FUNCTION AND REGULATION OF CD8 T CELLS DURING *MYCOBACTERIUM TUBERCULOSIS* INFECTION IN A MURINE MODEL

by

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ABSTRACT

Although more doses have been given of the tuberculosis vaccine (BCG) than any other vaccine in history, tuberculosis remains the second leading cause of death due to an infectious agent worldwide. Our understanding of the immune response to the bacterium is incomplete, which has partly been due to a lack of known bacterial T cell epitopes. In recent years, several epitopes have been identified, which has enabled the use of new reagents and assays to study T cell responses. Furthermore, development of recombinant bacteria expressing model antigens has facilitated the use of many more reagents and techniques, as well as allowing us to compare *M. tuberculosis*-specific T cell responses to those induced by other infectious agents. The aim of this thesis was to better understand the CD8 T cell response to *M. tuberculosis* and identify factors that regulate the response. We showed that, unexpectedly, the primary CD8 T cell response was more effective than the memory CD8 T cell response to *M. tuberculosis*, in that primary cells secreted higher levels of IFN-γ and were more cytotoxic. We also showed that cytotoxicity and secretion of IFN-γ were carried out by distinct *M. tuberculosis*-specific CD8 T cells, which indicates that there is a functional defect in these cells that is not seen in other infections. Contrary to chronic viral infections, however, *M. tuberculosis*-specific CD8 T cells did not appear to become exhausted, despite the chronicity of the infection. We further showed that the

function of CD8 T cells was influenced by various factors, such as interactions with pre-existing memory cells, the quality of CD4 T cell help and the environment that the cells were primed in. Manipulation of these factors can increase the quality of the immune response, giving multifunctional CD4 and CD8 T cells, as well as CD8 T cells capable of simultaneously being cytotoxic and secreting IFN-γ, without altering the bacterium. This indicates that modifications in delivery of tuberculosis vaccines may result in improved efficacy. The work presented may contribute to advances in the development of new tuberculosis vaccines, as well as improvement in efficacy of the existing vaccine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 INTRODUCTION

1.1 HISTORY OF TUBERCULOSIS IN HUMANS

Tuberculosis (TB) is primarily a lung disease, caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), a bacterium that has coexisted with humans for millennia. The first records of *M. tuberculosis* causing disease in humans come from 9000 year old skeletal remains from Israel [1]. Rampant TB in the human population has been documented from the time of Hippocrates (around 400 BC) and *M. tuberculosis* was the cause of the white plague of the 18^{th} and 19^{th} centuries [2, 3]. The TB pandemic was controlled to a great extent in developed countries through the development of antibiotics and improvement in public hygiene and nutrition, while the rate of infection remained high in less prosperous areas. TB received little attention until there was a resurgence in developed countries in the 1980s and 1990s, in part due to the spread of the human immunodeficiency virus (HIV) [4, 5]. In 1993 the World Health Organization (WHO) declared TB a global health emergency and since then a tremendous effort has been made to control the infection. Despite this, there are eight to ten million new cases of active TB annually. Approximately two million people die every year, making TB the second leading cause of death due to an infectious agent [6].

1.2 INFECTION OUTCOME

It is estimated that a third of the human population (approximately two billion people) is infected with *M. tuberculosis*, the majority (about 90%) of whom are able to contain the infection (termed latent infection) and show no signs of disease (Figure 1D and F). Induction of *M. tuberculosis*-specific adaptive immune responses (discussed in chapter 1.4) in these people yields a positive response to the purified protein derivative (PPD) skin test, although the skin test does not discriminate between persons latently harboring bacteria and those who may have eventually cleared the infection [7]. Vaccination with Bacille Calmette-Guerin (BCG, discussed in chapter 1.7.1) also induces a positive PPD skin test due to cross-reactive immune responses [8].

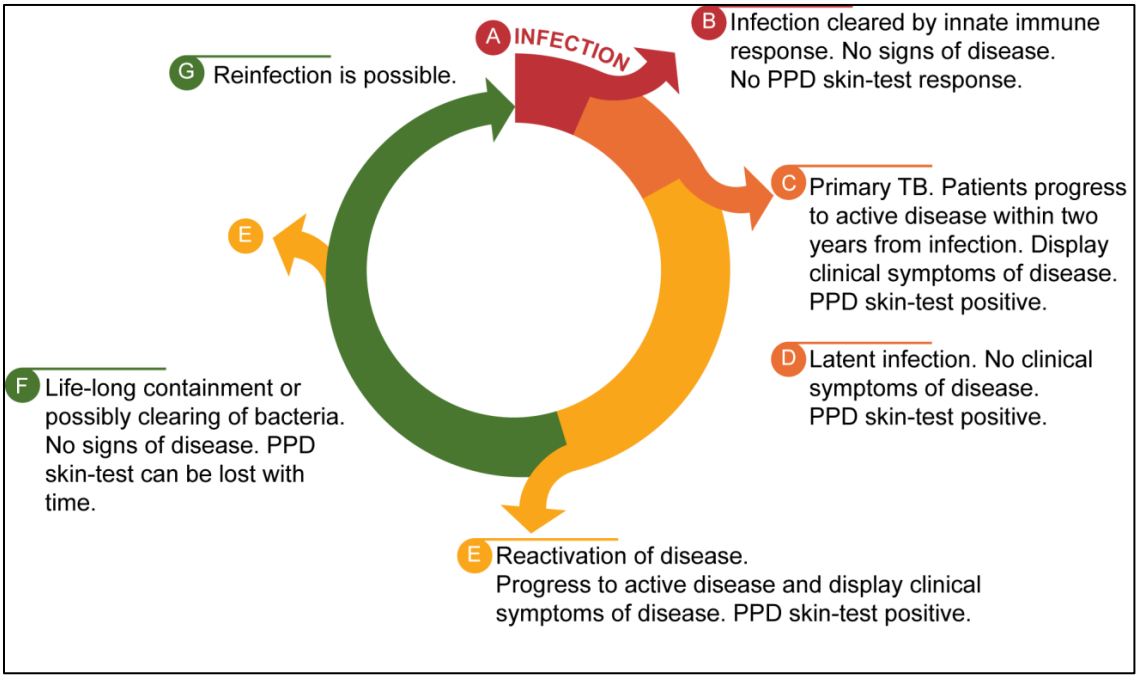


Figure 1 *M. tuberculosis* infection course in humans.
People exposed to the bacterium (A) can clear the infection (B) or be colonized (C to F). 5-10% of persons develop primary TB within two years from infection (C), while the majority are able to contain the infection without clinical symptoms (D). About 10% of latently infected individuals lose control of the infection at some point and progress to active disease (E), while the remainder contains the infection life-long (F). Previous exposure to *M. tuberculosis* does not necessarily prevent re-infection (G).

About 5% of infected individuals are unable to contain the initial infection and progress to active TB within two years of infection, although this percentage is higher in young children and immunocompromised persons [2, 9, 10] (Figure 1C). In addition to a positive PPD skin test, these individuals show clinical symptoms such as cough, weight loss and positive bacterial cultures [11], although the specific symptoms can depend on the site of disease (discussed below).

Of those who contain the initial infection and develop latent infection, there is a 10% life-time risk of reactivation and progression to active TB (Figure 1E). For unknown reasons, the rate of reactivation is higher in adolescents and young adults than in other age groups [9]. Immunosuppression, e.g. due to HIV-infection, is also a strong risk factor for reactivation (10% annual risk of reactivation) [2, 10, 12]. Thus, while the majority of persons are able to contain the bacterial infection, there is an enormous reservoir of persons harboring bacteria and the infection can reactivate for no apparent reason, although factors such as immunosuppression, malnutrition, and age are strong risk factors [9].

To further complicate the scenario, the symptoms of TB can vary greatly, making the infection difficult to identify. Pulmonary TB is the most common form of disease, of which the hallmark symptom is coughing. However, the bacteria can infect and cause disease in almost any part of the body, such as the meninges, lymphatic system, genitourinary system and bones. Each site of disease can manifest in different symptoms, e.g. pain and inflammation in the respective area, but more common symptoms are weight loss, fatigue and fever [11, 13]. While extrapulmonary TB is less infectious than pulmonary TB (discussed in chapter 1.3.4), this form of disease is often more severe [9, 11]. The risk of developing extrapulmonary TB is higher in immunosuppressed people and young children compared to healthy adults [13].

1.3 INNATE IMMUNE RESPONSE

M. tuberculosis bacteria are transmitted by aerosolized droplets that are expelled by persons with active pulmonary TB [2, 14]. Upon inhalation of these droplets, the bacteria are deposited in alveolar spaces where they can be phagocytosed by macrophages (Mϕ) and dendritic cells (DC) (innate immune cells) [15-17].

1.3.1 Phagocytosis

Bacteria can be phagocytosed by Mϕ and DC through a variety of receptors (reviewed in [18]). It has not been fully established what effect the route of internalization has on bacterial survival [19], although it can affect the phenotype of the phagocyte [20-22]. In some cases, the phagocytic cells can clear the bacteria [14] leaving no measurable trace of the infection, i.e. the person has a negative PPD skin test (Figure 1B). Other times, the bacteria are able to escape killing by the innate immune response and can replicate in the phagocytic vesicles. To accomplish this, the bacteria inhibit phagosomal maturation (i.e. acidification and fusion to lysosomes), an important antibacterial mechanism of the innate immune cells, while allowing fusion to early endosomal compartments containing iron and nutrients [23]. Phagosomal acidification is inhibited by exclusion of ATPases from the phagosome [24] while phago-lysosome fusion is prevented by inhibiting maturation of the phagosome, e.g. by retention of Rab5 (an early endosomal protein) and exclusion of Rab7 (a late endosomal protein) [25].

1.3.2 Recognition of infection

Although *M. tuberculosis* can inhibit killing by phagocytes, the infected cells can recognize the infection through binding of bacterial components (pathogen associated molecular patterns, PAMP) to pattern-recognition receptors (PRR), such as Toll-like receptors (TLR) and nucleotide oligomerization domain-like receptors (NLR). While TLR signaling in response to *M. tuberculosis* is the best characterized of the PRRs, the individual TLRs appear not to be essential for the host [26]. However, myeloid differentiation factor 88 (MyD88), the common adaptor molecule for the TLRs bound by *M. tuberculosis* components, is essential for host survival [27]. This indicates that the importance of individual PRRs may be masked by redundancy between them [28], but the phenotype of MyD88 deficient mice may also be due to its role in interleukin (IL)-1 receptor signaling [29].

Binding of *M. tuberculosis* components to PRR matures the phagocytes, and induces a downstream signaling cascade leading to production of microbicidal effector molecules, such as reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) and antimicrobial peptides [30-33], although they are not sufficient to control the infection without involvement of the adaptive immune response [34]. PRR signals also lead to production of chemokines that recruit leukocytes to the site of infection [17] and cytokines that determine the type of adaptive immune response that is induced.

1.3.3 Cytokines and chemokines of the innate immune cells

Mϕ and DC express various cytokines in response to the bacterium [35-38] which influence the priming of the adaptive immune response. Inflammatory cytokines, such as IL-1,

IL-12 and tumor necrosis factor (TNF), promote a Th1-type adaptive immune response and are essential for control of the bacterium [39-41]. Other cytokines, such as IL-4 and IL-10, promote a Th2-type response that results in decreased control of the bacterium [42, 43]. The immature immune response of young children is thought to increase their risk of developing extrapulmonary TB by skewing the immune response towards a Th2-type response (reviewed in [9, 44]). In addition to Th1-type and Th2-type responses, a recently identified subset of T cells, called Th17, are induced in response to IL-23. During primary infection, there is an early influx of IL-17 producing cells [45], but they do not appear to be essential for bacterial control [46].

The type of immune response induced by *M. tuberculosis* infection is influenced by factors such as bacterial components, some of which induce a Th1-type response while others induce Th2-type responses [38]. The resulting immune response is likely to be a mosaic, and the balance between the two determines the infection outcome. Parallel responses to other infectious agents can also influence the cytokines produced. Parasite co-infections have been implicated in reactivation of latent *M. tuberculosis* infection [47] as well as decreased efficacy of BCG vaccination [48, 49] by skewing the T cell response towards a Th2-type response.

Although both DC and Mϕ can be infected with *M. tuberculosis* and can prime T cell responses (serve as antigen-presenting cells), there are differences in how the cells respond to the bacterium. While Mϕ remain phagocytic and do not migrate out of the lung [21, 50], DC lose phagocytic potential [50] and upregulate expression of CCR7 and CXCR4, chemokines needed for migration to secondary lymphoid organs [51]. DC also express higher levels of costimulatory molecules and major histocompatibility molecules (MHC), needed for priming of naive T cells [35, 52].

DC and Mϕ responses also differ once T cells have been primed. Bacteria can replicate unhindered in both DC and Mϕ until they are activated by IFN-γ and TNF, secreted by Th1-type T cells. This allows Mϕ, but not DC, phagosomes to acidify and fuse to lysosomes, as well as increase the expression of RNI ([35], reviewed in [53]). The Mϕ are then able to kill intracellular bacteria, while activation of DC allows the cells to control bacterial expansion, but not kill them [35]. This indicates that Mϕ are specialized to kill bacteria, while DC bridge the gap between the innate and adaptive immune responses.

1.3.4 Granulomas

Granulomas are formed in response to chronic antigen stimulation, where infected cells are surrounded by uninfected Mϕ and DC. Inflammatory cytokines and chemokines secreted by the infected cells can activate the uninfected cells, to make them refractory to the bacterial inhibition of phagosome acidification and their fusion to lysosomes [54, 55]. The uninfected Mϕ and DC are surrounded by a cuff of lymphocytes [56]. Granulomas benefit the host by enclosing the bacteria and preventing their spread, and failure to form or maintain the granuloma structure is thought to dramatically increase the risk of developing TB. However, granulomas can also be beneficial to the bacteria, which can survive in this hypoxic environment for decades [57-59].

The spectrum of granulomas that are formed in humans ranges from calcified granulomas (where bacteria may be contained or eliminated) to necrotic granulomas that cause tissue destruction. If the tissue destruction is close to bronchi, bacteria can enter the airways and be transmitted by aerosolized droplets into the patient's environment (reviewed in [60]). This allows spread of the infection, and is the reason why pulmonary TB is more infectious than extrapulmonary TB.

Formation of the granuloma is the result of a complex interplay of host- and bacterial interactions that is not fully understood. Recent work in the zebrafish model, discussed in chapter 1.6.4, indicates that granulomas are formed through interaction between the bacteria and host innate immune responses, and that granuloma formation is beneficial to bacterial growth [61-63]. However, there are significant differences in the structures of granulomas in different models. Most notably, mouse granulomas are less structured than those seen in non-human primates and humans, and while TNF is required for both formation and maintenance of granulomas in mice [41, 64], TNF-depletion in non-human primates does not appear to affect granuloma structures [65]. However, while the granuloma structure is intact in TNF-depleted primates, TNF is important for control of the infection. This became apparent in patients undergoing therapy to decrease TNF-mediated inflammation in autoinflammatory diseases such as rheumatoid arthritis and Crohn's disease. Inhibition of TNF signaling in these patients greatly increased the risk of reactivation of TB and of developing extrapulmonary TB [66, 67]. While the effect of TNF-inhibition may be pleiotropic, one possible effect is decreased cytotoxicity of CD8 T cells, discussed in chapter 1.4.1.2.

1.4 ADAPTIVE IMMUNE RESPONSE

Mature DC transport live bacteria to secondary lymphoid organs, where they can present bacterial antigens to the adaptive immune cells. While this transport appears to be important for dissemination of the bacterium [68], it is essential for priming of T cell responses [69-71] and a delay in dissemination correlates with decreased control of infection [69, 72].

1.4.1 T cells

Priming of T cell responses to *M. tuberculosis* is delayed compared to many other infections [73, 74]. However, this priming coincides with control of bacterial expansion [75], which emphasizes their importance in combating *M. tuberculosis*. After priming, T cells migrate to infected tissues to exert their effector functions (discussed below). T cell responses appear to be primed in the mediastinal lymph node (the lung-draining lymph node), as this is the tissue where effector function is first observed [72]. Studies using adoptive transfer of *M. tuberculosis*-specific T cell receptor-transgenic CD4 T cells further support this conclusion, as the cells start to proliferate in the lymph node before appearing in the lungs [70, 71]. However, this model depends on transfer of non-physiological numbers of transgenic T cells, which can influence the behavior of the cells [76, 77].

1.4.1.1 CD4 T cells

CD4 T cells are essential for control of the bacterium [78, 79]. This is underscored in HIV-infected persons, where loss of CD4 T cells is associated with drastically increased risk of reactivation of TB [12]. CD4 T cells are important producers of IFN-γ, whose production peaks around three to four weeks post-infection [80]. IFN-γ activates antigen-presenting cells, and boosts expression of MHC and costimulatory molecules, and is the most critical factor identified in *M. tuberculosis* control, as IFN-γ-deficient mice succumb to the infection earlier than any other immuno-deficient mice [81]. In CD4 T cell-deficient mice, other cells can compensate for the lack of CD4 T cell-mediated IFN-γ by increasing production of the cytokine so that normal levels are reached by four weeks post-infection. However, this is not sufficient to save the mice

from death [79]. Thus, early production of IFN-γ, and possibly other functions of the CD4 T cells, are essential for the immune response against the bacterium.

Recent work using *Leishmania major* indicates that vaccines that induce CD4 T cells capable of secreting multiple cytokines (IL-2+IFN-γ+TNF+, multifunctional cells) give better protection than vaccines that induce CD4 T cells with fewer functions [82]. This appears to hold true for viral infections as well [83, 84], but has not been definitively demonstrated for mycobacterial infections [85-87]. It has not been established which immune responses are associated with protection during *M. tuberculosis* infection in humans, as the only current intervention that significantly lowers the bacterial load is long-term therapy with antibacterial drugs.

CD4 T cells also shape the CD8 T cell response by providing "CD4 T cell help" to DC, although the exact nature and requirement of this help are controversial and appear to depend on the infectious agent [88-91]. One of the outcomes of CD4 T cell help is activation of the DC, which is accompanied by increased expression of costimulatory molecules and cytokines, and results in improved priming of CD8 T cells. In *M. tuberculosis* infection, CD8 T cell responses can be primed in the absence of CD4 T cells [79], but they are less cytotoxic [92], indicating that CD4 T cell help is important for determining the quality of the CD8 T cell response.

CD4 T cells can also dampen the immune response, either by secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) [93], or through a mechanism requiring cell-cell contact [94]. These regulatory T cells, termed Tregs, attenuate the immune response to the bacterium, resulting in decreased inflammation [95] and a decrease in proliferation and effector function of T cells [96]. The literature indicates that Treg-mediated regulation of the immune response may be either beneficial [97] or detrimental [95, 98, 99] for

control of mycobacterial infection. More likely the answer lies somewhere in between, where a balance is struck between induction of immune responses, that can kill or control the bacteria, and suppression, that minimizes collateral damage (pathology) to the host.

1.4.1.2 CD8 T cells

Little is known about the role of CD8 T cells during *M. tuberculosis* infection. One reason is the dearth of known *M. tuberculosis* epitopes recognized by CD8 T cells [100]. In recent years, several *M. tuberculosis*-specific CD8 T cell epitopes have been identified [101, 102], enabling the use of numerous new techniques to measure CD8 T cell function, discussed in chapter 3. Furthermore, successful development of recombinant strains expressing exogenous epitopes [103] has allowed us to manipulate the mouse infection model to previously unprecedented levels to ask specific experimental questions, discussed in chapters 4 and 5.

One consequence of the high frequency of individuals co-infected with HIV and *M. tuberculosis* is that much has been learned about the role of CD4 T cells during *M. tuberculosis* infection. HIV-mediated depletion of CD4 T cells dramatically increases the risk of reactivation of TB [12], which confirms murine data on the importance of CD4 T cells in the control of infection [79]. We do not have a similar model of acquired CD8 T cell depletion in humans. However, CD8 T cell depletion in non-human primate models leads to rapid loss of control of the infection, which confirms the importance of CD8 T cells in TB ([104], Philana Ling Lin and JoAnne Flynn, unpublished results). This is not mirrored in the murine model [81], where CD8 T cells have a limited impact on the control of infection, observed mostly during chronic [78] and high-dose infections [105, 106]. However, the impact of CD8 T cells on bacterial load can be increased in mice through interventions such as DNA vaccines [107].

Thus, while the availability of immunological reagents and different mouse strains make the murine model exquisitely useful for basic research and discovery (discussed in chapter 1.6.1), the results need to be verified in other models that better represent humans. Alternatively, using "humanized" mice, i.e. mice that have been genetically altered to express certain human proteins, may give a more faithful picture of the human immune response. One such example is a mouse strain expressing granulysin, an antimicrobial protein that is stored in cytolytic vesicles. Granulysin can kill *M. tuberculosis*, and its expression correlates with protection against TB in humans [108, 109]. It will be interesting to see whether CD8 T cells expressing granulysin are better able to control the infection than CD8 T cells from wild-type (WT) mouse strains.

It is thought that the major role of *M. tuberculosis*-specific CD8 T cells is to eliminate infected cells that have become refractory to activating cytokines, such as IFN-γ [110]. The bacteria can then be taken up by uninfected Mϕ, which are still sensitive to IFN-γ and can better contain the bacteria [111]. While the importance of CD8 T cell-mediated cytotoxicity in mice is debated [112-114], it appears that cytotoxicity is important for control of TB in humans. It was recently shown that anti-TNF treatment decreased CD8 T cell mediated cytotoxicity in humans, by reducing the expression of perforin and granulysin [115]. This may be a mechanism of increased risk of reactivation of TB in patients undergoing anti-TNF treatment.

CD8 T cells can also secrete cytokines, such as IFN-γ and TNF, which help to activate Mϕ and DC, although mouse CD4 T cells appear to produce sufficient cytokines to contain the infection. It has been suggested that multifunctional CD8 T cells, i.e. cells secreting IFN-γ, IL-2 and TNF, may be more protective than cells with fewer functions [116]. However, very few *M. tuberculosis*-specific CD8 T cells appear to be multifunctional in humans [87] and non-human primates (Joshua T. Mattila and JoAnne Flynn, unpublished results). The impact of CD8 T cell-

mediated cytokine secretion in control of TB in humans and non-human primates is unknown. However, due to the importance of IFN-γ for control of TB, a vaccine that induces strong CD8 T cell-mediated cytokine production could benefit individuals co-infected with HIV and *M. tuberculosis*, where CD4 T cell-mediated secretion may be compromised.

1.4.2 Other cells

In addition to the cells discussed above, several other cell types respond during *M. tuberculosis* infection, although their role in controlling the bacterial infection is controversial. Examples include natural killer cells (NK), unconventional T cells (e.g. CD-1 restricted T cells and γδ T cells) and B cells. The B cell response illustrates the difficulty of establishing the role of the cells in controlling *M. tuberculosis* infection. During *M. tuberculosis* infection in both humans and mice, B cell responses have been shown to be protective [117], detrimental [44, 118] or contribute little to the infection outcome [119, 120]. It has also been suggested that B cells function in an antibody-independent manner, e.g. by presenting antigens to T cells [121], influencing the cytokine environment [117] or as a structural component needed for maintenance of the granuloma [118]. Thus, it remains to be conclusively established what role B cells play in control of infection.

