nevertheless, studies showed that CD8 T cells may be particularly crucial in some circumstances. In the 1980s, Orme and Collins showed that CD8 T cells from infected donor mice protected immunodeficient recipients from challenge. In this study, CD8 T cells conferred better protection in high dose aerosol infection, while CD4 T cells were more important in low dose aerosol infection [57]. These data

are supported by Chen and colleagues through experiments in NHP showing CD8 T cell depletion abolished BCG medicated protection in high dose challenge [78]. These results may be explained by the fact that CD8 T cells preferably recognize heavily infected cells [108]. Patankar and colleagues also found CD8 T cells produced more IFNγ when cultured with infected cells that have high MOI, while CD4 T cells responded to infected cells with low MOI and plateaued when cultured with heavily infected cells [107]. Collectively, these data suggest CD8 T cells play a role in immune surveillance and have greater control in heavily infected cells.

Whether CD8 T cells confer protection at particular time and location during Mtb infection is also postulated. The hypothesis that CD8 T cells are more important during the chronic phase of infection comes from observations that mice deficient in cytolytic pathways (i.e., perforin or Fas-FasL) lose control of bacterial burden only at later times [65, 101]. Similarly, van Pinxteren and colleagues found CD8 T cells were uniquely needed during latent infection [201]. In this study, anti-mycobacterial drugs were used after infection to establish a low bacterial burden, latent phase. CD8 T cell depletion, but not CD4 T cell depletion, during this phase increased bacterial burden significantly [201]. CD8 T cells may also be specialized to control systemic infection. Although there is no direct evidence for this theory, we observed 18b immunized MHCII KO CD8 T cells reduced bacterial burden in spleens, but not in lungs, of recipient mice after infection. A related result was reported previously by Feng and colleagues [60]. They found CD8 T cells from BCG immunized mice protected spleens of RAG KO mice from following infection,

but have limited control of bacteria burden in lungs [60]. Moreover, in experiments using anti-CD40 agonist antibody to provide a “help signal” to infected MHCII KO mice, we only observed reduced bacterial burden in spleens compared to untreated mice. These data suggest CD8 T cells exert better control in spleens than lungs. This differential protection between lung and spleen was previously reported by Sakai and colleagues who found that IFNγ had greater control of splenic bacterial burdens than burdens in lungs [37]. However, IFNγ mediated mechanisms are unlikely to explain the preferential protection exerted by CD8 T cells in spleen because CD4 T cells secrete more IFNγ. Other possible hypotheses include that CD8 T cells are more easily recruited to spleen, or they are better at recognizing infected cells in spleen.

A unique function of CD8 T cells described in many infections and cancer is their cytotoxic ability. Although CD8 T cells’ cytotoxic function in TB has been studied *in vitro,* to prove the requirement of these cytolysis pathways *in vivo* has shown to be challenging. Perforin, Fas, and FasL KO mice only succumb a little early than WT mice after infection [65, 101]. Therefore, to conclude the contribution of CD8 T cell cytotoxic ability in protective immunity is difficult, and the fact that NK and some CD4 T cells also express these cytotoxic molecules further complicates the explanation. However, the modest phenotypes in mice deficient in cytotoxicity pathways may also result from the lack of human granulysin gene ortholog. Granulysin is a cytotoxic granule protein that can directly lyse extracellular Mtb or kill intracellular Mtb through perforin dependent mechanism. Using scRNA-seq,

Gideon and Hughes et al. uncovered a previously unappreciated role of cytotoxic CD8 T cells in NHP. By comparing cellular compositions in restrictive vs. permissive granulomas, Gideon and Hughes identified cellular features that associated with Mtb control. Among these protective cells, a cluster of conventional T cells that expressed transcripts of multiple cytotoxic granule proteins and NK receptors showed the strongest correlation with protection. These cytotoxic CD8 T cells are also reported in human leprosy [75]. Interestingly, CD8 T cells expressing the widest variety of NK receptors secrete granulysin, granzymes, and perforin, while CD8 T cells with fewer NK receptors only express one or two of the cytotoxic granule proteins.

The importance of individual effector pathway in CD8 T cell mediated protection is not fully understood. The difficulty remains in the redundancy of effector functions within cytotoxicity pathways and between cytokine and cytolysis mediated pathways. Therefore, unbiased approaches including scRNA-seq may be particularly useful in studying these effector functions. Unraveling the requirement of effector pathways is needed to fully uncover the role of CD8 T cells role in immunity to Mtb.

Conclusion

A better delineation of protective immune responses against Mtb is the foundation to design better vaccines. This would facilitate identifying vaccine elicited responses that correlate with protection, and inform the evaluation of

potential candidates. To achieve this aim, we need to understand how different elements of our immune system interact. In this dissertation, I uncovered the significant role of CD4 T cell help in shaping CD8 T cell responses during TB. I identified “helped” vs. “helpless” CD8 T cell features, and showed helped CD8 T cells mediated better protection. These results and recent findings in NHP model collectively suggest CD8 T cell responses should be considered for vaccine design. We predict that vaccines which elicit both CD4 and CD8 T cell responses are more likely to be successful. Moreover, “help signals” can be included in the vaccine strategy to generate optimal CD8 T cell responses. The potential candidates for this purpose include anti-CD40 agonist, recombinant IL-2, and IL-21. This could be particularly important for designing effective TB vaccines for AIDS patients that have compromised CD4 T cells.

CHAPTER VI: MATERIALS AND METHODS

Experimental Model and Subject Details

Mice

IL-21R KO, P25 TCRtg, MHCII KO, and TCRα KO mice were purchased from Jackson Lab and bred locally. C57BL/6J, perforin KO, IFNγR KO mice were purchased from Jackson Lab. K^bD^b KO mice were a generous gift from Dr. Kenneth Rock (University of Massachusetts Medical School) [202]. Experimental mice were 7-12 weeks old and sex-matched. In adoptive transfer experiments, 7-12 weeks old, sex-matched TCRα KO mice from different litters were randomly assigned for receiving P25, CD8, or both cells. All animal studies were conducted using the relevant guidelines and regulations, and approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (UMMS) (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.

Mtb strain

The Erdman strain of *Mycobacterium* tuberculosis, which has been passaged through mice, was used for aerosol infection as described [203]. 18b was used for intravenous infection. H37Rv or CDC1551 strain was used for *in vitro* infection of macrophages. 18b and CDC1551 was provided by Dr. Christopher Sassetti (University of Massachusetts Medical School)

Method Details

Aerosolized Mtb infection of mice

Mice were infected by the aerosol route as previously described [16]. Frozen bacterial stocks were thawed and sonicated for 1 minute and then diluted into 5 ml of 0.01% Tween-80 in PBS. The diluted bacterial suspension was aerosolized to infect mice using a Glas-Col chamber (Terre Haute). The average number of bacteria delivered into the lung was determined for each experiment by plating lung homogenate from 4-5 mice 24 hours after infection and ranged between 40-150 CFU/mouse.

Intravenous 18b infection of mice

Frozen bacterial stocks were thawed, sonicated for 1 minute, and resuspended with 0.01% Tween-80 in saline. 200 ul of bacterial diluent containing 2 x 10^7 CFU of 18b was intravenously injected into mice. 2 mg/mouse of streptomycin was given intraperitoneally everyday for the first week of infection. The average number of bacteria delivered into the lung was determined for each experiment by plating lung homogenate on 7H10 plated contained 50 ug/ml of streptomycin from 3 mice 24 hours after infection and ranged between $10^{5.9}$-$10^{6.5}$ CFU/mouse.

Survival studies

Infected mice were monitored weekly in accordance with IACUC guidelines using the Body Condition Score (BCS) and serial weight determinations. Mice with a BSC score less than or equal to 2 or had lost 20% body weight were euthanized.

Bacterial burden in lung and spleen

Infected mice were euthanized at pre-determined timepoints, and the left lung or whole spleen were homogenized using 2 mm zirconium oxide beads (Next Advance) in a FastPrep homogenizer (MP Biomedicals). Tissue homogenates were serially diluted and plated on 7H11 agar plates (Hardy Diagnosis). For 18b infection, tissue homogenates were plated on 7H10 agar plates contained 50 ug/ml of streptomycin. CFU was enumerated after 19-21 days of incubation at 37°C and 5% CO_2.