1.5 TREATMENT OF TB

Before the development of effective TB drugs, the main approach to treating TB was to sequester patients in sanatoria. TB drugs, discovered between the 1940s and 1960s, enabled

clearing of infection, which greatly reduced infection rates as well as mortality and morbidity. Initially, recommended treatment duration was 24 months to ensure complete clearance of the bacteria [122], but subsequent advances have shortened the treatment to approximately 6 months [13]. While it is not fully understood why it takes so long to clear the bacteria, a granuloma-induced state of nonreplication and metabolic inertia is believed to make at least a subset of the bacteria phenotypically resistant to many drugs [123, 124]. In addition, noncompliance due to the long treatment duration and toxicity has led to increasing drug resistance of the bacterium [13], severely limiting treatment options for patients.

Despite pressing need for new TB drugs, no new first-line TB drugs have been added to the drug repertoire for the last 30 years [125]. In recent years, better understanding of the bacterium has led to the identification of several new drug candidates, some of which have reached clinical trials [123, 126].

1.5.1 Drugs from the 1940s

Streptomycin (SM), an inhibitor of protein synthesis in replicating bacteria, was discovered in 1944 and was the first drug to be used against TB [127, 128]. The same year, para-amino salt of salicylic acid (PAS), which likely inhibits bacterial cell-wall synthesis, was discovered to be effective against TB [127, 129]. In 1950 the British Medical Research Council published a paper showing that a two year regimen combining both drugs was more effective and decreased the risk of development of drug resistance compared to treatment with either drug alone [122, 130]. Both drugs are currently considered second-line TB drugs because of their toxicity [122, 124].

1.5.2 Drugs from the 1950s

Isonicotinic acid hydrazide (INH), which is thought to be an inhibitor of cell wall synthesis in replicating bacteria, was discovered in 1952 [129]. When added to the combination therapy of SM and PAS, this was more effective at clearing bacteria, and further decreased the risk of development of drug resistance [122]. Despite some toxicity associated with the drug, especially in HIV infected persons [124], INH is a first-line TB drug and is often prophylactically administered to people who are believed to be latently infected or have been exposed to persons with active TB [129].

Pyrazinamide (PZA) is another first-line drug that was shown to be effective against TB in 1952 [131], although the mechanism of action is unknown.

1.5.3 Drugs from the 1960s

Ethambutol (EMB), a metabolic inhibitor of replicating bacteria, was discovered in 1961 [129]. This drug replaced PAS in treatment regimens, as it reduced both toxicity and the duration of treatment to 18 months [122].

Rifampin (RIF), an RNA transcription inhibitor, was discovered in 1966 [129]. When added to INH, SM and EMB treatment regimens, RIF reduced the duration of treatment from eighteen months to about nine months. It was shown in the 1980s that adding PZA, a drug that was discovered in the 1950s, to INH and RIF treatment regimens could shorten treatment to as little as 6 months [122, 127].

Both EMB and RIF remain first-line drugs, but unfortunately RIF can interact with antiretroviral drugs, causing their rapid break-down. This complicates the use of the drug in HIV-infected persons [123].

1.5.4 Directly Observed Treatment (DOT)

The DOT treatment regimen was developed in 1959. DOT is a treatment strategy aiming at improving patient adherence, in which a person, such as a health-care provider or community volunteer, directly observes the patient taking their medicine. This has allowed patients to undergo treatment at home, instead of being isolated in sanatoria, away from their families for a year or more [127].

The treatment regimen has since been modified to Directly Observed Treatment, Short-Course (DOTS), a 6 month treatment course aimed at further improving patient adherence. The standard DOTS regimen has two phases. First is a daily four-drug intensive phase for two months, followed by a two to three times per week, two-drug continual phase for four months [123, 132]. The drug combinations and durations depend on bacterial drug susceptibility and how the patient responds to treatment, as well as the patient's history of infection.

1.5.5 Drug resistant bacteria

The current first-line drugs are INH, RIF, EMB, PZA and a rifamycin. Multidrug-resistant strains (MDR-TB) are defined as bacteria that are resistant to at least INH and RIF, while extensively drug-resistant strains (XDR-TB) are bacteria that are resistant to INH, RIF, a fluoroquinolone and at least one second-line injectable drug [14, 122]. Treatment of MDR-TB

and XDR-TB patients requires alternative drugs, many of which have severe side effects and have limited efficacy, and the treatment course is long [13]. Development of new TB drugs is believed to be especially beneficial to these individuals, many of whom have few or no alternative options.

1.5.6 New drugs

The Global Alliance for TB Drug Development, in collaboration with pharmaceutical companies, has three drugs in clinical trials, discussed below. All three drugs have a novel mechanism of action, which makes them exciting prospects for treating MDR-TB and XDR-TB patients who are resistant to other available drugs.

PA-824 is in phase II clinical trials. The drug kills both replicating and non-replicating bacteria by inhibiting cell-wall synthesis and causing respiratory poisoning through nitric oxide release, respectively [133]. The drug does not appear to interact with antiretroviral drugs [123].

TMC207 is in phase II clinical trials. The drug kills both replicating and non-replicating bacteria by inhibiting the mycobacterial ATP synthase [134]. Interaction with antiretroviral drugs is currently being tested.

Moxifloxacin is in phase III clinical trials. The drug kills replicating bacteria by inhibiting DNA gyrase [135]. This drug may be useful to significantly shorten the duration of treatment [136]. It does not appear to interact with antiretroviral drugs [137], making it useful for treatment of patients infected with HIV.

1.6 ANIMAL MODELS OF TB

There is great heterogeneity within the human population in the response to *M. tuberculosis*, ranging from life-long control, to intermittent lapse in control or active disease [138, 139]. There is also heterogeneity within individuals, who may harbor different types of granulomas within the same lung lobe [140], indicating a spectrum of bacterial control.

Mycobacteria can infect and cause disease in a wide range of animals [141], yielding an extensive selection of animal models in which to study the bacteria and their interactions with the hosts. Each animal model, some of which are mentioned below, reflects a particular aspect of human TB, and the suitability of the model depends on the experimental questions being asked.

1.6.1 Mouse

The mouse model is the most widely used in TB research. Although all mice are susceptible to *M. tuberculosis*, there are differences in susceptibility between mouse strains. C57BL/6 is the most commonly used mouse strain and is the most resistant, while C3H and DBA/2 are the most susceptible [142].

Like most humans, C57BL/6 mice are able to control *M. tuberculosis* and do not show signs of disease for one to two years. However, while humans have a low bacterial load and develop latent infection, mice have a high bacterial burden that causes a chronic progressive disease. This characteristic makes the mouse model particularly useful to study the effects of chronic antigen exposure on T cell function [139, 143]. Mouse granulomas lack the structural organization and caseous necrosis that are the hallmarks of human granulomas. Although the granulomas still serve to contain the infection, differences in their composition are likely to

affect the environmental conditions in the granuloma, such as the availability of oxygen and nutrients [126, 144].

The Cornell mouse model was developed to replicate human latent infection in mice. In this model, mice are treated with antibiotics to artificially simulate the paucibacillary state of humans [145]. There have been various alterations of the model, ranging from the infection route and inoculum dose to the duration of drug treatment [139]. While other animal models, such as rabbits, reflect human granulomas and latent infection better than mice, the mouse model has the advantage of being inexpensive, easily housed and manipulated, with a plethora of immunological reagents and mouse strains available [138].

1.6.2 Rabbit

Rabbits are relatively resistant to *M. tuberculosis* infection and eventually heal their lesions [146], although more virulent strains of *M. tuberculosis* can cause progressive disease [147]. Due to their resistance to *M. tuberculosis,* rabbits may be a good model for studying latent TB [139].

In contrast to *M. tuberculosis,* rabbits are highly susceptible to *M. bovis,* which induces pulmonary pathology similar to that seen in humans [138]. The structure of rabbit granulomas is similar to that of humans, and has both caseous necrosis and cavity formation [139, 143]. Cavity formation allows the bacterium access to the external environment, which increases the risk of bacterial transmission [139].

Rabbits are fairly expensive to house, due to the risk of disease transmission and space requirements [139, 143]. Furthermore, there are relatively few immunological reagents available, which limits their use as an animal model for TB.

1.6.3 Guinea pig

Guinea pigs are highly susceptible to *M. tuberculosis*. This animal model has been used to study the efficacy of antimycobacterial drugs and vaccines [148, 149], as vaccines and drugs need to be protective in a very susceptible host.

Guinea pig granulomas have similar organization and caseous necrosis as human granulomas, but unlike human granulomas they lack cavitation and are unable to control bacterial expansion, making them a useful model for dissemination and secondary granuloma formation characteristic of an unsuccessful host response [139, 143, 150].

There is a certain lack of immunological reagents for guinea pigs, but the benefits of the model include guinea pigs being relatively inexpensive and easy to house in a biosafety level 3 facility (BSL3) [150].

1.6.4 Fish

Goldfish and zebrafish have been used to study TB pathogenesis using *M. marinum*, a close relative of *M. tuberculosis*. For the first three weeks, fish embryos are transparent and have not yet developed an adaptive immune response [151, 152], allowing for real-time study of interaction between the pathogen and the innate immune response. Fish granulomas have a caseous center, which is a hallmark feature of human granulomas [152]. While there are still few immunological reagents available for fish, genetic and imaging tools have established this as a superb model for studying early events that are inaccessible in other models [151].

1.6.5 Non-human primate

Of all available animal models, the non-human primate immune response is the closest to the human response. The animals reproduce the full scale of human disease from latent infection to active disease and can transmit the bacterium as a human would [139]. Furthermore, they develop a spectrum of granulomas similar to that seen in humans [141, 153, 154]. The non-human primate model is the only animal model that faithfully represents latent TB and can be used to study both natural and induced reactivation of latent TB. This model is also the only model available for studies of *M. tuberculosis* and HIV co-infection, and it has been used to study the efficacy of antimycobacterial drugs and vaccines [143].

There are many immunological reagents for non-human primates available, and owing to the close evolutionary relationship between humans and non-human primates, many human immunological reagents can be used as well. However, the animals are very expensive to purchase and house, and owing to the genetic variability of the non-human primates, the host response to the bacterium can vary greatly. While this makes it more difficult to identify trends in immune responses, it reflects the diversity of the human population and spectrum of likely immune responses.

1.7 VACCINES

As stated above, antibiotic treatment of *M. tuberculosis* is long and difficult, especially MDR-TB and XDR-TB strains where there are few treatment options. As infected people display a spectrum of symptoms or are asymptomatic, determining who should be treated and for how

long can also be difficult. The most practical and effective strategy to TB control would be to prevent establishment of infection through an efficient vaccine. However, immune responses induced by previous exposure to mycobacteria, whether they are virulent or not, are not sufficient to prevent re-infection. Thus, an effective vaccine would ideally elicit a better immune response than what is induced by natural mycobacterial infection [125].

For the last 88 years, BCG (discussed below) has been the gold-standard vaccine, with over 3 billion doses administered [155]. Better understanding of *M. tuberculosis* and its pathogenesis have led to the development of new vaccine candidates, some of which have reached clinical trials (discussed below).

Some of the vaccines being developed are aimed at replacing BCG (pre-exposure vaccines). However, BCG is an important factor in limiting severe disease in children and cannot ethically be withheld. Thus, a new vaccine will have to be tested in conjunction with BCG and confer significantly more protection in order to be considered as a replacement for BCG. Other vaccines are aimed at boosting immune responses (post-exposure vaccines).

The efficacy of TB vaccines is measured as absence of disease. However, only a small portion of people progress to overt disease, and this progression can occur decades after the initial infection. Thus, testing vaccine efficacy involves large cohorts and a time-span of decades.

1.7.1 BCG

BCG is an inexpensive, live vaccine that was developed by serial passing of *M. bovis* for 13 years [15]. During this time, the bacteria lost several genetic loci and became attenuated. The vaccine is given intradermally to infants in TB-endemic areas soon after birth and has been

shown to protect children by decreasing the risk of severe, often fatal, childhood forms of disease. However, the vaccine does not prevent infection with *M. tuberculosis*.

Efficacy in adults is variable, ranging from 0-80% protection against pulmonary TB [122, 125, 156, 157]. Exposure to environmental mycobacteria, which induces cross-reactive immune responses, has been hypothesized as a reason for the variable efficacy of BCG [157, 158]. BCG needs to replicate and disseminate in the host in order to confer protection, but pre-existing immune responses to mycobacteria can result in rapid clearing of the bacteria [15]. BCG vaccination of adults in order to boost the immune response to mycobacteria has proven unsuccessful, presumably because pre-existing memory responses to *M. tuberculosis*, BCG or environmental mycobacteria rapidly cleared the vaccine [159, 160]. However, if BCG-induced memory responses can clear the vaccine, but not prevent infection or disease, this indicates that the vaccine efficacy is limited to begin with.

1.7.2 Recombinant BCG

The virulence of the BCG::RD1 recombinant BCG strain was increased by adding back the Region of Difference 1 (RD1), a genetic region that was lost during attenuation of *M. bovis*. This strain is more protective than the parental BCG strain in mice and guinea pigs [161, 162]. However, increased virulence may make this vaccine unsafe, especially in areas where HIV-induced immunosuppression is common and the need for improved vaccines is the greatest. The same argument holds true for attenuated strains of *M. tuberculosis* that are being developed as vaccine candidates, where the risk of reversion to increased virulence is likely to prevent widespread use of the vaccine [163].

Another recombinant BCG vaccine strain being developed, rBCG::ureC-llo^{+}, has decreased virulence compared to the parental strain [164], which may improve safety of the vaccine in HIV-infected individuals. The bacteria lack urease, which prevents them from arresting maturation of the phagosome. The bacteria express listeriolysin O (LLO), which is activated by the low pH of the mature phagosome. LLO damages the phagosomal membrane, allowing increased escape of the bacteria and bacterial antigens into the cytosol, which may improve priming of *M. tuberculosis*-specific CD8 T cell responses [164, 165]. This vaccine strain is scheduled to enter Phase I clinical trials.

1.7.3 Viral vectors

Viral vectors can induce strong cell-mediated immune responses without adjuvants [156]. The Modified Vaccinia Ankara virus expresses antigen 85A (MVA85A), an immunodominant mycobacterial antigen that is present in BCG as well as *M. tuberculosis* [166]. The MVA85A vaccine induces strong immune responses in humans [85] and is more protective than BCG in mice [167]. However, one disadvantage of using this vector is that some people may have pre-existing antibodies against the viral vector due to its use in other vaccines [168, 169], which could affect vaccine efficacy [170]. The MVA85A vaccine has entered Phase IIb clinical trials, the furthest of all new TB vaccines, and is one of the most promising novel vaccine candidates.

The AERAS-402 vaccine is an adenovirus-based vector that expresses a fusion protein of *M. tuberculosis*-derived antigen 85A, 85B and TB10.4. The vaccine backbone is adenovirus type 35, as pre-existing immune responses to it are relatively low [171], but as with the vaccinia-based vector, this vector is also being developed for use against several other diseases [172, 173]. This vaccine is currently in Phase II clinical trials.

1.7.4 Recombinant protein vaccines

Recent advances in adjuvant development have been crucial to advancing development of recombinant protein vaccines [156]. One of the most important advantages of using recombinant protein vaccines is that they are not based on an infectious agent, and thus are likely to be safer for immunocompromised individuals. Immune responses are induced against specific highly immunogenic antigens; Mtb32 and Mtb39 in the GSK M72 vaccine [174], and ESAT-6 and antigen 85B in the SSI HyVac4 vaccine [175].

Protection against *M. tuberculosis* is likely to require concerted immune responses against a wide range of antigens [125]. Thus, the narrow range of immune responses induced by both recombinant protein vaccines and viral vectors is likely to limit these vaccine strategies to post-exposure vaccines. GSK M72 and HyVac4 are currently in Phase II and I clinical trials, respectively.

2.0 STATEMENT OF THE PROBLEM

The goal of this study was to investigate the function and regulation of CD8 T cells during *M. tuberculosis* infection in mice. This cell subset has received relatively little attention from TB researchers, as it has a smaller impact on the control of infection than CD4 T cells in mice. Recent work, however, has shown that CD8 T cells have an essential role in controlling the infection in non-human primates, a much superior model for human TB. Interest has been reawakened in CD8 T cells, and TB vaccine development is increasingly targeting this cell type. However, little is known about the function of the cells and what would constitute a protective CD8 T cell response.

Infection with *M. tuberculosis* or vaccination with BCG leaves a person with memory CD4 and CD8 T cells that appear to be unable to prevent further infection. This indicates that in order to achieve a sterilizing vaccine (i.e. a vaccine that clears the bacteria or prevents establishment of infection) a different vaccine vector or alteration of the bacterial vaccines may be needed. However, as we don't know which T cell functions are desirable, this approach is "hit-or-miss". A better understanding of what shapes the immune response and identification of a protective phenotype of T cells could provide "an end to a means", i.e. a specific phenotype for vaccine candidates to induce.

We hypothesized that the function of *M. tuberculosis*-specific CD8 T cells induced during natural infection was not optimal, and that the function of the cells could be altered by

changing the environment that the cells develop in. To accomplish the work presented in this thesis, we generated a recombinant *M. tuberculosis* strain that expresses ovalbumin epitopes (*M. tuberculosis*-ova). This allowed examination of ova-specific CD4 and CD8 T cell responses in mice, and enabled us to use the plethora of ova-specific reagents available. Using this bacterial strain, we first studied CD8 T cell responses induced during primary and secondary infections (chapter 3). Next, using a strategy of WT *M. tuberculosis* infection followed by *M. tuberculosis*-ova infection, we examined the function of primary CD8 T cells induced in the presence of memory cells (chapter 4). To examine the effect that the environment has on the function of T cells, we adoptively transferred ova-specific CD4 and CD8 T cells into mice at different times post-infection (chapter 5). Finally, we examined the effect of an inhibitory molecule, Qa-1, on CD8 T cell responses (chapter 6).

3.0 CYTOTOXICITY AND SECRETION OF IFN-γ ARE CARRIED OUT BY DISTINCT CD8 T CELLS DURING *M. TUBERCULOSIS* INFECTION

This chapter has been adapted from a published study (Einarsdottir, T., et al., *Cytotoxicity and Secretion of Gamma Interferon are Carried Out by Distinct CD8 T Cells during Mycobacterium tuberculosis Infection*. Infect. Immun., 2009. **77**(10): p. 4621-4630) with permission from the editor.

3.1 INTRODUCTION

M. tuberculosis, the pathogen causing TB, is an escalating global health threat that is spread between people by aerosolized droplets. While a subset of people develop primary disease and are infectious, most people contain the infection through a successful immune response (Figure 1). Typing of *M. tuberculosis* strains has shown that people can be infected simultaneously or sequentially with different strains of *M. tuberculosis* [176, 177]. Thus, immune responses that are sufficient to contain an initial infection may be unable to prevent establishment of subsequent *M. tuberculosis* infections. In addition, persons treated for TB with antimycobacterial drugs can be re-infected and develop disease [178]. This is true in animal models as well [179-181]. This suggests that memory responses generated during previous

mycobacterial infections are not generally capable of protecting against new infections or disease.

It is currently unknown which, if any, immune functions can protect against establishment of infection. In terms of T cell responses, IFN-γ and TNF can activate infected Mϕ to induce antimicrobial activity, while cytolysis of infected cells can kill the bacterium or release it to be taken up by healthy cells that are better able to contain it [182, 183]. Most studies on the role of CD8 T cells during *M. tuberculosis* infection have focused on either IFN-γ secretion or cytotoxicity. A few studies have examined both functions, but not on a single cell basis [184-186]. These studies indicated that CD8 T cell mediated IFN-γ secretion and cytotoxicity peak in lungs at four weeks post-infection. IFN-γ secretion subsequently decreases, while the results differ as to whether cytotoxicity decreases. This difference may be due to variation between mouse strains and epitopes, or the techniques used to assess cytotoxicity.

In the current study, we demonstrate that most *M. tuberculosis*-specific CD8 T cells generated during primary infection were limited to either secretion of IFN-γ or cytotoxic function. There was a higher fraction of CD8 T cells with cytotoxic potential compared to those that produced IFN-γ. The CD8 T cell memory response was similarly exclusive in function. Despite increased degranulation, cells responding during secondary infection were less cytotoxic than cells responding during primary infection. Induction of superior quality CD8 T cells may provide benefit during *M. tuberculosis* infection, but a more detailed understanding of the complex T cell response to *M. tuberculosis* is necessary for the development of future preventive and therapeutic strategies.

3.2 MATERIALS AND METHODS

3.2.1 *M. tuberculosis-ova* cloning

The culture filtrate protein 10 (CFP10) gene (Rv3874, 225 bp) and 228 bp upstream sequence containing the CFP10 promoter were PCR amplified from *M. tuberculosis* genomic DNA and cloned into the pJL37 cloning vector [187]. An 87 bp ovalbumin (ova) gene fragment containing OTI- and OTII-recognized epitopes was PCR amplified from a larger fragment and cloned into pJL37-CFP10. Restriction enzymes (Roche, Indianapolis, IN) and T4 DNA ligase (Invitrogen, Carlsbad, CA) were used, according to manufacturers' protocols. Plasmids were transformed into DH5α cells (Invitrogen) by 42˚C heat shock for 30 seconds and purified with High Pure Plasmid Isolation Kit (Roche), according to manufacturer's protocols. The CFP10-ova insert was cloned into pMH94 integration vector [188] and electroporated [189] into *M. tuberculosis* Erdman strain (originally obtained from the Trudeau Institute, Saranac Lake, NY), using a Gene Pulser II (Bio-Rad, Hercules, CA). pJL37 and pMH94 plasmids were kindly provided by Graham Hatfull.

3.2.2 PCR

AccuPrime (Invitrogen), with primers from Integrated DNA Technologies (Coralville, IA) was used for PCR (95ºC [20sec], 45ºC [30sec], 68ºC [1min], 32 cycles, 68ºC [7min]), unless otherwise stated.

3.2.2.1 Primers for PCR amplification of CFP10

CACCTCTAGAGCTCGCGCAGGAGCGTGAAGAAG (sense, CFP10-XbaI 5'), TATACATATGGAAGCCCATTTGCGAGGACAGCG (antisense, CFP10-NdeI 3').

3.2.2.2 Primers for PCR amplification of large ova fragment

Overlapping primers were designed with UpGene DNA Codon Optimization Algorithm [190]. The primers were annealed and amplified at 94ºC (30 sec), 52ºC (30 sec), 72ºC (30 sec), 55 cycles.

AACCGGGATCCGGCTCGGAGC (s1), TAATGAATTCTCAGTGATGGT (as1), AGCTGGAGAGCATCATCAACTTCGAGAAGCTGGGCTCGGAGT (s2), GGTGGTGGTGGCCGCAGGTGGCGAAGTTGTACACGGCCTCG (as2), CGCTGAAGATCTCGCAGGCCGTGCACGCCGCGCACGCCGAGA (s3), AGCCGCGGCCGGCCTCGTTGATCTCGGCGTGCGCGGCGTGCA (as3), TCAACGAGGCCGGCCGCGGCTCGAAGGCCGTGTACAACTTCG (s4), CGGCCTGCGAGATCTTCAGCGACTCCGAGCCCAGCTTCTCGA (as4).