Lung cell preparation

Lungs were perfused with 10 ml of cold RPMI (Gibco) before removal. Single cell suspensions were prepared by homogenizing lungs using a GentleMACS tissue dissociator (Miltenyi), digesting with 300 U/ml collagenase (Sigma) in complete RPMI (10% FBS, 2 mM L-Glutamine, 100 units/ml Penicillin/Streptomycin, 1 mM Na-Pyruvate, 1X Non-essential amino acids, 0.5X Minimal essential amino acids, 25 mM of HEPES, and 7.5 mM of NaOH) at 37°C for 30 minutes, and followed by

a second run of dissociation using the GentleMACS. Suspensions were then sequentially filtered through 70 µm and 40 µm strainers.

Intravascular staining

Mice were injected intravenously with 0.2 ug/mouse of anti-CD45-AF647 in 200 ul of injection medium (2% FBS in PBS) 2 minutes before euthanizing with CO_2. Lungs were then perfused and removed about 3 minutes after anti-CD45-AF647 injection. Lymphocytes from blood collected in RPMI containing 40 U/ml of heparin were isolated using Lympholyte® (CEDARLANE), and analyzed by flow cytometry to confirm uniform staining with anti-CD45-AF647.

Flow cytometric analysis

Cells were first stained with Zombie Fixable Viability dye (Biolegend) for 10 minutes at room temperature (RT). For surface staining, cells were stained with antibodies in autoMACS running buffer (Miltenyi) containing 5 ug/ml of anti-mouse CD16/32 (BioXcell) for 20 minutes at 4°C. In some experiments, $TB10.4_{4\text{-}11}$ and/or $32A_{309\text{-}318}$ tetramers were used with other antibodies. To measure transcription factor (TF) expression, cells were fixed and permeabilized for 30 minutes at RT, followed by staining for 30 minutes with antibodies to the different TF at RT using the Foxp3/TF staining buffer set (ThermoFisher). Samples were acquired on Aurora (Cytek) or MACSquant (Miltenyi). Flow data were analyzed using FlowJo v10.7.1, and FlowJo plugins UMAP v3.1 and PhenoGraph v3.0. For UMAP

projections of WT/KO CD8 T cells (i.e., Fig 3C and S2C), 2000 CD45iv^{-} CD44^{+}CD62L^{-} CD8 T cells/mouse from 5 WT and 5 MHCII KO mice were concatenated to one FCS file before creating UMAP projections. In UMAP projections comparing two models (i.e., Fig 5D), 2000 CD45iv^{-}CD44^{+}CD62L^{-} CD8 T cells/mouse from 5 WT and 5 KO, and 6 helped and 4 helpless mice from adoptive transfer model were concatenated and created UMAP projections. K= 300 and K= 400 were used in PhenoGraph analysis in Fig 3D and Fig 5B, respectively, and the resulting clusters were overlayed onto UMAP projections.

Adoptive transfer model

Spleens and lymph nodes from C57BL/6J mice were mechanically disrupted onto 70 μm strainers using the plungers of 3 ml syringes. CD8 T cells were then purified from cell suspensions using CD8a (Ly-2) microbeads (Miltenyi) and autoMACS separator (Miltenyi). Preparing cell suspension with same method, CD4 T cells were purified from spleens and lymph nodes of P25 mice using MojoSort^{TM} CD4 isolation kit and magnet (Biolegend). Purities of cells were determined for each experiment using flow cytometry. 2-5 million of CD8 T cells were transferred into TCRα KO mice with or without distinct congenic marked 10^5 P25 cells before infecting with Erdman.

Adoptive transfer with infected CD8 T cells

Spleens of Mtb or 18b infected WT or MHCII KO mice were mechanically disrupted onto 70 μm strainers using the plungers of 3 ml syringes. CD8 T cells were then purified from cell suspensions using CD8a (Ly-2) microbeads (Miltenyi) and autoMACS separator (Miltenyi). Purities of cells were determined for each experiment using flow cytometry. 2-5 million of CD8 T cells were transferred into TCRα KO mice. TCRα KO mice received WT or MHCII KO CD8 T cells, and TCRα KO mice didn't receive cells were challenged with aerosolized Mtb.