3.2.2.3 Primers for PCR amplification of 87 bp ova fragment

CACCCATATGATCTCGCAGGCCGTG (OTI-NdeI 5', sense), GGTGGAATTCGCGGCCGGCCTCGTT (OTII-EcoRI 3', antisense)

3.2.2.4 PCR amplification for screening and sequencing

Sense: CFP10-XbaI 5' and Antisense: OTII-EcoRI 3'. High Pure PCR Product Purification Kit (Roche) was used, and the product sequenced by the University of Pittsburgh Sequencing Core facility (Pittsburgh, PA).

3.2.3 Mice and bacterial infections

Six- to eight-week old C57BL/6 and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1) mice were purchased from Jackson Laboratories (Bar Harbor, Maine), while OTI and OTII T cell receptor transgenic mice on a C57BL/6 RAG1-/- background (kindly provided by Louis D. Falo) were bred at the University of Pittsburgh Biotechnology Center (Pittsburgh, PA). Mice were kept under specific pathogen-free conditions in a BSL3 facility. OTI and OTII cells were purified from naïve spleens using MACS cell separation technology (Miltenyi, Auburn, CA), according to manufacturer's protocol. $5*10^3$ OTI and OTII cells in PBS (Sigma, St. Louis, MO) were adoptively transferred to WT C57BL/6 or CD45.1 mice. 24 hours later, the mice were infected with either WT *M. tuberculosis* or *M. tuberculosis-ova*, using a nose-only exposure aerosolizer (Intox, Moriarty, NM) [191]. This delivered approximately 10-20 colony forming units (CFU) per lung, estimated by plating of whole lung homogenate 24 hours post-infection on 7H10 media agar (Difco, Sparks, MD) and incubating at 37ºC, 5% CO_2 for 3 weeks. Four to twelve mice were used per time-point per experimental procedure. Experiments were performed at least twice. Animal protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.4 Antibiotic treatment

For secondary challenge experiments, infected mice were treated for three months with 0.1g/L isoniazid (Sigma), 0.1g/L rifampin (rifampicin, Sigma) and 8g/L pyrazinamide (Acros, Geel, Belgium) in drinking water. Plating of whole lung homogenates confirmed that no bacteria could be cultured from the lungs after antibiotic treatment.

3.2.5 PD-1 signal blocking treatment

Treatment was initiated during the chronic phase of infection (eight weeks post-infection) and was continued for four weeks. Mice were injected intraperitoneally twice a week with anti-PD-1 (200µg, clone 29F.1A12) and anti-PD-L1 (200µg, clone 10F.9G2X) antibodies, or with PBS, as in [192]. The antibodies were kindly provided by Gordon J. Freeman.

3.2.6 Harvesting of lungs and lymph nodes

Organs were crushed through a 40µm cell strainer with a 5ml syringe plunger to obtain single-cell suspensions. An aliquot was used to determine bacterial load by plating serial dilutions on 7H10 media agar. For the remainder of the samples, red blood cells were lysed with an NH_4Cl/Tris solution and washed in DMEM (Sigma). Cells were resuspended in T cell medium (DMEM supplemented with 10% FBS [Sigma], 1mM Na-pyruvate [Sigma], 2mM L-glutamine [Invitrogen], 25mM HEPES [BioWhittaker, Walkersville, MD] and 50µM 2-ME [Sigma]) and counted based on trypan blue exclusion (Sigma).

3.2.7 ELISpot assay

Bone-marrow derived dendritic cells (DC) were used as antigen-presenting cells in ELISpot assays. Mouse bone-marrow was cultured for six days in DMEM media supplemented with 10% FBS, 2mM L-glutamine, 1000U/ml GM-CSF (kindly provided by Binfeng Lu) and 20ng/ml IL-4 (PeproTech, Rocky Hill, NJ). Cells were split and media added on day three. DC

were either left uninfected or infected with *M. tuberculosis* at a multiplicity of infection of 3 for 24 hours in media lacking IL-4.

ELISpot plates (MultiScreen HTS 96-well, Millipore, Billerica, MA) were coated with anti-mouse IFN-γ antibody (10μg/ml, clone R4-6A2) in PBS. $5*10^4$ cells were co-incubated for 48 hours with $4*10^4$ DC. 4μg/ml peptide [GAP (GAPINSATAM) or ova (SIINFEKL)] (GenScript, Piscataway, NJ) was added to DC to stimulate antigen-specific responses, while *M. tuberculosis*-infected DC were used to stimulate total *M. tuberculosis*-specific responses. Uninfected DC and media were used to determine background levels of IFN-γ secretion. Biotinylated anti-mouse IFN-γ antibody (5μg/ml, clone XMG1.2), streptavidin-conjugated peroxidase (PK6100, Vector Laboratories, Burlingame, CA) and AEC substrate (PK4200, Vector Laboratories) were used to detect IFN-γ. Spots were counted on an ImmunoSpot automatic ELISpot reader (Cellular Technology, Shaker Heights, OH).

3.2.8 Flow cytometry

Cells were incubated with GAP (GAPINSATAM) or ova (SIINFEKL) tetramer-PE (Beckman Coulter, Fullerton, CA) for 30 min at 4ºC in T cell medium. The cells were then washed and incubated at 37ºC for 5 hours in T cell medium containing 3μM monensin (Sigma), anti-CD107a and CD107b FITC antibody, anti-CD28 antibody (clone 37.51, 1μg/ml) and anti-CD3 antibody (clone 145-2C11, 0.1μg/ml). Cells were then washed and stained with anti-CD8 PerCP antibody in 20% mouse serum (Gemini, West Sacramento, CA) in FACS buffer (PBS with 0.1% BSA [Sigma] and 0.1% NaN_3 [Sigma]) for 15 minutes, washed and fixed in 2% paraformaldehyde (Sigma). Cells were permeabilized in permeabilization buffer (FACS buffer

containing 0.2% saponin [Calbiochem, Gibbstown, NJ]) and stained with anti-IFN-γ-PE-Cy7, anti-granzyme B-APC (Invitrogen). Samples were read on a FACS Aria (BD Biosciences, San Jose, CA) and analyzed with FlowJo (TreeStar, Ashland, OR) software. Antibodies were obtained from BD Biosciences, unless otherwise stated.

3.2.9 *In vivo* cytotoxicity assay

Uninfected splenocytes were labeled with 0.4mM or 5mM CFSE (Molecular Probes, Eugene, OR) for 15 minutes at 37ºC. The reaction was quenched with T cell medium, and cells washed in 20% FBS in PBS. $CFSE^{low}$ cells were pulsed for 1 hour with 20μg/ml peptide in PBS (GAP or ova for antigen-specific killing, LCMV gp33 to 41 for controls).

$CFSE^{high}$ and $CFSE^{low}$ cells were mixed 1:1 and approximately $5*10^7$ cells injected into the tail veins (four mice/group/time-point). Mice were sacrificed 18 hours later and cell homogenates harvested from lungs and lymph nodes. Cells were washed in FACS buffer and fixed in 4% paraformaldehyde. Samples were read on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software. Peptide-specific lysis was measured by loss of $CFSE^{low}$ cells compared to $CFSE^{high}$ cells (ratio averaged per group).

%peptide-specific lysis= 100 – 100*[(%$CFSE^{low}$/%$CFSE^{high}$) from GAP or ova group / (%$CFSE^{low}$/%$CFSE^{high}$) from LCMV group].

3.2.10 Statistical analysis

GraphPad Prism 4.01 (GraphPad Software, La Jolla, CA) was used for graphing and statistical analysis. Unpaired two-sided t tests were used for two-group analysis, while two-sided,

nonparametric Spearman rank correlation coefficient tests were used to determine correlation. P values of <0.05 (*) and 0.01 (**) were considered significant and highly significant, respectively.

3.3 RESULTS

3.3.1 Generation of *M. tuberculosis*-ova strain for analysis of the CD8 T cell response

A scarcity of known *M. tuberculosis*-specific CD8 T cell immunodominant epitopes in C57BL/6 mice [193] led us to generate a recombinant *M. tuberculosis* (*M. tuberculosis-ova*) expressing ova epitopes fused to culture filtrate protein 10 (CFP10). CFP10 is a small protein that forms a heterodimer with early secreted antigenic target 6-kDa (ESAT-6), and together they are secreted by *M. tuberculosis* [194]. CFP10 epitopes are not efficiently presented by MHC class I molecules in C57BL/6 mice and thus do not induce CD8 T cell responses [186]. However, CFP10 elicits robust CD8 T cell responses in humans [195], non-human primate [196, 197] and the C3H mouse strain [186], indicating that CFP10 has access to the antigen presentation pathway. For this reason, CFP10 was chosen as a carrier protein for the ova epitopes. This construct enables us to take advantage of the numerous ova-specific immunologic reagents available, as well as to compare ova-specific responses in the context of *M. tuberculosis* infection to those in other infections where recombinant ova-expressing strains have been used. Here we used this construct to compare responses to two different CD8 T cell epitopes: ova and

an endogenous *M. tuberculosis* CD8 T cell epitope (GAPINSATAM, designated GAP, from Rv0125 [198]).

Ova epitopes 257 to 264 (SIINFEKL) and 323 to 339 (ISQAVHAAHAEINEAGR) are recognized by H-2b restricted OTI transgenic CD8 T cells and I-Ab restricted OTII transgenic CD4 T cells, respectively [199], as well as non-transgenic T cells in C57BL/6 mice. *M. tuberculosis-ova* was generated by fusing OTI and OTII recognized sequences downstream of the intact CFP10 gene and upstream regulatory sequences. The CFP10-ova fusion gene was inserted into pMH94, a vector that integrates into the bacterial genome [188] (Figure 2A and B). Integration of the pMH94CFP10ova plasmid did not alter the growth rate or survival of the bacterium *in vitro* or *in vivo*, compared to the parent *M. tuberculosis* Erdman strain (data not shown). *M. tuberculosis-ova*, but not WT *M. tuberculosis*, induced ova-specific responses, as demonstrated by IFN-γ ELISpot assay (Figure 2C, $P<0.01$). Ova-specific CD4 T cell responses were always weaker than ova-specific CD8 T cell responses. Endogenous T cell responses to GAP (CD8) and ESAT-6 peptide amino acids 1 to 20 (CD4) were not altered by the plasmid integration, nor were any differences in survival of mice inoculated with *M. tuberculosis-ova* and WT *M. tuberculosis* observed.

In summary, we generated a recombinant *M. tuberculosis* strain expressing ova epitopes, expanding the repertoire of peptides for detection of CD8 T cells during *M. tuberculosis* infection.

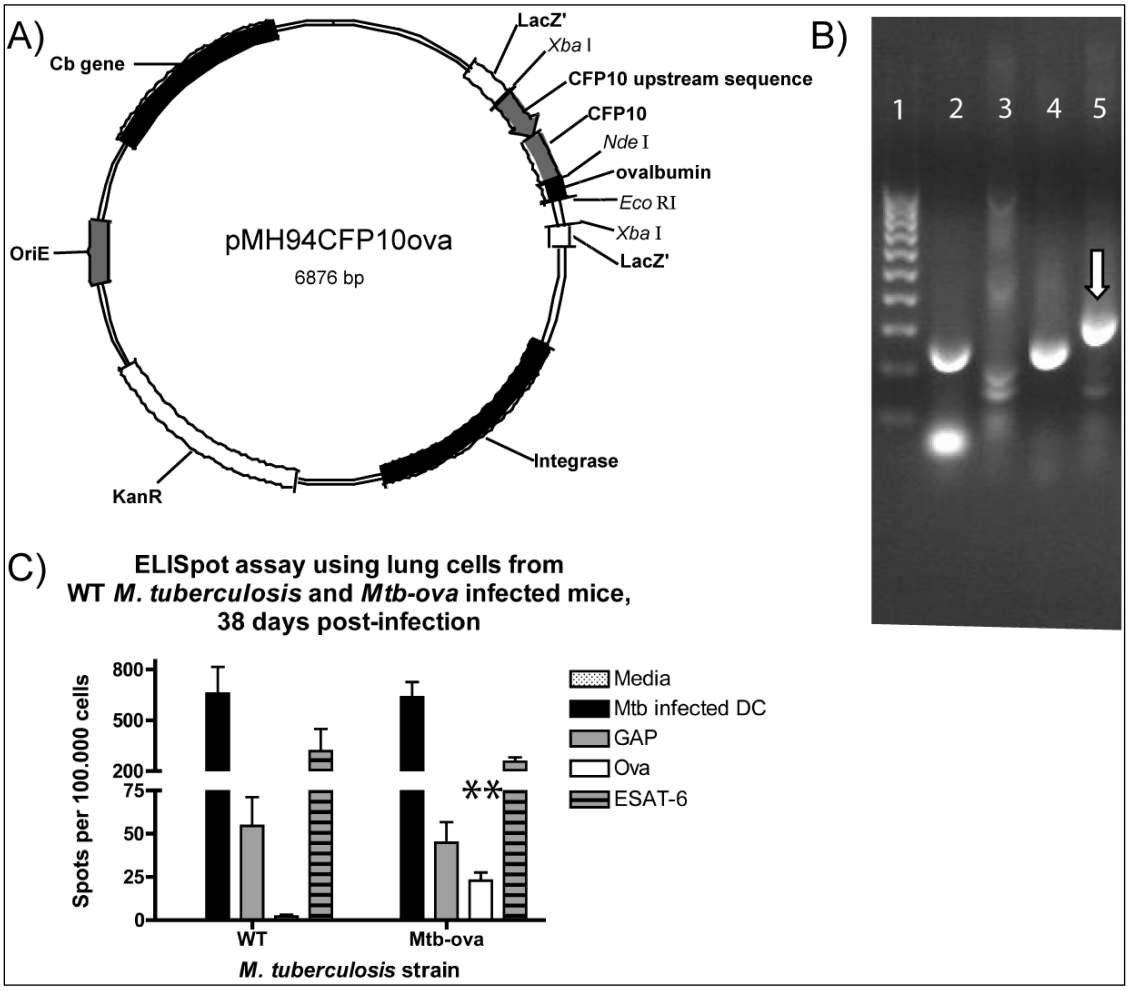


Figure 2 Generation and screening of *M. tuberculosis-ova*.
A) Map of pMH94CFP10ova integration vector, drawn in Vector NTI suite 8 (InforMax Inc). CFP10 and the upstream sequence were inserted into pMH94, followed by the ova sequence. The plasmid lacks an origin of replication in *M. tuberculosis*, and is lost unless it is integrated in a site-specific manner into the genome by the integrase via the attP site. Recombinant colonies were selected based on kanamycin resistance (KanR). B) PCR screening for integration of pMH94CFP10ova into *M. tuberculosis*. Lane 1 shows a 100 bp DNA ladder (Invitrogen). Lanes 2 (WT *M. tuberculosis*) and 4 (*M. tuberculosis-ova*) show positive controls, screening for endogenous copies of CFP10 (225 bp). Lanes 3 (WT *M. tuberculosis)* and 5 (*M. tuberculosis-ova*) screen for pMH94CFP10ova with primers specific for the ends of the CFP10-ova fusion gene (312 bp, indicated by the arrow). The figure has been edited to remove irrelevant lanes. C) ELISpot assay measuring IFN-γ secretion in response to *M. tuberculosis*, endogenous T cell epitopes (GAP and ESAT-6), ova CD8 T cell epitope and background levels (media). The data are the means ± standard errors of mean (SEM) of four mice. Ova-specific CD8 T cell responses induced by *M. tuberculosis-ova* were statistically different (**, $P<0.01$) from responses induced by WT *M. tuberculosis*, while other responses were not statistically different.

3.3.2 Expansion of CD8 T cells specific for GAP and ova during primary infection with *M. tuberculosis-ova*

In C57BL/6 mice infected by aerosol with a low dose of *M. tuberculosis*, primary T cell responses can be detected two to three weeks post-infection. We used MHC class I tetramers to quantify GAP- and ova-specific populations of CD8 T cells. In infected mice, GAP- and ova-specific CD8 T cells comprised about 2 to 10% of the primary CD8 T cell response in the lungs and 1 to 13% in the lymph nodes (Supplementary Figure 1A and B). This response was sustained for at least seven months post-infection. GAP- and ova-specific CD8 T cell responses were similar, peaking first at three weeks post-infection and then contracting as bacterial expansion was controlled (Figure 3A to C, [200]). GAP- and ova-specific CD8 T cells expanded again late in infection in the lung, which was associated with an increase in bacterial load (Figure 3C, $P<0.05$, week sixteen versus twenty-eight post-infection). CD8 T cell expansion late in infection was observed previously [200].

In summary, GAP and ova induced similar primary CD8 T cell expansion, which were detected in both acute and chronic phases of infection in the lung and, to a lesser extent, in the mediastinal lymph node.

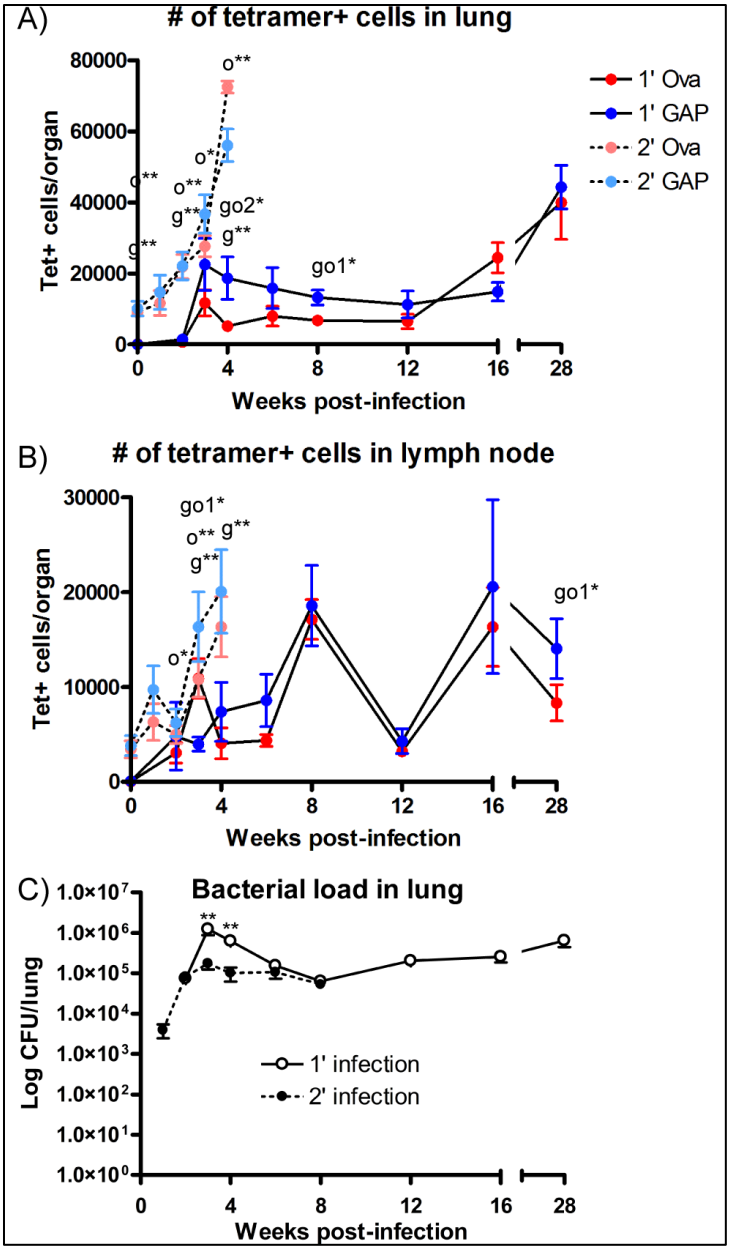


Figure 3 *M. tuberculosis-ova* induces similar GAP- and ova-specific responses.
Based on percent tetramer positive CD8 T cells (Supplemental Figure 1A and B) and live cell numbers (data not shown), the number of tetramer positive cells in A) lungs and B) lymph nodes were calculated. The frequency of tetramer-specific cells during primary (1') and secondary (2') infections was significantly different 0, 2, 3 and 4 weeks post-infection for ova-specific cells (o*) and 0, 2 and 4 weeks post-infection for GAP-specific cells (g*). The frequency of ova and GAP-specific cells was significantly different at eight weeks (lung) and twenty-eight weeks (lymph node) post-primary infection (go1*) and four weeks (lung) post-secondary infection (go2*). C) Bacterial load in lungs. Primary and secondary infections were statistically different at three and four weeks post-infection ($P<0.01$). Data are mean ± SEM of four (A and B) to twelve (C) mice per time-point.

3.3.3 GAP- and ova-specific CD8 T cell function during primary infection

To characterize the effector functions of individual *M. tuberculosis*-specific CD8 T cells over the course of infection, we examined CD8 T cell mediated cytotoxicity and cytokine production on a single cell basis using flow cytometry. For this, we used intracellular cytokine staining for IFN-γ and surface staining for CD107 (Supplemental Figure 2A). CD107a and CD107b are lysosomal proteins that are transiently exposed to the surface upon degranulation, which is indicative of cytotoxicity [201]. Incubation of the cells with anti-CD107 antibody during stimulation provides an assay to assess which cells degranulated during the incubation period.

Two weeks post-infection GAP- and ova-specific CD8 T cells started expanding in the lymph nodes (Figure 3B); by three weeks post-infection primed GAP- and ova-specific CD8 T cells had expanded and migrated to the lungs (Figure 3A). By three weeks post-infection 25% of GAP- and ova-specific CD8 T cells in lungs had acquired the ability to be cytotoxic (i.e. CD107+), while little degranulation was detected in the lymph nodes (Supplemental Figures 3A to B and 4A and B). Approximately 15% of GAP- and ova-specific CD8 T cells in both lungs and lymph nodes acquired the ability to secrete IFN-γ. Less than 5% of the cells were able to both degranulate and secrete IFN-γ, indicating that distinct populations of cells carry out these functions.

By four weeks post-infection there was an increase in the frequency and number of cytotoxic cells in the lungs, while there were fewer IFN-γ secreting cells (Figure 4A and B). However, IFN-γ production peaked at four weeks post-infection as measured by ELISpot assay using *M. tuberculosis*-infected DC (Supplemental Figure 5) or peptide pulsed DC as stimulators

(Figure 5A). The difference in results is likely due to the differences in the methods: ELISpot assay includes stimulation by antigen-pulsed or infected DC for two days, while intracellular cytokine staining occurs during a short (5 hour) stimulation with tetramer and anti-CD3/CD28 antibody. The longer stimulation may induce production of IFN-γ from cells that are not immediately ready to produce it. There may also be differences in the sensitivity of the assays [202].

In vivo cytotoxicity assays (Figure 5B) showed a peak in cytotoxic activity against GAP- and ova peptide-loaded cells in the lungs at four weeks post-infection. Cytotoxicity decreased after the four-week peak, but then slowly increased during the chronic phase of infection. Comparison of the pattern of GAP-specific cytotoxicity and the frequency of CD107+ GAP-specific cells in lungs over the course of infection (Figure 5C) indicates that CD107 surface expression is a good indicator of cytotoxicity during primary *M. tuberculosis* infection.

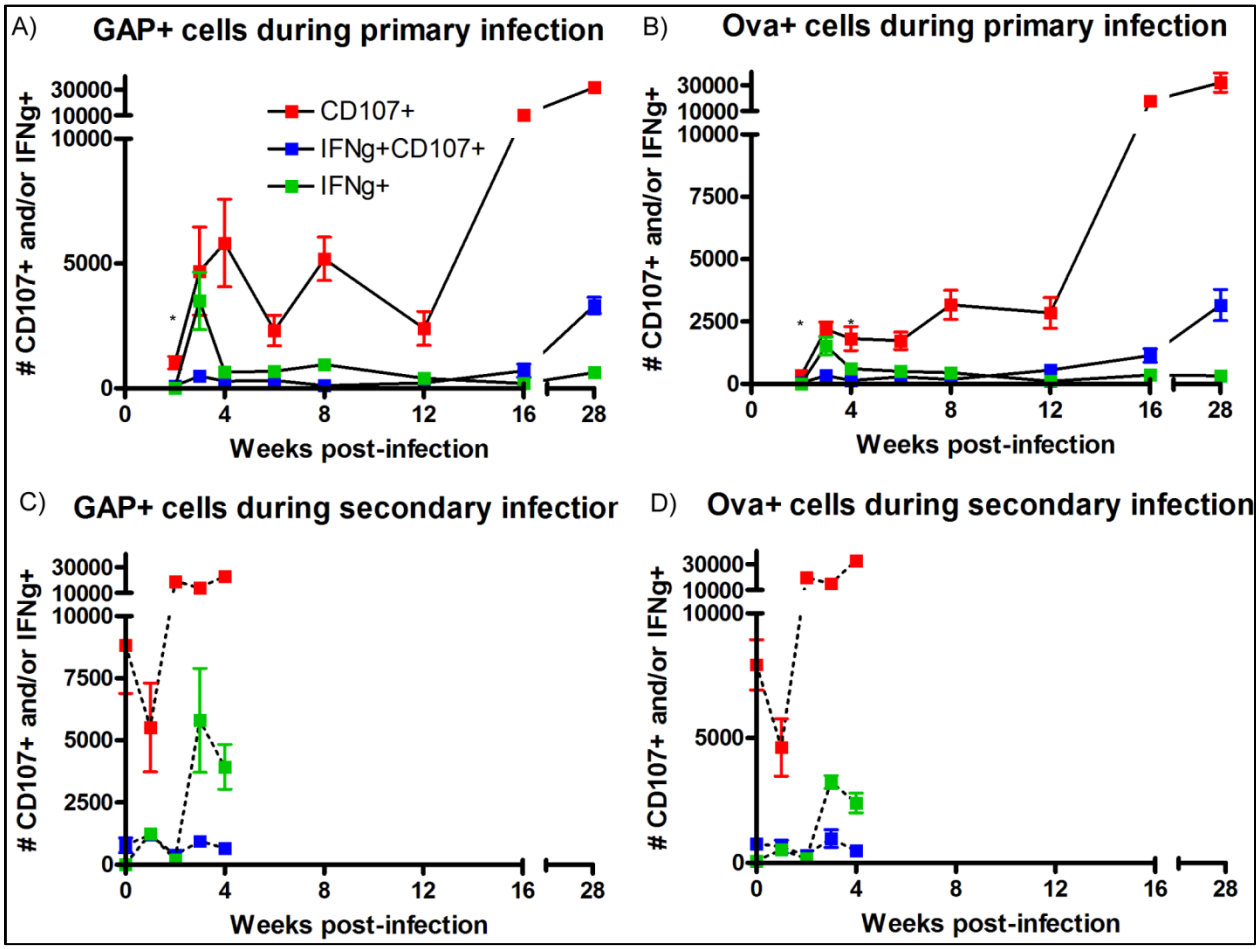


Figure 4 IFN-γ secretion and degranulation are carried out by distinct populations of cells in lungs.

Based on GAP and ova-specific CD8 T cell numbers from Figure 3A and percent IFN-γ+ and CD107+ cells (Supplemental Figure 3A and B), numbers of cells per organ were calculated. A) and B) are primary infection, C) and D) are secondary infection. A) and C) are GAP, B) and D) are ova. The data are mean ± SEM of four mice per time-point. There was a significant difference in the frequency ($P<0.05$) and number ($P<0.05$) of IFN-γ+CD107+ cells between primary and secondary infections for GAP-specific cells at two weeks post-infection and ova-specific CD8 T cells at two and four weeks post-infection.

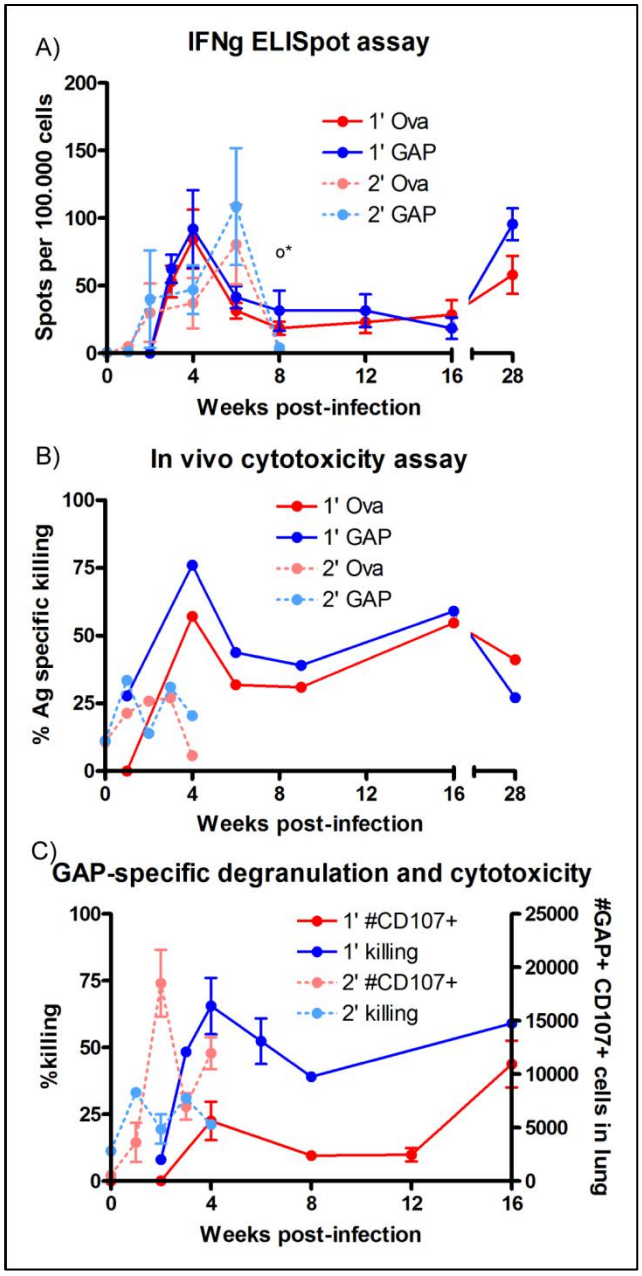


Figure 5 Functional assays for GAP- and ova-specific cytotoxicity and IFN-γ secretion.
A) ELISpot assay, where lung cells were incubated with DC pulsed with GAP or ova peptide (SIINFEKL) for 48 hours. There was no background level of IFN-γ secretion, measured by incubating cells with either T cell media or unpulsed, uninfected DC (data not shown). Data are mean ± SEM of four mice per time-point. There was no statistical significance between primary and secondary infections, except for ova-specific cells at eight weeks post-infection ($P<0.05$). B) *In vivo* cytotoxicity assay in lung. Data are the average of four mice per group. C) GAP-specific cytotoxicity versus the frequency of CD107+ GAP-specific CD8 T cells in lung. There was a significant correlation ($P<0.05$) between cytotoxicity and CD107 surface expression during primary infection, as measured by the Spearman non-parametric test, while there was no significant correlation during secondary infection.

By seven months post-infection, expansion of CD107+ and IFN-γ+CD107+ T cells was observed (Figure 4A and B, Supplemental Figure 3A and B, Supplemental Figure 5A and B). These data indicate a dynamic response to *M. tuberculosis* over the acute and chronic phases of infection, with changes in the effector functions of CD8 T cells. However, the assays are not able to discriminate whether cellular function changes over time or whether new cells appear with different functions.

In summary, cells primed in the lymph nodes primarily secreted IFN-γ, but lost the ability to secrete IFN-γ after the first month of infection and later acquired cytotoxic function. Effector cells migrating to the lungs retained the phenotype expressed in lymph node, or acquired cytotoxic function. Again, IFN-γ was mostly lost after the first month of infection. Finally, cytotoxicity and secretion of IFN-γ were carried out by distinct cell populations.

3.3.4 Blocking PD-1 signaling during primary infection

PD-1 is a negative costimulatory molecule, whose expression is upregulated following T cell activation [192]. High expression level of PD-1 has been implicated in CD8 T cell exhaustion in several chronic infections in humans, e.g. HIV [203, 204] and HCV [205], as well as in animal models, e.g. LCMV [192]. Using congenic strain markers, we examined temporal expression of PD-1 by CD45.2+ OTI T cells adoptively transferred into CD45.1+ mice. The frequency of *M. tuberculosis*-specific cells expressing PD-1 increased in both lungs and lymph nodes following infection, and continued to increase as infection progressed (Figure 6A). Geometric mean fluorescence intensity (MFI), however, was highest immediately upon priming, after which PD-1 levels remained low (Figure 6B, Supplemental Figure 2G and H).

Administration of PD-1 blocking antibody during chronic infections [192, 203, 206, 207] has been shown to improve CD8 T cell function and lower viral loads. Thus, we examined whether intraperitoneal administration of blocking antibodies against PD-1 and PD-L1 would significantly alter CD8 T cell function, specifically the ability of CD8 T cells to make IFN-γ and be cytotoxic. Treatment was initiated eight weeks post-infection, which we define as the beginning of chronic phase, and was continued over a four week period. This treatment did not produce cells capable of being both cytotoxic and secreting IFN-γ (Figure 6C, Supplemental Figure 2D), indicating that PD-1 is not involved in maintaining the functional distinction of cytotoxic cells and cells that secrete IFN-γ. Treatment did not significantly alter the frequency of CD107+ and IFN-γ+ nor bacterial load (Figure 6D).

In summary, while the frequency of PD-1 expressing cells increased with time, expression levels on a per-cell basis decreased, indicating that newly primed cells expressed PD-1 and that its expression then decreased. Blocking PD-1 signaling had no appreciable effect on CD8 T cell function as measured here. It is possible that blocking PD1 signaling at a different time point, either earlier or later in infection, may affect CD8 T cell function.

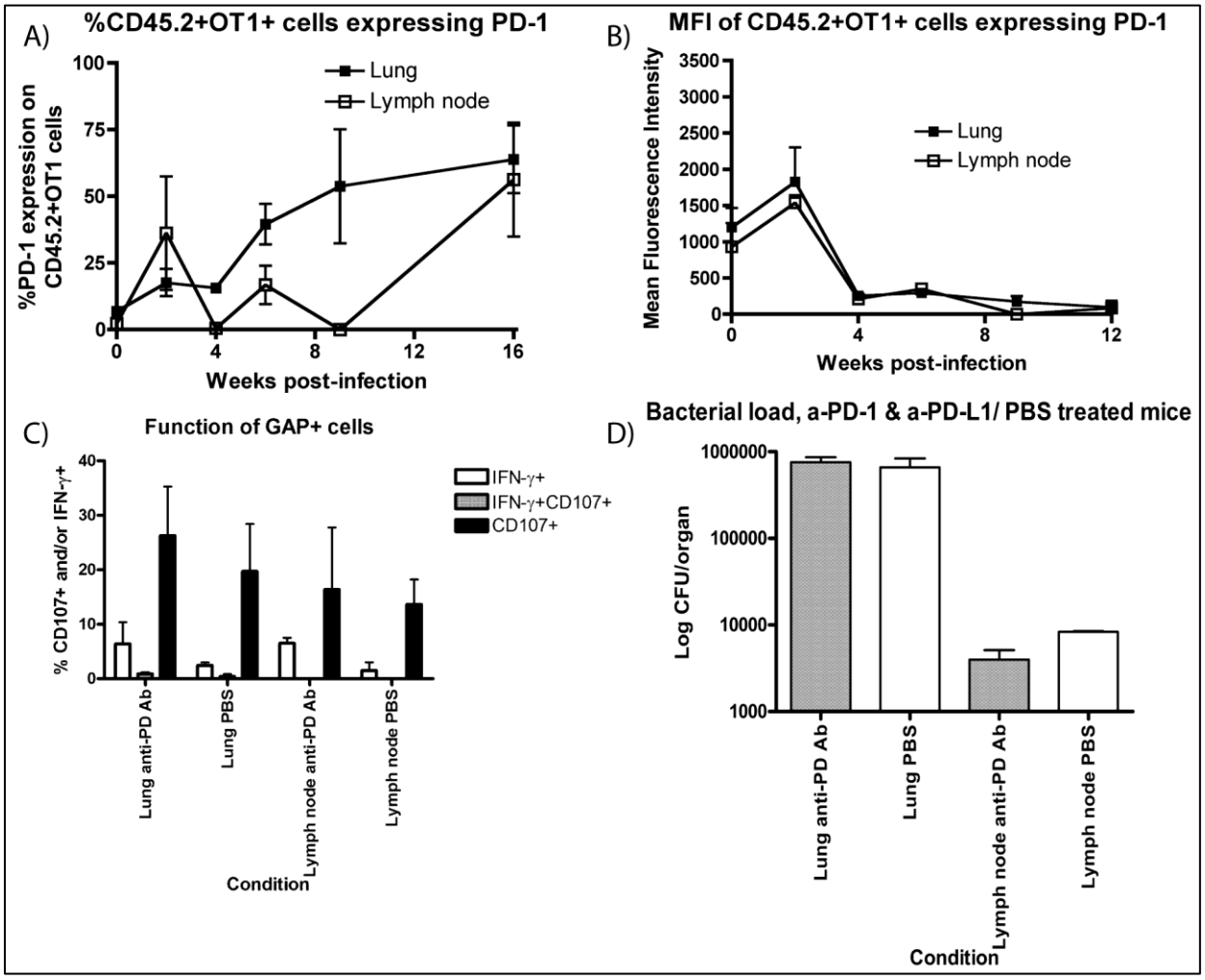


Figure 6 Blocking PD-1 signal *in vivo* does not generate IFN-γ+CD107+ cells.
$5*10^3$ OTI cells (CD45.2) were adoptively transferred to CD45.1 mice, which were subsequently infected with *M. tuberculosis-ova*. Using anti-CD45.2 antibodies, responses of these cells were examined over the course of infection. A) Percent CD45.2+ OTI+ cells expressing PD-1, B) geometric mean fluorescence intensity (MFI) of PD-1 staining in A). (C and D) Mice treated with anti-PD-1 and anti-PD-L1 antibodies, or with PBS, for four weeks. Ab, antibody; a-, anti-. C) GAP+ CD8 T cells expressing IFN-γ and surface CD107. D) Bacterial load in lungs and lymph nodes. Data are mean ± SEM for three or four mice per experimental group.

3.3.5 Expansion of CD8 T cells specific for GAP and ova epitopes during rechallenge with *M. tuberculosis-ova*

As a model for studying memory T cell responses, mice were infected with *M. tuberculosis* for two months and then treated with antibiotics in drinking water for three months, after which no bacteria could be cultured from whole lung homogenates. Mice were then re-infected and sacrificed at weekly time-points. Recalled memory T cells can be examined for the first two weeks post-infection. After that time the response is likely to consist of both memory and primary T cells, since bacteria are not cleared, allowing for priming of new T cells.

GAP- and ova-specific memory CD8 T cell expansion was significantly larger in terms of number of cells than primary CD8 T cell expansion to these epitopes (Figure 3A and B, $P<0.01$), and they represented a greater proportion of the total CD8 T cell response (Supplemental Figure 1, $P<0.01$ for ova, $P<0.05$ for GAP). This indicates that GAP and ova CD8 T cell epitopes are presented during both primary and secondary infections. Memory T cell expansion against *M. tuberculosis* was associated with an earlier control of bacterial numbers in the lung (Figure 3C, $P<0.01$). This protection was transient, as previously published [208], indicating that memory CD8 T cells induced by infection in this model do not confer significant long-term protection against subsequent *M. tuberculosis* infection. *M. tuberculosis*-specific CD8 T cells did not contract despite control of bacterial infection, reaching higher numbers in the lungs than during primary infection (Figure 3A). Masopust et al. showed, using heterologous infections, that cells contracted more slowly with each subsequent infection [209]. Based on this, chronic antigen exposure during *M. tuberculosis* infection may cause the slow contraction of T cells.

In summary, both GAP and ova memory CD8 T cells are present in the lungs at higher levels during *M. tuberculosis* re-infection, indicating a more rapid and robust response to secondary challenge.

3.3.6 Function of CD8 T cells specific for GAP and ova during rechallenge with *M. tuberculosis-ova*

Before re-infection of mice, residual *M. tuberculosis*-specific CD8 T cells in the lungs had a cytotoxic phenotype (CD107+) (Figure 4C and D, Supplemental Figure 3C and D, Supplemental Figure 5C and D). There was a continued expansion of CD107+ cells in the lungs following re-infection. IFN-γ secreting cells appeared by two weeks post-infection, measured by ELISpot (Figure 5A). As in primary infection, almost no cells were observed that both secreted IFN-γ and degranulated (Figure 4C and D, Supplemental Figure 3C and D, Supplemental Figure 5C and D). By three weeks post-infection, CD8 T cells expressing IFN-γ were observed by flow cytometry. These cells are presumably newly primed, since they correspond to the peak of these cells in primary infection. After three weeks post-infection cytotoxicity, as measured by the *in vivo* cytotoxicity assay, decreased (Figure 5B) despite continued expansion of CD107+ cells (Figure 4C and D). The lack of correlation between GAP-specific cytotoxicity and the frequency of CD107+ GAP-specific cells in lungs (Figure 5C) suggests that although memory CD8 T cells are capable of degranulation, they are less efficient at killing targets during secondary infection.

To summarize, cytotoxicity is the main function of *M. tuberculosis*-specific memory CD8 T cells in the lungs, as appears to be the main function of primary CD8 T cell responses. By four weeks post-infection, however, the memory CD8 T cell response was altered compared to primary responses in that cytotoxicity had decreased.

3.3.7 Cytotoxic effector molecules

To address a potential cause of decreased cytotoxicity during secondary infection, we examined expression of perforin and granzyme B (Supplemental Figure 2I). They are the primary cytotoxic effector molecules, stored in lytic vesicles, during *M. tuberculosis* infection [210]. While granzyme B protein levels were low in both primary and memory GAP-specific CD8 T cells in unstimulated samples (Figure 7A), incubation with anti-CD3 and anti-CD28 antibodies could increase expression in both primary and memory cells (Figure 7B, $P<0.05$ at weeks two and six post-infection). Perforin levels, on the other hand, were high in both primary and secondary unstimulated GAP-specific CD8 T cells (Figure 7A).

Both primary and memory GAP-specific CD8 T cells had high levels of perforin, while granzyme B could be induced in both. Thus, a lack of effector molecules in recalled CD8 T cells is unlikely to account for the reduction in actual cytotoxicity during the later memory response.

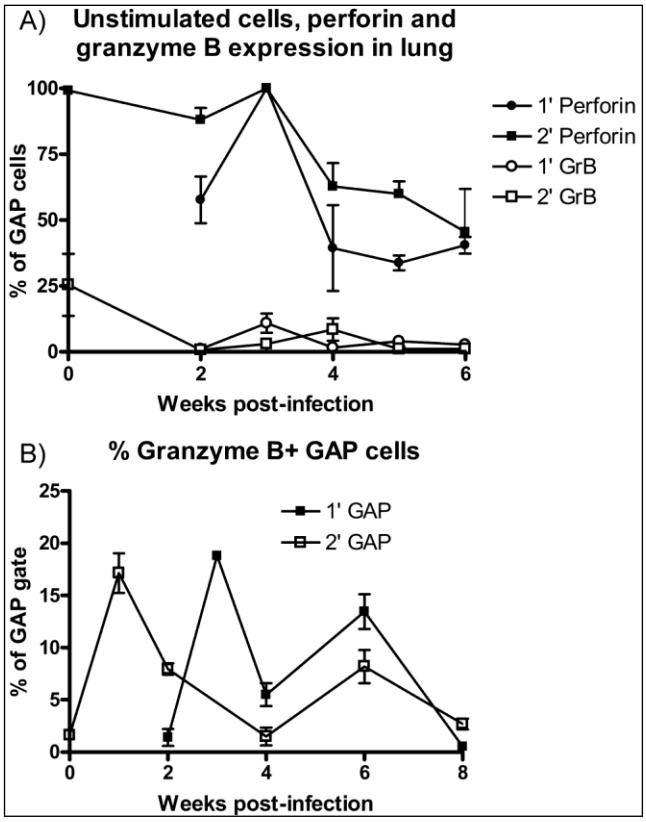


Figure 7 CD8 T cells express perforin, while granzyme B expression can be induced.
A) Cells were stained, without incubation, for perforin (FITC, clone dG9) and granzyme B (GrB), using fixative and protocol from Apo-Active 3 (Cell Technology, Mountain View, CA). B) Cells were stained for granzyme B after a 5 hour incubation, as described in Materials and Methods. Data are mean ± SEM of three or four mice per experimental group.

3.3.8 Degranulation and IFN-γ secretion are highest in IFN-γ+CD107+ cells

Although IFN-γ+CD107+ cells were a very small population during primary infection, these cells produced the most IFN-γ (Figure 8A, IFN-γ+CD107+ versus IFN-γ+CD107- cells at four weeks post-infection, $P<0.05$). The quantity of IFN-γ secreted on a per-cell basis decreased during secondary infection (Supplemental Figure 2B and C, Figure 8A IFN-γ+CD107+ cells at four weeks post-infection. 1' versus 2' responses, $P<0.05$), while the frequency of secreting cells was slightly increased (Figure 4A and B, four weeks post-infection. 1' versus 2' responses, $P<0.05$).

During primary infection, CD107 MFI remained constant and there was little difference between CD107+ and IFN-γ+CD107+ cells until late in infection, when there was increased degranulation, especially by IFN-γ+CD107+ cells (Figure 8B). Both numbers (Figure 4A and B, four weeks post-infection. 1' versus 2' responses, $P<0.01$) and MFI (Supplemental Figure 2E and F, Figure 8B, $P<0.01$) of degranulating cells were higher during secondary infection, while actual *in vivo* cytotoxicity was decreased compared to primary infection (Figure 5B and C).

In summary, IFN-γ+CD107+ cells may be the best effector cells because of their increased secretion of IFN-γ. However, they constituted a small population and their contribution to control of *M. tuberculosis* thus small, while less efficient cells constituted the bulk of the response.