In vitro CFU assay

Purified CD8 T cells were cultured with infected macrophages to quantify their ability to inhibit Mtb growth. The assay is described in following sections:

Preparation of Mtb: CDC1551 was grown in 7H9 media (supplemented with 10% OADC, 0.05% of Tween-80, and 0.2% glycerol) until an OD_{600} = 0.6-1, washed with RPMI, and opsonized with TB coat (RPMI 1640 containing 1% heat-inactivated FBS, 2% human serum, and 0.05% Tween-80). The bacteria were passed through 5 μm filter to remove clumps and then enumerated by microscopy using a Petroff-Hausser counting chamber. The concentration was adjusted to provide a multiplicity of infection (MOI) of 2.

Macrophage collection and infection: Thioglycolate-elicited peritoneal macrophages (TG-PMs) were obtained by peritoneal lavage 3-5 days after intraperitoneal injection of mice with 3% thioglycolate, and purifying from the

lavage using CD11b microbeads (Miltenyi) and LS columns (Miltenyi). Purified TG-PMs were plated in Nunc™ Up-Cell™ 12-well plates (10^6/well), and once adhered, infected with CDC1551 overnight. Infected TG-PMs were detached and washed with cold complete RMPI, and re-seeded as 10^5/well in 96-well flat bottom plate before CD8 T cells were added.

CD8 T cell isolation and co-culture: CD8 T cells were purified from the lungs of infected mice at indicated timepoints using CD8a (Ly-2) microbeads (Miltenyi) and an autoMACS separator (Miltenyi). The purity of cells was determined for each experiment and was typically 95%. Purified CD8 T cells were added to infected TG-PMs at a ratio 1: 2 (CD8 T: TG-PM) or as indicated. TG-PMs were lysed with 1% Triton X-100 after 4-6 days of co-culture, and CFU was determined by plating serial dilutions of the lysate on 7H10 or 7H11 plates (Hardy Diagnosis). Percent inhibition by CD8 T cells was calculated as: 100 x (Mtb net growth without CD8 T cell – Mtb net growth with CD8 T cell)/ Mtb net growth without CD8 T cell.

Cytokine production by CD8 T cells

Lung CD8 T cells were isolated and cultured with macrophages infected with CDC1551 or H37Rv, as described above with the modifications that an MOI=5 was used and CD8 T cells were added into infected TG-PMs at a 1:1 ratio. Culture supernatants were collected after 18-24 hours of co-culture, and IL-2, IFNγ, and TNF were assayed using LEGENDplex™ (Biolegend).

RNA-seq

CD8 T cells were collected and purified from lungs of infected mice 8wpi as described above. Purified CD8 T cells from individual lungs were suspended in RNAprotect® cell reagent (Qiagen) and the RNA extracted using RNeasy® mini kit (Qiagen). RNA samples were sequenced by GENEWIZ. Reads were aligned to the mouse genome and differential expression analysis performed by GENEWIZ. Gene Ontology analysis was performed with genes filtered with p-adjusted value (padj) < 0.05 and $|\log_2$ fold-change$| \geq 1$, or filtered with padj < 0.05 independently of fold-change using R (v 3.6.1) package clusterProfiler (v3.14.3) [204]. The top 10 enriched pathways with the highest padj value were presented. GSEA was performed using the Broad institute software and published datasets of CD8 transcriptome from Gene Expression Omnibus (GEO) [205, 206]. Results comparing with datasets GSE30962 and GSE9650 were shown.

In vivo antibody treatment

100 ug of anti-CD40 (clone FGK45) was given intraperitoneally into infected MHCII KO mice once every week for 7 weeks. 100 ug of anti-Lag3 (clone C9B7W) antibody was given intraperitoneally into infected TCRα KO mice received WT or MHCII KO CD8 T cells every three days for 3 weeks. 200 ug of anti-CD4 was given intraperitoneally a day before and one day after 18b infection to deplete CD4 T cells in B57BL/6J mice, and 100 ug of anti-CD4 was continuously given every five days for 3 weeks.

Quantification and Statistical Analysis

Statistical analysis was performed using Graphpad Prism 8. P-values were calculated using unpaired t test, one-way ANOVA, or two-way ANOVA as indicated in the figure legends.

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