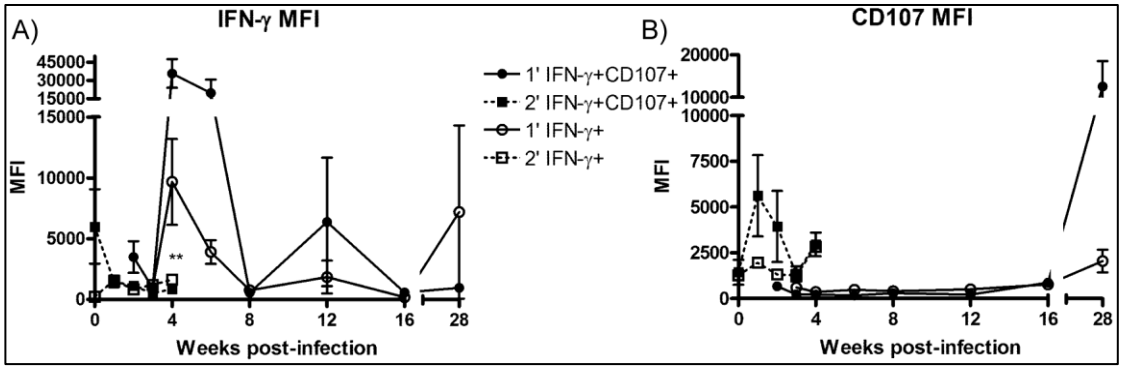


Figure 8 IFN-γ+CD107+ cells secrete more IFN-γ and degranulate more than cells limited to either function.

The geometric MFIs of CD107+ and IFN-γ+ GAP-specific cells was analyzed. A) IFN-γ MFI, B) CD107 MFI of cells that both secrete IFN-γ and degranulate or are limited to either function. Data are mean ± SEM of four mice per time-point. There were significant differences in both IFN-γ secretion and degranulation between primary and secondary infections ($P<0.05$) at three and four weeks post-infection. IFN-γ+CD107+ cells expressed significantly more IFN-γ four weeks post-infection than IFN-γ+CD107- cells ($P<0.01$) while there was no significant difference in degranulation between IFN-γ+CD107+ and IFN-γ-CD107+ cells.

3.4 DISCUSSION

In this study, we characterized the multifunctional nature of *M. tuberculosis*-specific CD8 T cell responses in the murine model. Using a recombinant strain expressing ova epitopes (*M. tuberculosis-ova*), ova-specific CD8 T cell responses were shown to be comparable to CD8 T cell responses to an endogenous *M. tuberculosis* epitope (GAP) over the course of both primary and secondary infections in C57BL/6 mice. *M. tuberculosis-ova* persistence in the host is comparable to that of the WT strain, despite the addition of a CD8 T cell epitope to CFP10. The GAP- and ova-specific CD8 T cell responses are comparable to, albeit weaker, than those seen against *M. tuberculosis*-specific CD8 T cell epitopes such as TB10.3/4 [184, 185]. There has been a lack of good reagents to study CD8 T cell responses in C57BL/6 mice, even though it is the most commonly used mouse strain in TB research. Advances in the use of tetramers and new assays for function of CD8 T cells, as well as the use of the recombinant *M. tuberculosis-ova* strain and ova-specific reagents, will allow for more in-depth studies of CD8 T cell responses in TB.

IFN-γ secretion and cytotoxic function have been traditional measurements of CD8 T cell responses. Study of CD8 T cell responses to *M. tuberculosis* infection has been complicated by the fact that CD8 T cells are a minor contributor of IFN-γ, compared to CD4 T cells ([211]; our unpublished results) and until recently, *in vitro* expansion of *M. tuberculosis*-specific CD8 T cells was required to measure cytotoxic function [200]. In other systems, it has been reported that induction of IFN-γ secretion requires more antigen than cytotoxicity [212]. While *M. tuberculosis* expansion is contained by four weeks post-infection, we previously showed that antigen accumulates in the lungs as the infection progresses [200]. This indicates that the lack of

antigen exposure is likely not the cause of the low number of IFN-γ secreting CD8 T cells. This is supported by the finding that co-incubation in an ELISpot assay with antigen-pulsed DC was not sufficient to induce IFN-γ secretion from CD8 T cells after the peak of infection at four weeks (Figure 5A). It is possible that IFN-γ secreting cells contracted, leaving cells primed for other functions, that cells with different functions entered the lungs, or that IFN-γ secretion was blocked. This block may be relieved as bacterial infection is cleared by antibiotics, as memory cells could respond by making IFN-γ, although the per-cell amount was reduced compared to that produced during primary infection (Figure 8A).

Studies examining CD8 T cell mediated cytotoxicity in TB have generated conflicting results. Previous work from our laboratory, using an *in vitro* cultured cells from C57BL/6 mice to assay for cytotoxicity, indicated that CD8 T cells lost cytotoxic function by four weeks post-infection [200]. Work by Kamath et al., using *in vivo* cytotoxicity assay in BALB/c mice, indicates that CD8 T cells retain their cytotoxic function throughout the infection [186]. It is likely that *in vitro* culture of cells led us to underestimate the frequency of cytotoxic CD8 T cells in infected lungs, as cells from the lungs can be highly activated [213] and prone to apoptosis *in vitro*. Recent work by Billeskov et al. [184], using the *in vivo* cytotoxicity assay in C57BL/6 mice, indicated that cytotoxicity peaks at four weeks post-infection and decreases slightly with time. We observed decreased cytotoxicity after four weeks, tracking a different CD8 T cell population, followed by an increase late in primary infection as the frequency of degranulating cells increased. The frequency of cells expressing surface CD107 correlated well with cytotoxicity during primary infection but not during secondary infection, where cytotoxicity was decreased compared to that seen during primary infection despite an increase in the frequency of degranulating cells. Wolint et al. [214] showed that cytotoxicity by memory cells was related to

effector molecules in lytic granules rather than degranulation. Partial CD8 T cell exhaustion caused by a lack of effector molecules may be the cause of decreased cytotoxicity during secondary *M. tuberculosis* infection, although the cells expressed perforin, and granzyme B expression could be induced by antigen stimulation. It is possible that antigen-pulsed targets used for *in vivo* cytotoxicity assays did not stimulate the cells sufficiently to induce granzyme B expression.

An unexpected finding was that, on a single cell basis, the vast majority of CD8 T cells induced by infection with *M. tuberculosis* were not capable of both secreting IFN-γ and degranulating. This contrasts with data from other chronic infections, such as HIV-1, where CD8 T cells chronically exposed to antigen are able to perform both functions [215]. During chronic LCMV infection, cells appear to be primarily cytotoxic while CD8 T cells from acutely infected mice retain the ability to be both cytotoxic and secrete IFN-γ [216]. Presumably, the phenotype of cytotoxicity only is due to constant antigen exposure during chronic LCMV infection, leading to exhaustion of the CD8 T cells. However, it is unlikely that distinct CD107+ and IFN-γ+ cell populations that arise during the initial phase of *M. tuberculosis* infection are due to exhaustion from chronic antigen exposure, as the inoculum is low and the bacterium slow growing, while the functional division, i.e. IFN-γ secretion or degranulation, is maintained from the initial priming events of the adaptive immune response. Additionally, while the frequency of cells expressing PD-1 increased over the course of *M. tuberculosis* infection, which has been correlated to CD8 T cell exhaustion in other infections [192, 204], the expression levels decreased following priming, and administration of blocking antibodies for four weeks had no significant effect on CD8 T cell function or the bacterial load (Figure 6).

In summary, *M. tuberculosis* infection yielded CD8 T cells that were to a large extent unable to both be cytotoxic and secrete IFN-γ and memory responses that were less functional than primary responses. Elucidating the cause of the poor quality of CD8 T cell responses may enable generation of improved vaccines against *M. tuberculosis*.

4.0 MEMORY CELLS INFLUENCE THE NUMBER AND QUALITY OF NEW CD8 T CELLS DURING *M. TUBERCULOSIS* INFECTION

4.1 INTRODUCTION

As discussed in chapter 3, primary adaptive immune responses are induced approximately two weeks post-infection in the murine model. Bacterial expansion is contained around four weeks post-infection, after which the bacterial load remains steady. Memory cells respond faster and contain the bacterial expansion at approximately two weeks post-infection [103, 217] but are neither able to prevent infection nor prevent disease. The same holds true for humans who have been previously vaccinated with BCG or infected with *M. tuberculosis* as these people can still be infected with *M. tuberculosis* and develop disease, despite the presence of memory cells [218, 219] (Figure 1G). We showed in chapter 3 that *M. tuberculosis*-specific memory CD8 T cells are less functional than primary CD8 T cells in that they secrete less IFN-γ and are less cytotoxic in mice [103]. This finding underscores the importance of primary CD8 T cell responses.

Both viral and bacterial infection models have shown that pre-existing memory cells can influence new CD8 T cell responses [220-225]. Primary and memory cells can respond during *Listeria monocytogenes* [220, 222, 224, 225] and lymphocytic choriomeningitis virus (LCMV) [222, 223] infections. However, the larger the memory response, the smaller the primary

response and priming of new CD8 T cell responses is dependent on the dose of infection [225]. On the other hand, memory cells can enhance the quantity and quality of new CD8 T cell responses through improved CD4 T cell help [224].

Due to the extent of BCG vaccination and *M. tuberculosis* infection in the human population, there is a very large pool of people who have formed memory responses to the bacteria, which can influence immune responses upon further infection or vaccination. In this study, we examined whether pre-existing memory influences the quality and quantity of new CD8 T cell responses. Our experimental system (described below) does not require adoptive transfer of cells. This limits cell manipulation and ensures that CD8 T cell responses were induced from a physiologically relevant number of progenitor cells that are in their natural niche.

Our findings indicate that a sufficiently high bacterial inoculum was required for primary CD8 T cell responses to be induced in the lungs when there was pre-existing memory. This did not hold true for the lymph nodes, where a lower bacterial load could prime larger CD8 T cell responses. Memory responses could affect the quality of the new CD8 T cell responses. CD8 T cells producing IFN-γ were more multifunctional when they were primed in the presence of memory cells, and they secreted significantly more IL-2 than CD8 T cells primed in naive mice.

Taken together, these results indicate that there is significant interaction between memory cells and newly primed CD8 T cells, which can influence both the quality and quantity of the new CD8 T cell responses.

4.2 MATERIALS AND METHODS

4.2.1 Mice

C57BL/6 mice (six to eight week old) were obtained from Jackson Laboratories and kept in a BSL3 facility under specific pathogen-free conditions. Three to four mice were used per time-point. The University of Pittsburgh Institutional Animal Care and Use Committee approved all animal protocols used in this study.

4.2.2 Bacterial infections

Mice were infected using a nose-only exposure aerosolizer (Intox) with either WT *M. tuberculosis* Erdman strain or *M. tuberculosis*-ova [103], a recombinant *M. tuberculosis* Erdman strain expressing CD4 and CD8 ovalbumin epitopes. Depending on the concentration of the bacterial inoculum, infection delivered approximately 2-150 CFU per lung, determined by plating whole-lung homogenates at 24 h post-infection on 7H10 medium agar (Difco) and incubation at 37°C in 5% CO_2 for three weeks.

4.2.3 Viral infections

The replication deficient Adenovirus-ova vector was produced and provided by the University of Pittsburgh Vector Core Facility. Mice were infected intratracheally with $5*10^{10}$ particles, based on a dose escalation study for induction of responses.

4.2.4 Antibiotic treatment

For secondary challenge experiments, *M. tuberculosis* infected mice were treated with 0.1 g/liter isoniazid (Sigma), 0.1 g/liter rifampin (rifampicin, Sigma), and 8 g/liter pyrazinamide (Acros) in drinking water for three months. In our experience this treatment clears the infection, as no bacteria could be cultured from the lungs, determined by plating of whole-lung homogenates. Treatment was initiated early (two weeks) or late (two months) after infection to model acute and chronic infections, respectively.

4.2.5 Harvesting of lungs and lymph nodes

Single-cell homogenates were obtained by crushing lungs and lymph nodes through a 40-µm cell strainer with a 5-ml syringe plunger. An aliquot was plated on 7H10 medium agar to determine the bacterial load. Red blood cells were lysed with an NH_4Cl-Tris solution and washed in DMEM (Sigma), after which cells were resuspended in T cell medium and counted based on trypan blue exclusion (Sigma).

4.2.6 Flow cytometry

Cells were incubated with GAP-phycoerythrin (GAPINSATAM) and ova-allophycocyanin (SIINFEKL) tetramers ([103], Beckman Coulter) for 30 min at 4°C in T cell medium, after which the cells were washed and resuspended in T cell medium containing 3 µM monensin (Sigma), anti-CD107a and anti-CD107b fluorescein isothiocyanate antibody, anti-CD28 antibody (clone 37.51, 1 µg/ml), and anti-CD3 antibody (clone 145-2C11, 0.1 µg/ml).

Cells were incubated at 37°C for 5 h, then washed and stained for 15 min with anti-CD8-pacific blue and anti-CD4-alexa fluor 700 antibodies in 20% mouse serum (Gemini) in FACS buffer. Cells were washed and fixed in 2% paraformaldehyde (Sigma).

Cells were permeabilized in permeabilization buffer, after which cells were stained with anti-IFN-γ-phycoerythrin-Cy7, anti-IL-2 Peridinin Chlorophyll Protein Complex (eBioscience, San Diego, CA) and anti-TNF-biotin, followed by streptavidin-allophycocyanin-Cy7 antibody. Samples were read on an LSR II (BD Biosciences) and data analyzed with FlowJo software (Tree Star). Unless otherwise stated, all antibodies were obtained from BD Biosciences.

4.2.7 Statistical analysis

Graphing and statistical analysis were performed using GraphPad Prism 4.01 (GraphPad Software). For two-group analysis, unpaired, two-sided *t* tests were used. P values of <0.05 (*), <0.01 (**) and <0.001 (***) were considered significant, very significant and highly significant, respectively. Spearman's rank correlation was used to determine correlation between bacterial load and the frequency of cells. *P* values of <0.05 and r^2 coefficients approaching 0.5 were considered significant.

4.3 RESULTS

4.3.1 Induction of ova-specific CD8 T cells in the presence and absence of pre-existing memory cells

To study primary CD8 T cell responses induced in the presence of memory cells, we infected mice with WT *M. tuberculosis*, which induces GAP-specific CD8 T cell responses [103]. The mice were then treated with antibiotics to clear the infection and re-infected with *M. tuberculosis*-ova, a recombinant *M. tuberculosis* that induces ova-specific CD8 T cell responses in addition to GAP-specific responses [103]. Using tetramers, GAP-specific memory responses and ova-specific primary responses could be simultaneously examined (Figure 9A). For comparison, we also infected mice with *M. tuberculosis*-ova (Figure 9B) and examined ova-specific primary responses that developed in the absence of memory cells.

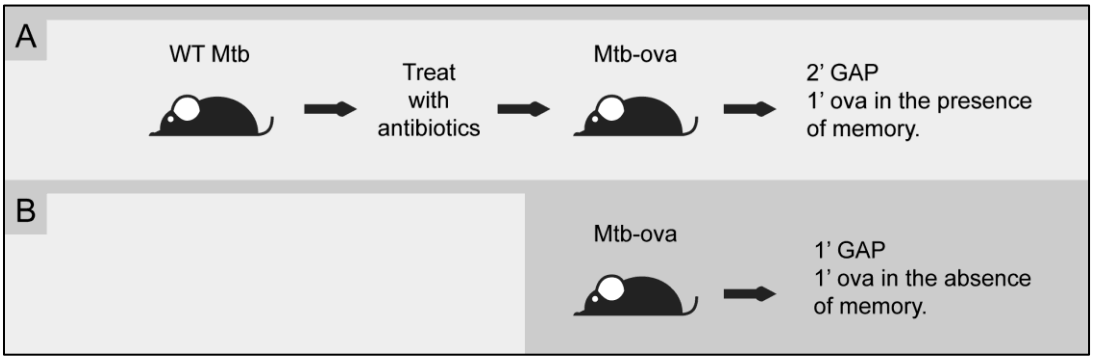


Figure 9 Experimental design to examine primary ova-specific responses induced in the presence or absence of memory cells.
A) To examine ova-specific responses that develop in the presence of memory, mice were infected with WT *M. tuberculosis*, which induces primary GAP-specific responses. The mice were then treated with antibiotics for three months to clear the infection, after which the mice were infected with *M. tuberculosis*-ova, which induces responses to both GAP (memory response) and ova (primary response). B) To examine ova-specific responses that develop in the absence of memory, mice were infected with *M. tuberculosis*-ova, which induces primary responses to both GAP and ova.

The *M. tuberculosis* infection inoculum determines at what level the bacterial load reaches a plateau in murine lungs [226]. To examine the relationship between bacterial load and induction of CD8 T cells, we infected mice with a range of *M. tuberculosis*-ova inocula. During primary infection, there was not always a direct correlation between CD8 T cell numbers (termed "Primary cells") and bacterial load (Figure 10A to D). In most experiments, higher bacterial load induced larger CD8 T cell responses in the lung (Figure 10A and B, red squares). Low bacterial burden could, however, induce larger responses in both lung (data not shown) and lymph node (Figure 10C and D, blue squares), although this was not statistically significant. It is unknown what factors, besides bacterial load, determine the magnitude of the CD8 T cell responses.

When data were pooled from eight independent experiments (Figure 11A to C) with a range of inocula (2-150 CFU, a physiologically relevant range of infection), the correlation (r^2) between bacterial load in the lungs and the number of *M. tuberculosis*-specific CD8 T cells was weak. There was no significant correlation between bacterial load and CD8 T cell numbers in the lymph nodes (data not shown).

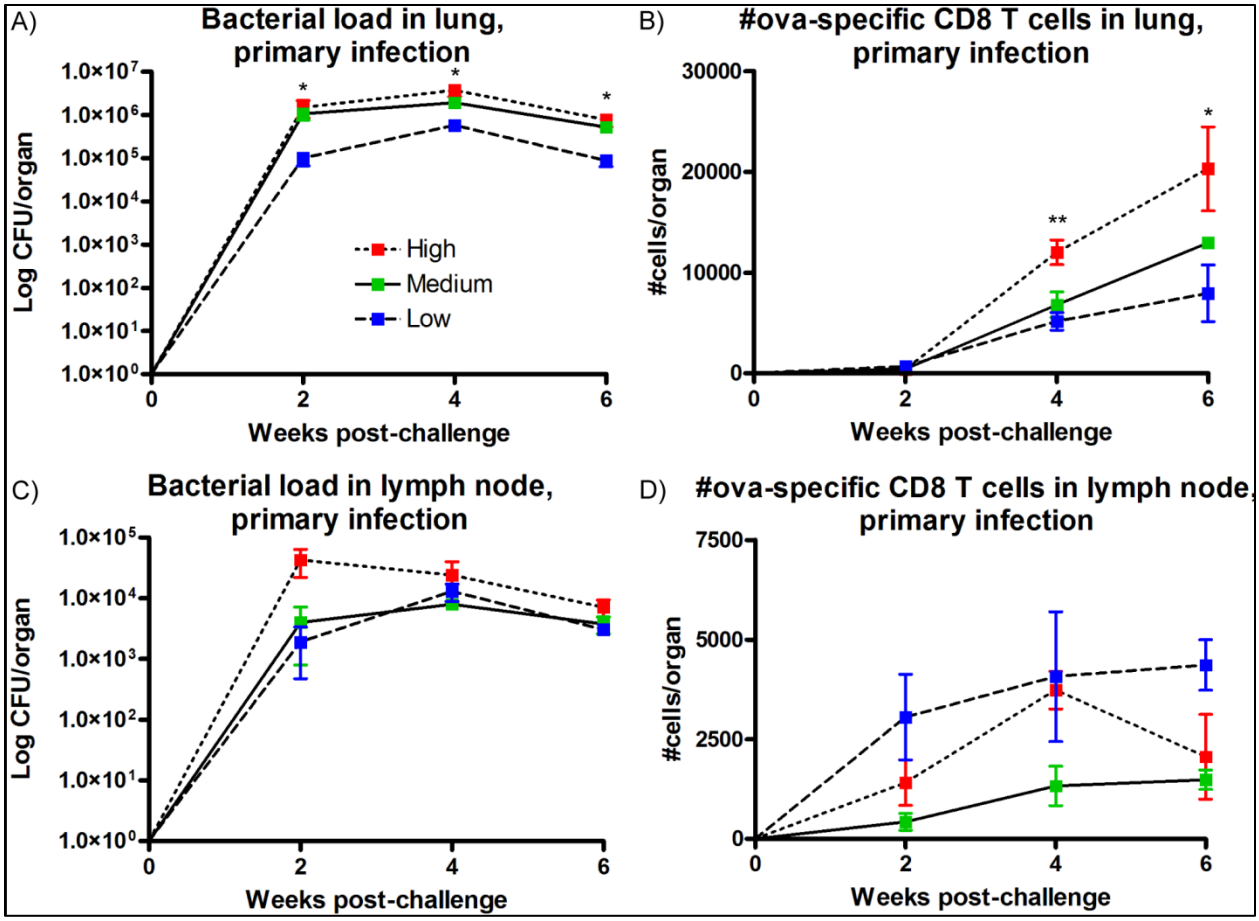


Figure 10 Bacterial load and induction of ova-specific CD8 T cells during primary infection.

(A and C) Bacterial load during primary infection in the lungs (A) and lymph nodes (C). (B and D) Number of ova-specific CD8 T cells in the lungs (B) and lymph nodes (D), calculated by multiplying ova-tetramer positive cells by the frequency of live cells (data not shown). The mice received a high (130 CFU, red squares), medium (30 CFU, green squares) or low (2 CFU, blue squares) inoculum, determined by plating of lung homogenates 24 hours after infection. The high group had significantly higher bacterial burdens than the low group in lungs ($P<0.05$ at two weeks post-infection, $P<0.02$ at four and six weeks post-infection). There were significantly more ova-specific CD8 T cells in lungs of the high group than in the low group in lungs ($P<0.005$ at four weeks post-infection, $P<0.05$ at six weeks post-infection). Data are the means ± standard errors of the means from test results for three mice per time-point.

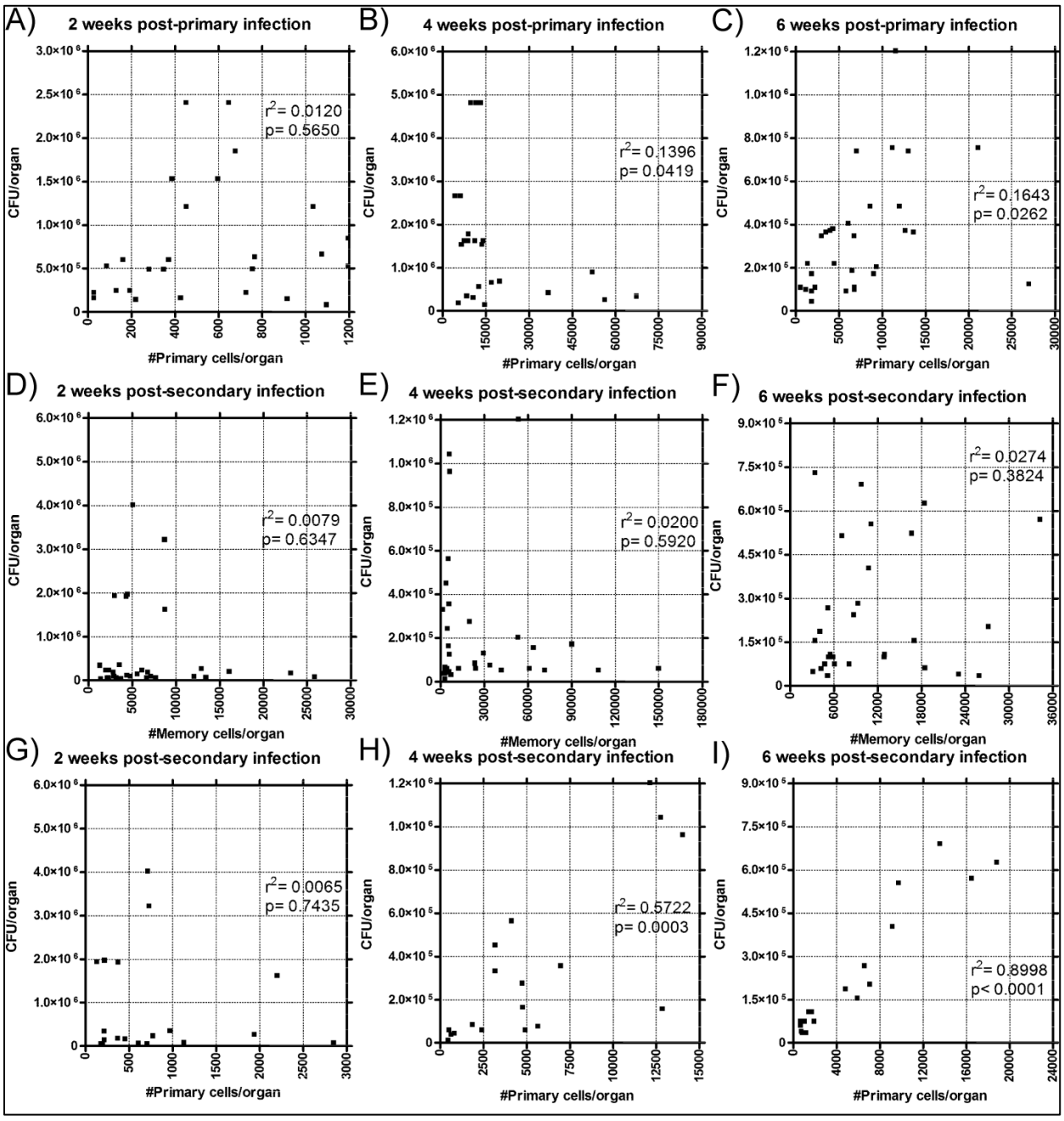


Figure 11 Correlation between bacterial load and induction of CD8 T cell responses in the lungs.

Data was pooled from eight experiments where mice received infection inocula ranging from 2-150 CFU. A) to C) are primary CD8 T cell responses, induced during a primary infection. D) to F) are secondary CD8 T cell responses, induced during a secondary infection. G) to I) are primary CD8 T cell responses, induced during a secondary infection. A), D) and G) are two weeks post-infection, B), E) and H) are four weeks post-infection and C), F) and I) are six weeks post-infection.

During secondary infection, there was no significant correlation between memory recall (termed "Memory cells") and bacterial load in the lungs (Figure 11D to F) or lymph nodes (data not shown). There was, however, a correlation between bacterial load and the number of ova-specific CD8 T cells (primary CD8 T cell response induced in the presence of memory cells) in the lung (Figure 12A to B, red squares). The higher the bacterial load, the larger the ova-specific CD8 T cell response was in the lung. This did not hold true for the lymph node (Figure 12C to D), where lower bacterial load could prime larger ova-specific CD8 T cell responses (blue squares). The mice with the lowest bacterial burden (Figure 12, blue squares) induced ova-specific CD8 T cells in the lymph nodes, but not in the lungs. GAP-specific recall was normal in these mice (Figure 12B and D, purple diamonds). This indicates that a sufficient bacterial load is required to induce new CD8 T cell responses in the lungs. When data were pooled, the correlation between the number of ova-specific CD8 T cells primed in the presence of memory and bacterial load was strong after two weeks post-infection in the lungs (Figure 11G to I) but not in the lymph nodes (data not shown).

In summary, while lower bacterial load in the lymph nodes can result in priming of larger CD8 T cell responses, a sufficiently high bacterial burden is required to detect new CD8 T cell responses in the lungs when there is pre-existing memory. Induction of CD8 T cell responses in the absence of pre-existing memory and the recall of memory cells appear to be less dependent on bacterial burden.

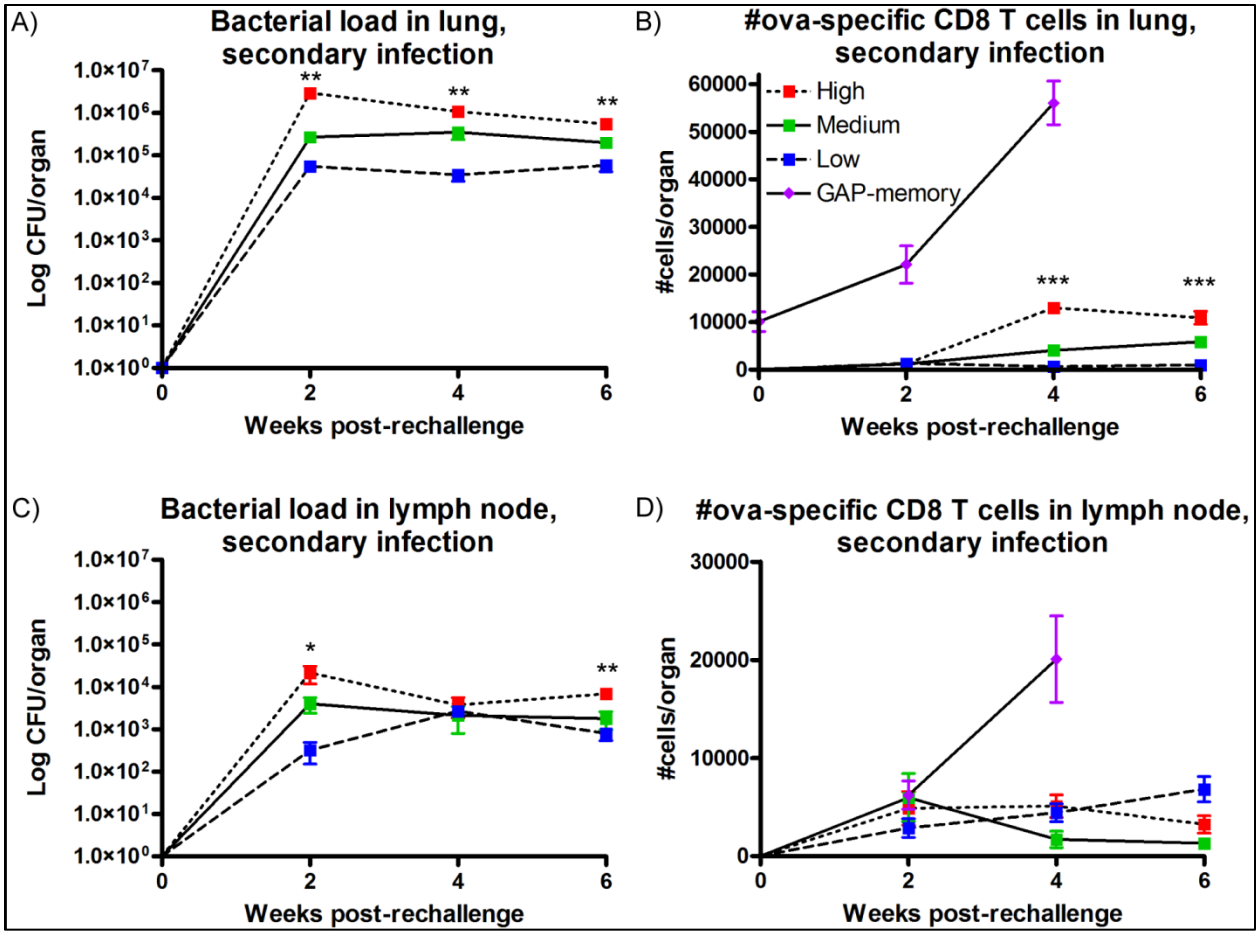


Figure 12 Bacterial load and induction of ova-specific CD8 T cells during secondary infection.

(A and C) Bacterial load during secondary infection in the lungs (A) and lymph nodes (C). (B and D) Number of ova-specific CD8 T cells in the lungs (B) and lymph nodes (D), calculated by multiplying ova-tetramer positive cells by the frequency of live cells (data not shown). The mice received a high (130 CFU, red squares), medium (30 CFU, green squares) or low (2 CFU, blue squares) inoculum, determined by plating of lung homogenates 24 hours after infection. The high group had significantly higher bacterial burdens than the low group in lungs ($P<0.005$ at week two post-infection and $P<0.001$ at four and six weeks post-infection) and lymph nodes ($P<0.05$ at week two post-infection and $P<0.002$ at six weeks post-infection). There were significantly more ova-specific CD8 T cells in the high group than in the low group in lungs ($P<0.0001$ at four and six weeks post-infection). Data are the means ± standard errors of the means from test results for three mice per time-point.

4.3.2 Effect of bacterial load on the function of CD8 T cells

To examine whether the bacterial burden had a signficant effect on the function of the cells, we examined the correlation between bacterial load and secretion of cytokines (IFN-γ, TNF and IL-2) or degranulation of CD8 T cells. To do this, we pooled data from the experiments above to get a range of bacterial loads (2-150 CFU)(Table 1). There did not appear to be a strong correlation between the bacterial load and function in any of the CD8 T cell groups. When there was a significant correlation, it was mostly early, at two weeks post-infection. No r^2 coefficient approached 0.5, which we consider a relevant cut-off for significant correlation.

In summary, there does not appear to be a strong correlation between bacterial load and the functions we studied when looking at a range of low-dose infections. This may, however, be due to the limited number of data points, despite pooling of data, or the relatively narrow range of bacterial inocula used.

		Lung			Lymph node		
Function	Weeks post-infection	**Memory**	**Primary with memory**	**Primary without memory**	**Memory**	**Primary with memory**	**Primary without memory**
IFN-γ	2	0.3667			0.1774		0.2315
	4			0.1119			
	6				0.2010		
CD107	2	0.1342	0.1322		0.2670		
	4	0.3987					
	6	0.2596	0.1801			0.2956	
TNF	2			0.1933	0.1280		0.3350
	4						
	6			0.1438			
IL-2	2	0.1886		0.3125	0.2667		0.1167
	4		0.1588	0.1805			
	6			0.3799			

Table 1 Correlation between bacterial load and function.
Data were pooled from eight independent experiments, with infection inocula ranging from 2-150 CFU. Where there was a significant correlation between bacterial load and function ($P<0.05$), the correlation coefficients (r^2) are shown to indicate the strenght of the correlation. Where the correlation between bacterial load and function were not significant ($P>0.05$), the boxes are grey.

4.3.3 Effect of memory cells on the quality of new CD8 T cell responses

To examine the effect of pre-existing memory cells on the quality of new CD8 T cell responses, we compared the function of cells primed in the presence and absence of memory. Cells primed in the presence of memory secreted more IL-2 and less TNF in both lungs (Figure 13A and B) and lymph nodes (data not shown). However, there was little difference in the expression of IFN-γ and degranulation between the two groups in the lungs (Figure 13C and D) and lymph nodes (data not shown). No CD8 T cells were able to simultaneously degranulate and secrete IFN-γ, and there was no difference in the geometric mean fluorescence intensity of CD107, indicating that cytotoxicity was not affected by the presence of memory cells (data not shown). Other infection models have indicated that multifunctional T cells, i.e. cells able to simultaneously secrete IFN-γ, IL-2 and TNF, are more protective than T cells secreting fewer cytokines [82, 227], although this has not been definitively shown for *M. tuberculosis* [86, 116]. When we examined the frequency of IFN-γ+ CD8 T cells that were multifunctional, there were significantly more ova-specific CD8 T cells simultaneously secreting IFN-γ, IL-2 and TNF in the lungs when cells were primed in the presence of memory cells, while there was no significant difference in the lymph nodes (Figure 14).

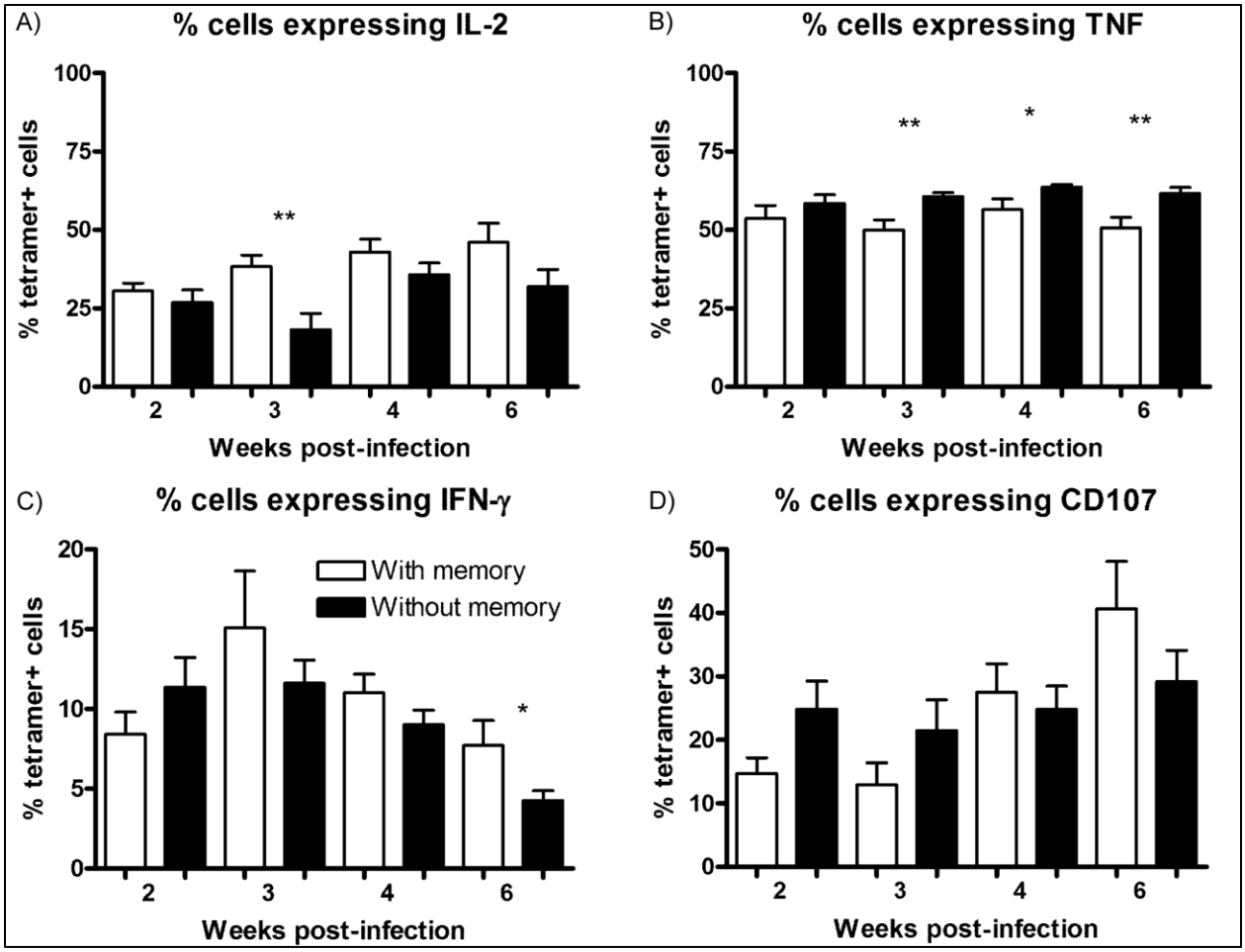


Figure 13 Function of primary CD8 T cells, primed in the presence or absence of memory cells.

A) IL-2 secretion, B) TNF secretion, C) IFN-γ secretion and D) degranulation by CD8 T cells in the lungs. White bars are CD8 T cells primed during secondary infection, while black bars are cells primed during primary infection. Cells primed in the presence of memory secreted significantly more IL-2 ($P<0.006$, week three post-infection) and IFN-γ ($P<0.02$, week six post-infection), and significantly less TNF ($P<0.002$, week three post-infection, $P<0.02$, week four post-infection and $P<0.004$, week six post-infection). Data are the means ± standard errors of the means from test results for three mice per time-point.

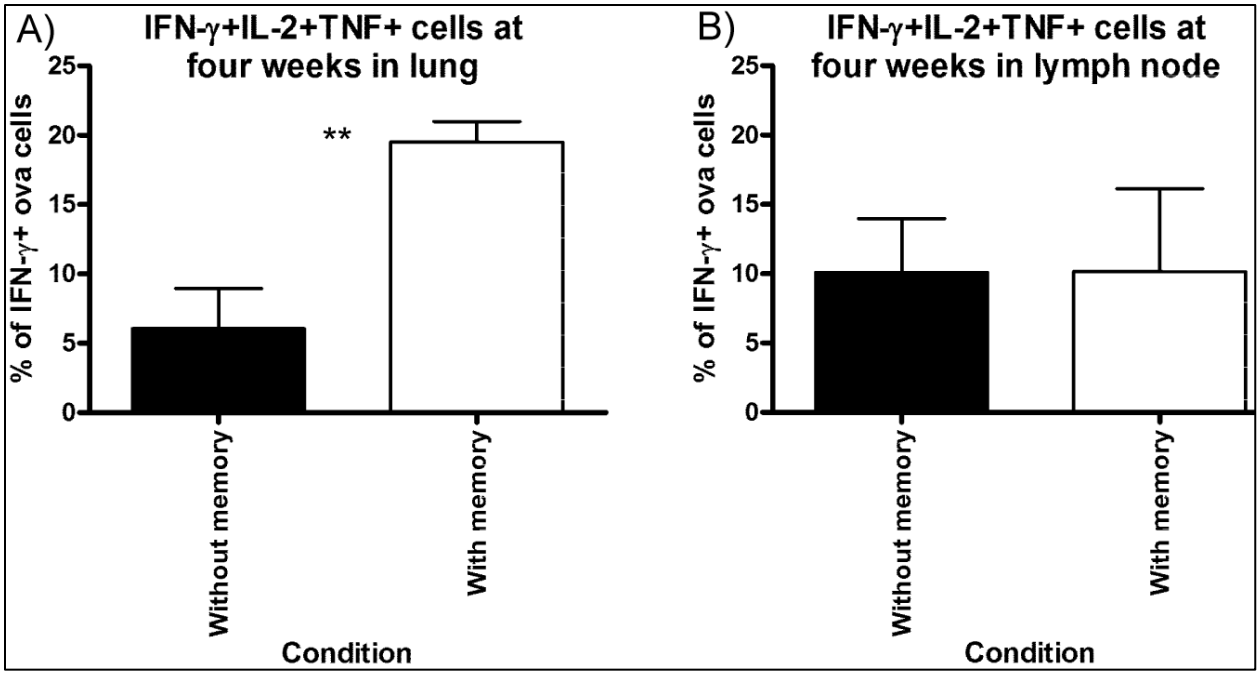


Figure 14 Multifunctional CD8 T cells primed in the presence or absence of memory cells. Frequency of IFN-γ producing cells capable of simultaneously secreting IL-2 and TNF as well in the lungs (A) and lymph nodes (B) at four weeks post-infection. White bars are CD8 T cells primed during secondary infection, while black bars are cells primed during primary infection. IFN-γ producing cells primed in the presence of memory were significantly more multifunctional in the lungs ($P<0.002$), while there was no significant difference in the lymph nodes. Data are the means ± standard errors of the means from test results for three mice per time-point.

One possible difference between the CD8 T cells primed with and without memory cells is the quality of CD4 T cell help they receive. However, the requirement for and mechanism of CD4 T cell help appears to depend on the model system [88, 90, 228-230]. One mechanism is ligation of CD40L to CD40 on DC. This increases antigen-presentation and expression of co-stimulatory molecules on the DC, which enables it to prime CD8 T cell responses [231]. However, activation of CD8 T cell responses during *M. tuberculosis* infection is independent of CD40L [40], as *M. tuberculosis* heat shock protein 70 serves as a ligand for CD40 [232]. Thus, other mechanisms of CD4 T cell help are more likely to be involved. Two alternative mechanisms of CD4 T cell help are secretion of IFN-γ [229] and IL-2 [230]. We therefore compared CD4 T cell cytokine production in primary and memory CD4 T cells and found that memory CD4 T cells expressed significantly more IFN-γ at 2 weeks post-infection (Figure 15A) and IL-2 at 4 weeks post-infection (Figure 15B) in the lungs. No significant difference was found in TNF expression. There was no significant difference in CD4 T cell function in the lymph nodes, where the cells are likely to be primed and where there is the greatest requirement for CD4 T cell help. However, memory cells respond faster than primary cells, and could thus provide increased CD4 T cell help in the lymph nodes during secondary infection, even though the cytokine profile is the same as that of primary CD4 T cells.

In summary, CD8 T cells primed in the presence of memory secreted more IL-2, less TNF, and were more multifunctional. One possible mechanism of this observed difference is improved and earlier CD4 T cell help by memory CD4 T cells, which secreted IFN-γ earlier than CD4 T cells induced during primary response.

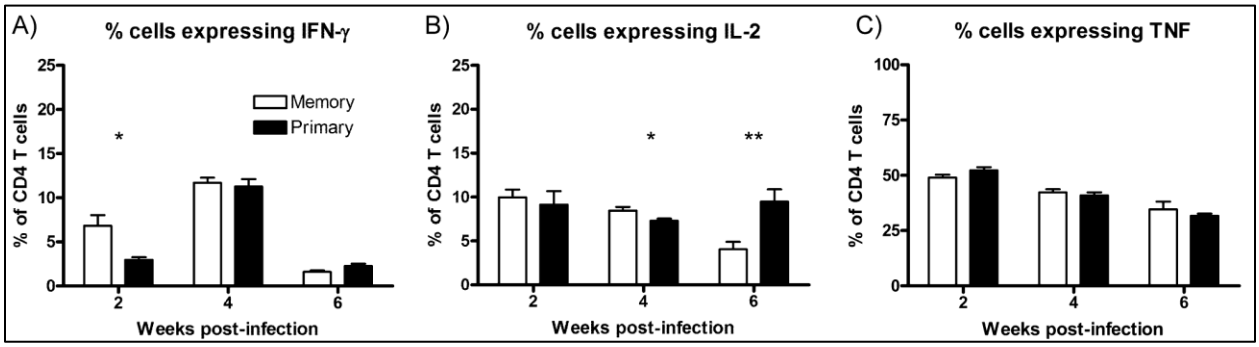


Figure 15 Cytokine production by CD4 T cells during primary and secondary infections.
A) IFN-γ secretion, B) IL-2 secretion and C) TNF secretion by CD4 T cells in the lungs during primary infection (black bars) and secondary infection (white bars). Memory CD4 T cells secreted significantly more IFN-γ ($P<0.01$, week two post-infection) and more ($P<0.03$, week four post-infection) and less ($P<0.008$, week six post-infection) IL-2 than primary CD4 T cells in the lungs, while there was no significant difference in the lymph nodes. Data are the means ± standard errors of the means from test results for three mice per time-point.

4.3.4 Quality of memory and primary cells formed during acute infections

We next examined whether altering the quality of the memory responses could influence the quality of newly primed CD8 T cells. This is based on work comparing chronic and acute LCMV infections in mice. Wherry et al. showed that LCMV-specific memory CD8 T cells generated during acute infection are functionally superior to those generated during chronic infection [233]. Thus, we examined whether the duration of primary *M. tuberculosis* infection influenced the quality of the memory CD8 T cell recall, and whether this in turn affected the quality of newly primed CD8 T cells.

Our first strategy was to model an acute *M. tuberculosis* infection by initiating antibiotic treatment early (two weeks post-infection, "acute" group) instead of our standard late initiation (two months post-infection, "chronic" group). Bacteria were undetectable in lymph nodes by two weeks after the initiation of drug treatment and by four weeks in the lungs (Figure 15A and B), although both the acute and chronic groups were treated with antibiotics for three months to ensure that bacteria were cleared, based on previous experience. To study the development of memory CD8 T cells, we examined their function during antibiotic treatment, at eight weeks after initiation of drug treatment. Memory CD8 T cells developed similarly in both groups (Figure 16C and D), which indicates that shortening the primary infection did not dramatically alter the phenotype of the cells.

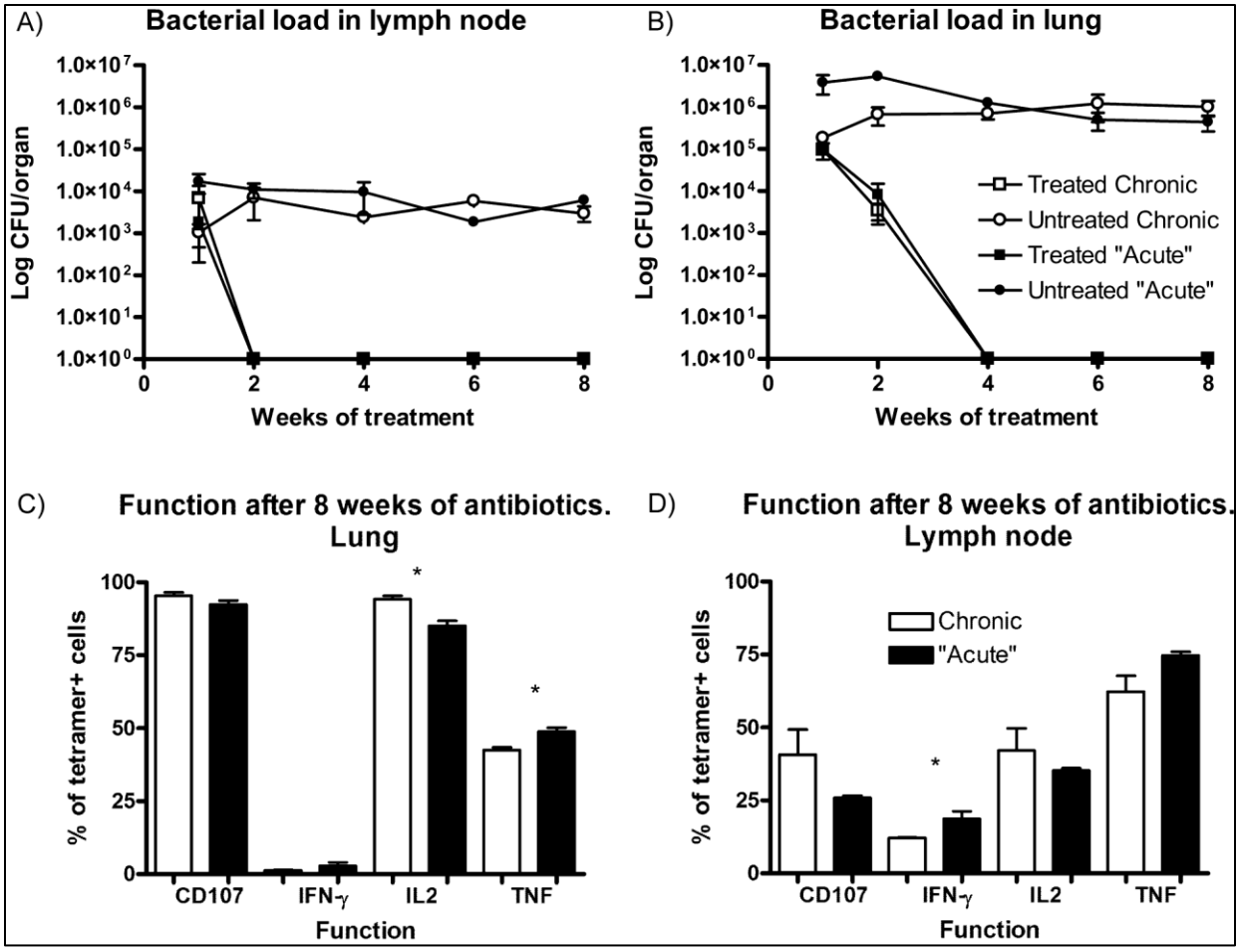


Figure 16 Clearing of bacteria and formation of memory cells during chronic and acute *M. tuberculosis* infections.

(A and B) Antibiotic treatment cleared detectable bacteria at a similar rate in mice that had been infected for two weeks (black squares) and two months (open squares), while bacterial levels reached a plateau in the lungs (A) and lymph nodes (B) of untreated mice (circles). Memory cells formed during acute infection secreted significantly less IL-2 ($P<0.01$, lungs) and significantly more TNF ($P<0.02$, lungs) and IFN-γ ($P<0.04$, lymph nodes), but the overall functional phenotype of the cells was similar. Data are the means ± standard errors of the means from test results for three mice per time-point.

Upon re-infection, there was no significant difference in the bacterial load (Figure 17A and B) or in the function of the memory cells generated under acute and chronic conditions (lungs and lymph nodes, data not shown). While there was little significant difference in the quality of primary cells (Figure 17C to F), cells primed in the presence of acute memory cells secreted more IL-2 at 2 weeks post-infection. However, at this early time-point the primary response had not expanded significantly (Figure 10B), which questions the biological significance of this finding. Thus, shortening the primary infection did not significantly alter the quality of the memory CD8 T cells or the newly primed CD8 T cells. However, as elimination of both live mycobacteria (Figure 16A and B) and bacterial antigens [234] is slow, this is not a true model of acute infection.

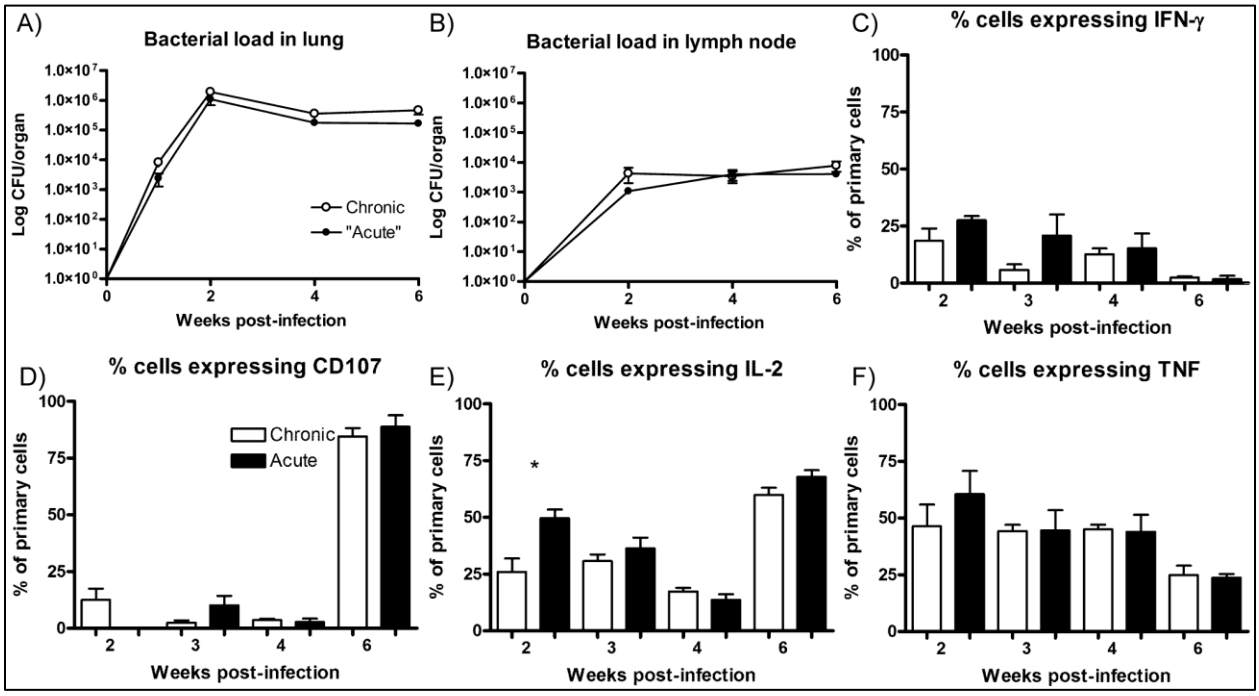


Figure 17 Bacterial load and function of primary CD8 T cells upon re-infection of mice. The duration of the primary infection had no significant effect on the bacterial load of the lungs (A) or lymph nodes (B) when mice were re-infected with *M. tuberculosis*. There was also no significant difference in the quality of the primary CD8 T cell response in lung (C to F) or lymph nodes (data not shown) that was induced during the secondary infection, except for IL-2 secretion ($P<0.03$, week 2 post-infection in lung). Data are the means ± standard errors of the means from test results for four mice per time-point.

Therefore, we next used a replication deficient adenovirus that expresses ova (Adeno-ova) to model an acute primary infection. We intranasally infected mice with Adeno-ova and let the mice clear the infection for two months, after which we challenged the mice with *M. tuberculosis*-ova or Adeno-ova. Adeno-ova induced primary ova-specific CD8 T cells by 10 days post-primary infection in the lungs (Figure 18A, 7 weeks before re-infection). Upon re-infection with Adeno-ova, ova-specific CD8 T cells expanded by 7 days, and contracted by 14 days. Little ova-specific response was seen in the lymph nodes (Figure 18B). When mice were re-infected with *M. tuberculosis*-ova (M.tb.), there was no significant difference in ova-specific recall whether the primary infection was with Adeno-ova or *M. tuberculosis*-ova (Figure 18A and B). Adeno-ova provided significantly less protection than *M. tuberculosis*-ova upon re-infection with *M. tuberculosis*-ova (Figure 18C and D). This is likely due to induction of CD4 and CD8 T cell memory to multiple antigens by *M. tuberculosis*-ova, while the majority of memory responses induced by Adeno-ova did not recognize mycobacterial antigens. Furthermore, there was no significant difference in the function of ova-specific memory cells induced by Adeno-ova and *M. tuberculosis*-ova infections (Figure 19).

In summary, shortening the primary infection did not significantly alter the quality of the memory CD8 T cells or of the newly primed CD8 T cells.

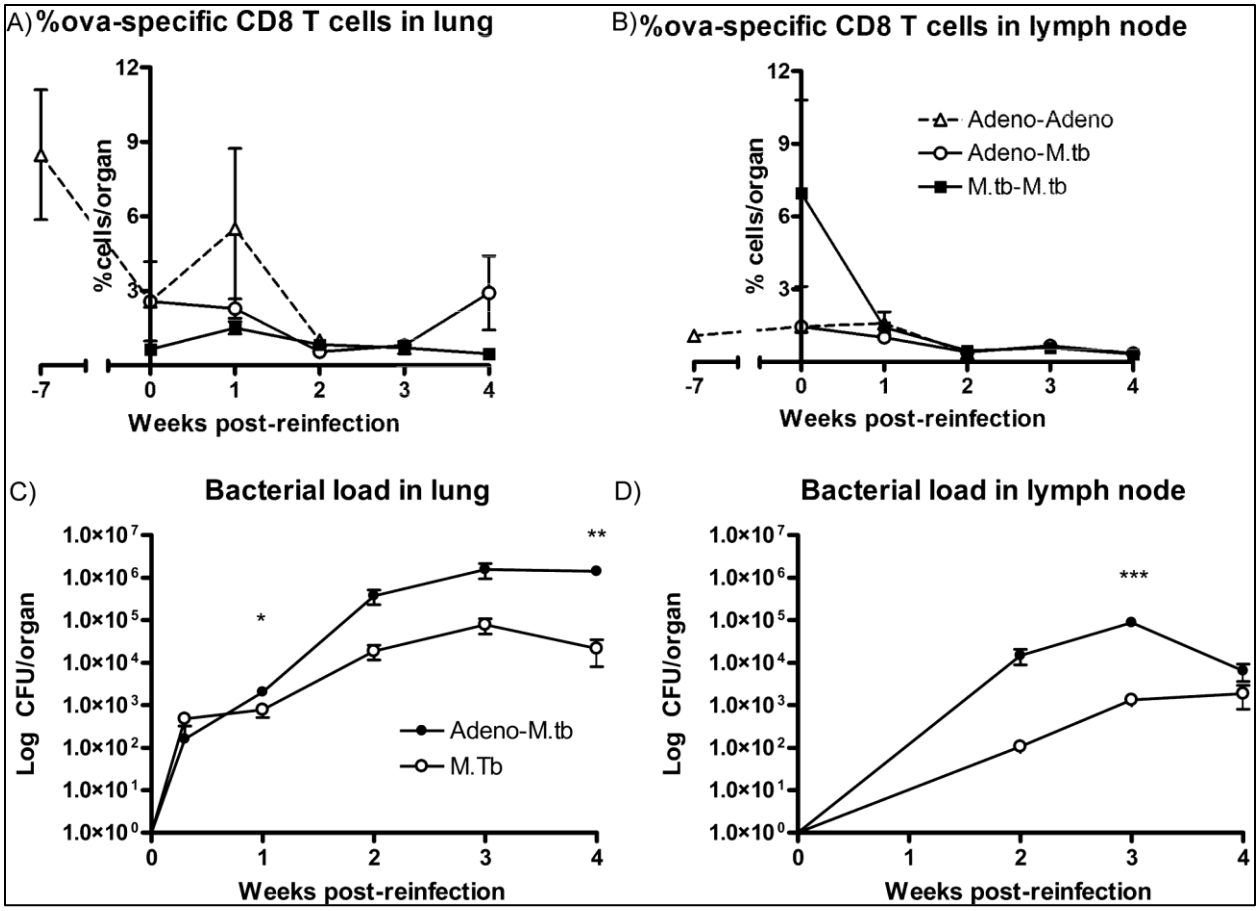


Figure 18 Expansion and contraction of ova-specific CD8 T cells in response to Adeno and *M. tuberculosis* infections.

There was no significant difference in the expansion of ova-specific CD8 T cells whether the cells were induced during Adeno or *M. tuberculosis* infections (A and B, lung and lymph node, respectively). Memory responses induced by *M. tuberculosis* provided significantly more protection in both lung (C, $P<0.04$, week 1 post-infection and $P<0.002$, week 4 post-infection) and lymph node (D, $P<0.0007$, week 3 post-infection) than memory responses induced by Adeno. Data are the means ± standard errors of the means from test results for three mice per time-point.

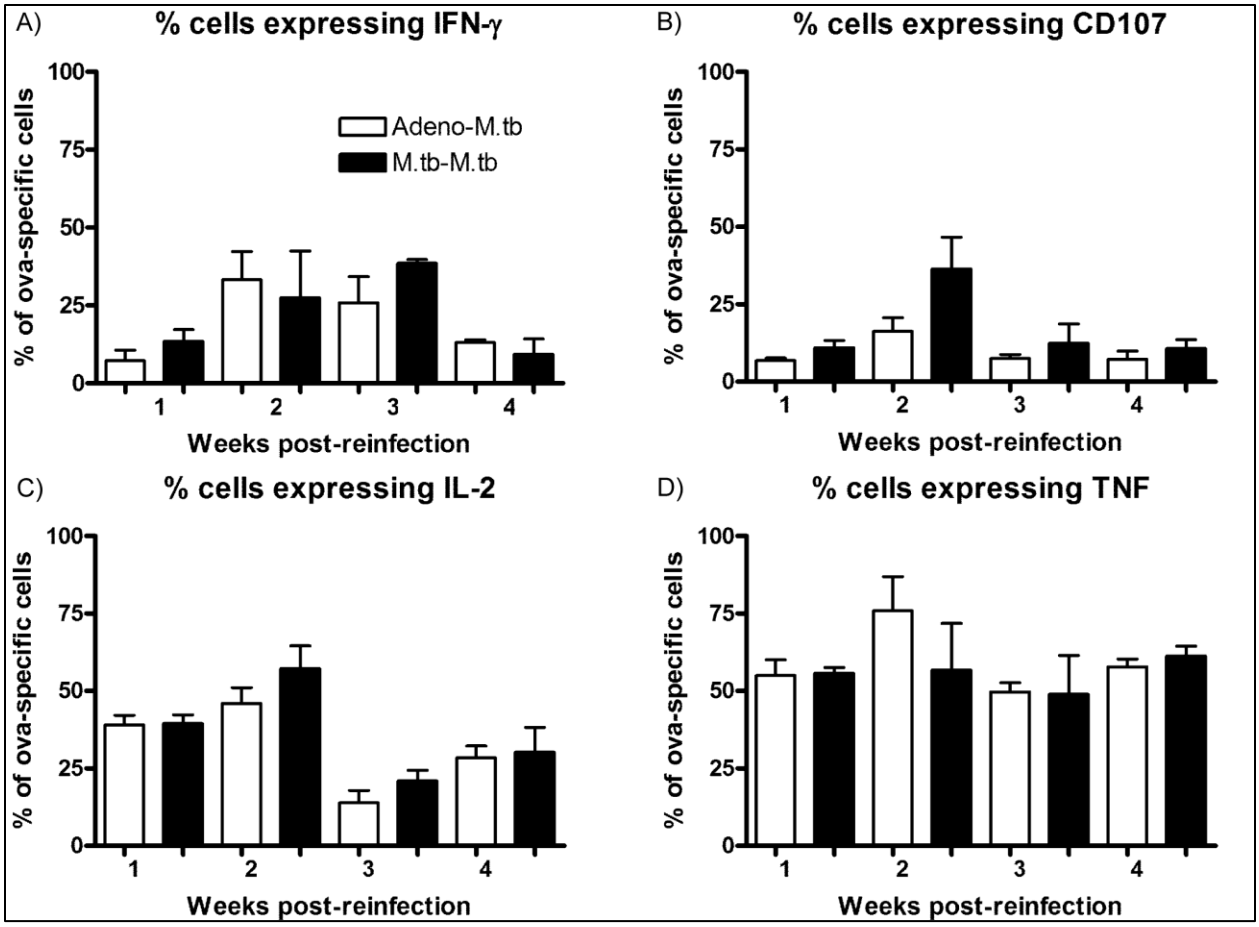


Figure 19 Function of ova-specific memory CD8 T cells, primed during Adeno and *M. tuberculosis* infections.

A) IFN-γ secretion, B) degranulation C) IL-2 secretion and D) TNF secretion memory CD8 T cells in the lungs during secondary infection with *M. tuberculosis*. White bars are CD8 T cells primed during Adeno infection, while black bars are cells primed during *M. tuberculosis* infection. There was no significant difference in function between the cells in the lungs (A to D) or lymph nodes (data not shown). Data are the means ± standard errors of the means from test results for three mice per time-point.

4.4 DISCUSSION

In this study, we examined the effect of pre-existing memory cells on the number and function of new CD8 T cells primed during *M. tuberculosis* infection. Memory responses contain the bacterial infection around two weeks post-infection, as new CD8 T cell responses begin to expand. This decreases the inflammation and availability of antigen compared to a primary infection, where there are no memory cells. Our data show that a low bacterial load could prime CD8 T cells in the lymph nodes in the presence of memory. However, a sufficiently high bacterial load was required to induce new CD8 T cell responses in the lungs. The data indicate that cells are primed in the lymph node during secondary infection and subsequently migrate to the lungs. Anis et al. [235] showed that CD4 T cells are primed in the lymph nodes in response to intranasal delivery of antigen and that pulmonary inflammation (in their study BCG infection) is required for the cells to migrate to the lungs after priming. Thus, in our experiments the lack of ova-specific CD8 T cell responses in the lungs when bacterial load was low may be due to insufficient antigen or inflammation in the lungs. T cells primed in the absense of pre-existing memory cells were less dependent on the bacterial load, which may be due to increased inflammation and availability of antigen. Memory responses were also less dependent on the bacterial load, which likely reflects their lower dependency on antigen stimulation [236].

The data show that the presence of pre-existing memory during priming of new cells did not alter the frequency of cells that degranulated or secreted IFN-γ. However, the frequency of multifunctional IFN-γ+ cells increased, which indicates that the quality of the responding cells may be improved. The presence of memory also led to increased IL-2 secretion and decreased TNF secretion. Increased IL-2 production may be indicative of increased proliferative potential

of the cells, although cells primed in the presence and absence of memory reached similar numbers during the time-frame of this study (Figure 10 and Figure 12). Furthermore, loss of IL-2 secretion has been shown to be an early event in CD8 T cell exhaustion during chronic viral infection [237]. Increased levels of IL-2 may thus be an early indicator of enhanced fitness of the cells. Increased IL-2 secretion has also been implicated in enhancing cytotoxicity of CD8 T cells by increasing the levels of perforin and granzymes, the effector molecules of cytotoxicity [238]. This will be a future area of study.

TNF is secreted by many cell types during *M. tuberculosis* infection [239]. While TNF is important for containment of the bacterium, high levels are detrimental and lead to pathology [240]. Whether the decreased TNF expression by cells induced in the presence of pre-existing memory results in decreased histopathology is difficult to determine, however, as the early curtailment of bacterial expansion by the memory response results in decreased histopathology. While the current study focused on endogenous responses in their native setting, future studies will use adoptive transfer of cells to examine whether priming in the presence of memory leads to a biologically significant difference and examine a wider range of functions.

Shortening the primary infection by early antibiotic treatment had surprisingly little effect on the quality of both the memory cells and primary cells. The data indicate that the chronicity of *M. tuberculosis* may not significantly influence the quality of the immune response, as has been suggested by some [102, 241]. Similarly, priming of ova-specific CD8 T cells by an acute heterologous infection did not significantly alter the ova-specific recall response to *M. tuberculosis*.

The data show that there is significant interaction between primary and secondary immune cells during *M. tuberculosis* infection, and that memory cells can increase the quality of

the primary T cell response. However, the cells can also shape the environment, e.g. by early containment of bacterial expansion, so that new T cell responses are not induced in the lungs when the inoculum dose is too low. As the lungs are the site of infection, it is likely that the most protection would be derived from *M. tuberculosis*-specific immune responses localizing to the lungs. This may be one of the reasons why attempts to boost BCG vaccinations have largely failed in humans [159, 160], as the vaccine is given intradermally and may result in accumulation of memory cells in locations other than the lungs. This is supported by a study by Chen et al. who showed that intranasal BCG vaccination conferred superior protection compared to subcutaneous vaccination in mice [242]. Cumulatively, these data may lead to improved vaccination strategies against TB and a better understanding of what shapes the immune response to *M. tuberculosis*.

5.0 PRIMING OF MULTIFUNCTIONAL CD4 AND CD8 T CELLS DURING *M. TUBERCULOSIS* INFECTION IN MICE

5.1 INTRODUCTION

Neither *M. tuberculosis* infection nor BCG vaccination induce immune responses that reliably prevent establishment of infection or prevent re-infection. In fact, people who have successfully cleared the bacteria with antibiotics can be re-infected with *M. tuberculosis* and develop disease [219]. For these reasons, it has been suggested that mycobacterial vaccines may not be able to induce significantly protective immune responses against *M. tuberculosis* [80, 125] and that alternative vaccine strategies should be pursued, such as subunit vaccines and virally based vaccines [85, 86]. However, the BCG vaccine cannot be easily replaced due to the essential role that it plays in decreasing the severity of childhood TB. For this reason, much effort is being put into developing post-exposure vaccines, which boost immune responses induced by BCG and other mycobacteria.

Despite the long coexistence of humans with *M. tuberculosis*, our understanding of the bacterium and the immune response required to contain the bacterium are limited. It is known that Th1-type immune responses, characterized by secretion of interferon-γ (IFN-γ) and tumor necrosis factor (TNF) by T cells, are essential for control of the bacterium [66, 243-246]. Furthermore, recently published work indicates that multifunctional T cells are beneficial [86,

116], as seen in other infection models [82, 227]. Multifunctional T cells are cells capable of simultaneously secreting IFN-γ, IL-2 and TNF.

The aim of this study was to better understand what shapes the immune response to *M. tuberculosis*, and examine whether the functional phenotype of the immune response can be altered, without the use of adjuvants or viral vectors. To do this, we infected mice with *M. tuberculosis*-ova, a recombinant strain that expresses ovalbumin epitopes. We then adoptively transferred naive ova-specific CD4 and CD8 T cells into the mice at different times post-infection and compared the responses to cells present from before infection. Our data show that the functional phenotype of the T cell response is not static, and that there is a significant difference in effector functions, depending on the environment in which the cell is primed. By manipulating the priming environment, we were able to significantly increase the percentage of multifunctional CD4 and CD8 T cells. Thus, while BCG provides necessary but insufficient protection, manipulation of the vaccination strategy may induce improved immune responses, without altering the bacterium.

5.2 MATERIALS AND METHODS

5.2.1 Mice

B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1) and B6.PL-*Thy1*^{a}/CyJ (Thy1.1) breeding pairs were obtained from Jackson Laboratories while C57BL/6 RAG1-/- background OTI and OTII T cell receptor transgenic mice were kindly provided by Louis D. Falo. The mice were bred at the

University of Pittsburgh Biotechnology Center and kept under specific pathogen-free conditions in a BSL3 facility.

MACS cell separation technology (Miltenyi) was used to purify OTI and OTII cells from naive spleens. $5*10^3$ cells of each T cell receptor specificity were adoptively transferred in PBS to recipient mice by tail-vein injection. Experiments were performed at least twice, with three to four mice used per group/time-point/experimental procedure. The University of Pittsburgh Institutional Animal Care and Use Committee approved all animal protocols in this study.

5.2.2 Bacterial infections

Mice were infected with a low bacterial inoculum, using a nose-only exposure aerosolizer (Intox). Mice were infected with *M. tuberculosis-ova* [103], a recombinant *M. tuberculosis* Erdman strain that expresses ovalbumin epitopes recognized by CD8 T cells (OTI epitope, 257-264) and CD4 T cells (OTII epitope, 323-339). 24 hours post-infection, whole lungs were homogenized and plated on 7H10 media agar (Difco) and incubated for three weeks at 37ºC, 5% CO_2 to estimate the inoculum dose, 20-150 colony forming units (CFU) per lung.

5.2.3 Single-cell suspensions

To obtain single-cell suspensions of harvested tissues, lungs and lymph nodes were crushed through a 40-µm cell strainer with a 5-ml syringe plunger. Bacterial load was determined by plating serial dilutions on 7H10 media agar plates. For the remainder of the samples, an NH_4Cl-Tris solution was used to lyse red blood cells, after which the cells were washed and

resuspended in T cell medium. Cells were counted and viability assessed using trypan blue exclusion (Sigma).

5.2.4 Cytokine and chemokine analysis

Tissues were crushed in 3ml of DMEM, cells pelleted and the tissue supernatants were filtered through a 0.45um syringe filter (Millipore). Bio-Plex beads (Bio-Rad) were used, according to manufacturer's instructions. Cytokines analyzed were: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IFN-γ and TNF. Chemokines analyzed were: GM-CSF, MIP-1β, RANTES, MCP-1, VEGF. Samples were read on a Luminex 100 IS Bio-Plex System machine (Luminex Corporation, Austin, TX).

5.2.5 Flow cytometry

Cells were incubated with CD8 ova (SIINFEKL) tetramer-phycoerythrin (Beckman Coulter) at 4ºC for 30 min in T cell medium. After washing, the cells were resuspended and incubated for 5 hours at 37ºC in T cell medium containing 3μM monensin (Sigma), anti-CD107a and CD107b fluorescein isothiocyanate antibody, anti-CD28 antibody (clone 37.51, 1μg/ml) and anti-CD3 antibody (clone 145-2C11, 0.1μg/ml) for nonspecific stimulation, or 4μg/ml OTI and OTII-specific peptides (GenScript) for ova-specific stimulation.

Cells were washed in FACS buffer and surface-stained for 15 min with anti-CD8-pacific blue, anti-CD4-alexa fluor 700 and anti-CD45.2 or anti-Thy1.2 antibodies in 20% mouse serum (Gemini). The cells were then washed and fixed in 2% paraformaldehyde (Sigma). For intracellular staining, the cells were permeabilized in permabilization buffer and stained with

anti-IFN-γ-phycoerythrin-Cy7, anti-IL-2 Peridinin-chlorophyll Protein Complex (eBioscience) and anti-TNF-biotin. After washing, cells were stained with streptavidin-allophycocyanin-Cy7 antibody. An LSRII (BD Biosciences) was used to read the samples, and the data were analyzed using FlowJo software (Tree Star). All antibodies were purchased from BD Biosciences, unless otherwise stated.

5.2.6 *In vivo* cytotoxicity assay

To measure ova-specific CD8 T cell mediated cytotoxicity, uninfected splenocytes were labeled with CFSE (Molecular Probes) at 0.4mM or 5mM concentrations for 15 min at 37ºC. Addition of T cell medium quenched the reaction, after which the cells were washed in 20% FBS in PBS. $CFSE^{low}$ cells were pulsed for 1 hour with 20ug/ml SIINFEKL peptide (for ova-specific killing) or LCMV gp33-41 peptide (for controls) in PBS. $CFSE^{high}$ cells were left unpulsed.

$CFSE^{high}$ and $CFSE^{low}$ cells were mixed at a ratio of 1:1, and approximately $5*10^7$ cells injected by tail-vein injection into recipient mice. 18 hours later, mice were sacrificed and organs homogenized. The cells were washed in FACS buffer and fixed in 4% paraformaldehyde.

A FACSCalibur (BD Biosciences) and FlowJo software were used to read and analyze the samples, respectively.

%ova-specific lysis=100– 100*([$\%CFSE^{ovalow}$/$\%CFSE^{high}$]/[$\%CFSE^{LCMVlow}$/$\%CFSE^{high}$]).

5.2.7 Priming of cells *in vitro*

OTI and OTII cells were purified from naive transgenic mice on the RAG1-/- background using MACS cell separation technology, as described above. $5*10^4$ cells were incubated with

tissue homogenates from infected mice. To obtain sufficient numbers, tissue homogenates from up to six mice were pooled, as needed. The cells were incubated for 5 days in T cell medium, without addition of any cytokines or antibiotics, after which they were stimulated and processed, as described in the flow cytometry section.

5.2.8 Graphing and statistical analysis

GraphPad Prism 4.01 (GraphPad Software) and SPICE 4.1.5 (developed by Mario Roederer, Vaccine Research Center, NIAID, NIH) were used for graphing and statistical analysis. Unpaired two-sided *t* test was used for parametric two-group analyses, while Mann-Whitney *t* test was used for statistical analysis of bacterial load. ANOVA with Bonferroni post-test was used for analysis of three and more groups. P values of <0.05 (*), <0.01 (**) and <0.001 (***) were considered significant, highly significant and extremely significant, respectively.

5.3 RESULTS

5.3.1 Expansion and contraction of cells present before infection

To follow the kinetics of the expansion and contraction of *M. tuberculosis*-specific cells, we adoptively transferred naive ova-specific cells from OTI and OTII mice to wild-type recipient mice prior to infection with *M. tuberculosis*-ova. To later identify the cells, the donor mice had a different congenic background (CD45.2 or Thy1.2) from the recipient mice (CD45.1 or Thy1.1). While host T cells can be renewed by emigration of naive T cells from the thymus, the

adoptively transferred cells do not have a source of renewal. Thus, we can follow their responses, from expansion to contraction.

The OTI and OTII cells started to expand about two weeks post-infection and peaked around three to four weeks post-infection (Figure 20A and B, bars). Both OTI and OTII cells had contracted by six weeks post-infection, leaving a small fraction of the cells behind. There were significantly more OTI cells remaining than OTII cells in the lungs at six weeks post-transfer. The overall host CD4 and CD8 T cell responses continued to expand (Figure 20A and B, lines) in the lungs, while the cell numbers reached a relatively stable level in the lymph nodes. The data indicate that CD4 and CD8 T cell responses to *M. tuberculosis* are composed of overlapping waves of cells. Thus, as the infection went through different phases, cells were continuously being primed in different environments. In early infection, inflammation was low (Figure 21A and B) and there were low amounts of bacterial antigen. As the infection progressed, the bacterial load peaked at about three weeks post-infection (Figure 20C and D) and inflammation increased (Figure 21A and B). Chemokine expression increased as well (Figure 21C and D), which helped to recruit cells to the tissues, while there was an increase (IL-6 and IL-13) or decrease (IL-10) in the expression of anti-inflammatory cytokines and cytokines that skew the T cell response towards a Th2 response (Figure 21E and F). Further progression of infection resulted in more inflammation and expression of chemokines, and the amount of bacterial antigen was likely to be further increased despite control of bacterial expansion, as the clearing of mycobacterial antigens is slow [75].

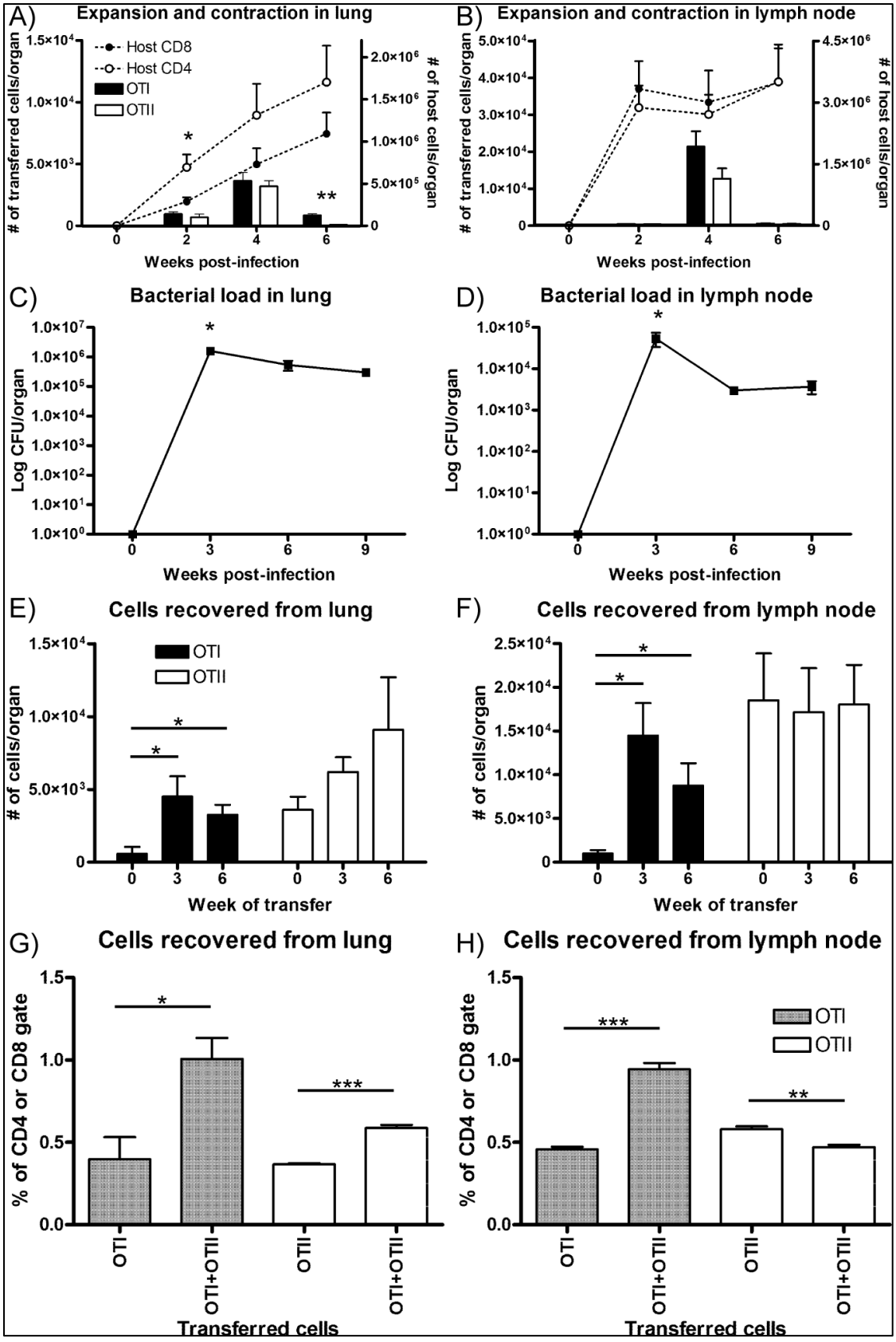

A)
Expansion and contraction in lung
Host CD8
Host CD4
OTI
OTII
# of transferred cells/organ
# of host cells/organ
Weeks post-infection
B)
Expansion and contraction in lymph node
C)
Bacterial load in lung
Log CFU/organ
D)
Bacterial load in lymph node
E)
Cells recovered from lung
# of cells/organ
Week of transfer
F)
Cells recovered from lymph node
G)
Cells recovered from lung
% of CD4 or CD8 gate
OTI
OTI+OTII
OTII
OTI+OTII
Transferred cells
H)
Cells recovered from lymph node

Figure 20 Expansion and contraction of adoptively transferred OTI and OTII cells in different environments.

(A and B) Expansion and contraction of OTI (black bars) and OTII (white bars) cells, adoptively transferred before infection, in the lungs (A) and lymph nodes (B). For comparison, host CD8 (black circles) and CD4 (white circles) T cell responses are shown in lines, with the right-hand scale. There were significantly more OTI cells remaining at six weeks post-infection in the lung than OTII cells ($P<0.002$), and host CD4 T cells responded faster than CD8 T cells ($P<0.04$, week 2 post-infection). (C and D) Live bacterial load peaked at three weeks post-infection in both the lungs (C) and lymph nodes (D) ($P<0.05$ and $P<0.03$, respectively). (E and F) Recovery of OTI and OTII cells adoptively transferred in at different times post-infection from the lungs (E) and lymph nodes (F). OTI cells (black bars) transferred in at three weeks post-infection expanded significantly more than OTI cells transferred in at other times ($P<0.04$ in lungs, $P<0.02$ in lymph nodes), while there was no significant difference in the frequency of OTII cells (white bars). (G and H) Recovery of OTI (grey bars) and OTII (white bars) cells, transferred together or separately at three weeks post-infection. OTI cells expanded significantly more in both the lungs (G, $P<0.03$) and lymph nodes (H, $P<0.0003$) when they were transferred with OTII cells, while OTII cells expanded more in the lungs ($P< 0.0005$) and less in the lymph nodes ($P<0.009$) when they were transferred with OTI cells. Data are the means ± standard errors of the means from test results for four mice per time-point.

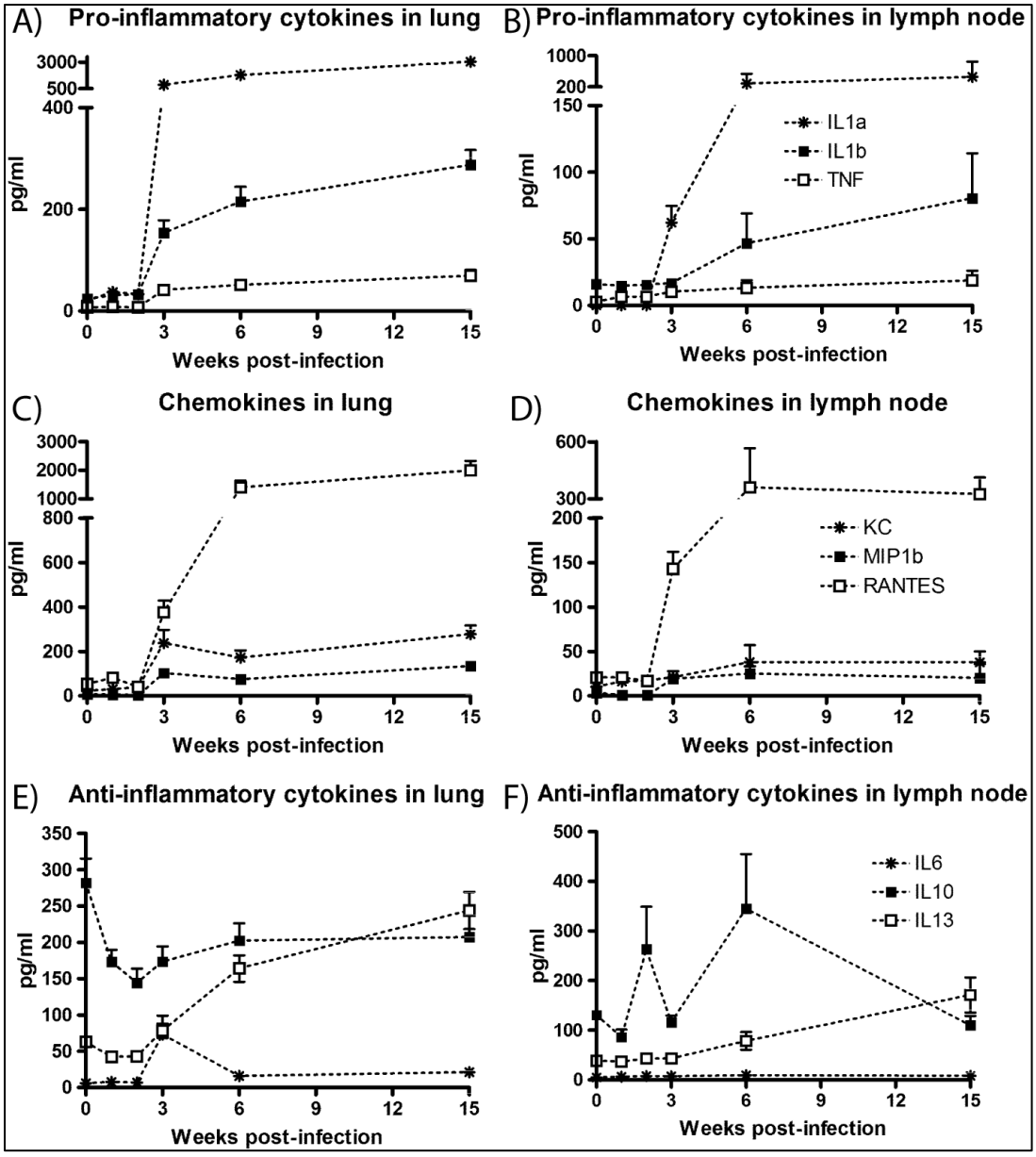


Figure 21 Cytokines and chemokines in infected organs.

Protein levels of pro-inflammatory cytokines (A and B), chemokines (C and D) and anti-inflammatory cytokines and Th2 cytokines (E and F) in tissue supernatants from the lungs (A, C and E) and lymph nodes (B, D and F), measured by Luminex. Results are shown for cytokines and chemokines with at minimum of 3-fold difference in levels between uninfected tissues and infected tissues. Data are the means ± standard errors of the means from test results for four mice per time-point.

5.3.2 Expansion and contraction of cells transferred after infection

We next examined expansion of cells that were adoptively transferred into mice at different times post-infection and recovered three weeks later. OTI cells transferred at three weeks post-infection expanded more than cells transferred at other times post-infection, as more cells were recovered from both lung and lymph node from this group (Figure 20E and F, black bars). Conversely, more OTII cells were recovered from lungs when cells were transferred later in infection, while there was no difference in numbers recovered from lymph nodes regardless of the timing of the transfer (Figure 20E and F, white bars). To examine whether OTI cell expansion was influenced by the presence of OTII cells, we transferred OTI and OTII cells, alone or together, at three weeks post-infection and examined the cell recovery (Figure 20G and H). OTI cells expanded significantly more when they were transferred with OTII cells, as more cells were recovered from both lung and lymph node. More OTII cells were recovered from lung when the cells were transferred with OTI cells, while fewer cells were recovered from the lymph node. This indicates that there is significant interaction between the cells, which influences their expansion.

5.3.3 Function of OTI cells

As we have shown previously [103], very few *M. tuberculosis*-specific CD8 T cells are able to both secrete IFN-γ and be cytotoxic. Instead, the cells are limited to either function, with the majority skewed towards cytotoxicity rather than secretion of IFN-γ. Cytotoxicity is indicated by surface expression of CD107, a lysosomal protein that is transiently exposed to the surface when lytic granules are released from the cell. To examine whether the phase of infection

influences the function of CD8 T cells, we compared the function of adoptively transferred OTI cells ("AT CD8"), transferred at different times post-infection, to endogenous ova-specific CD8 T cell responses (Figure 22, "Host CD8"). Their function was examined three weeks after transfer, when the cells should have responded (as demonstrated in Figure 20A and B).

As expected, host CD8 T cells were primarily cytotoxic (blue), with IFN-γ production (green) peaking early, at three weeks post-infection (first column of pie charts, Figure 22A and B). Very few cells were able to be both cytotoxic and secrete IFN-γ (red). OTI cells transferred before infection were almost exclusively cytotoxic (Figure 22C and D, first column of pie charts). When OTI cells were transferred after the peak of infection (six or fifteen weeks post-infection), significantly more cells were skewed towards IFN-γ production than in the host CD8 T cell response. As before, few, if any, cells were able to be both cytotoxic and secrete IFN-γ. The exception to this was cells adoptively transferred at three weeks post-infection (second column of pie charts), where unexpectedly some of the cells expressed both functions in the lungs and lymph nodes.

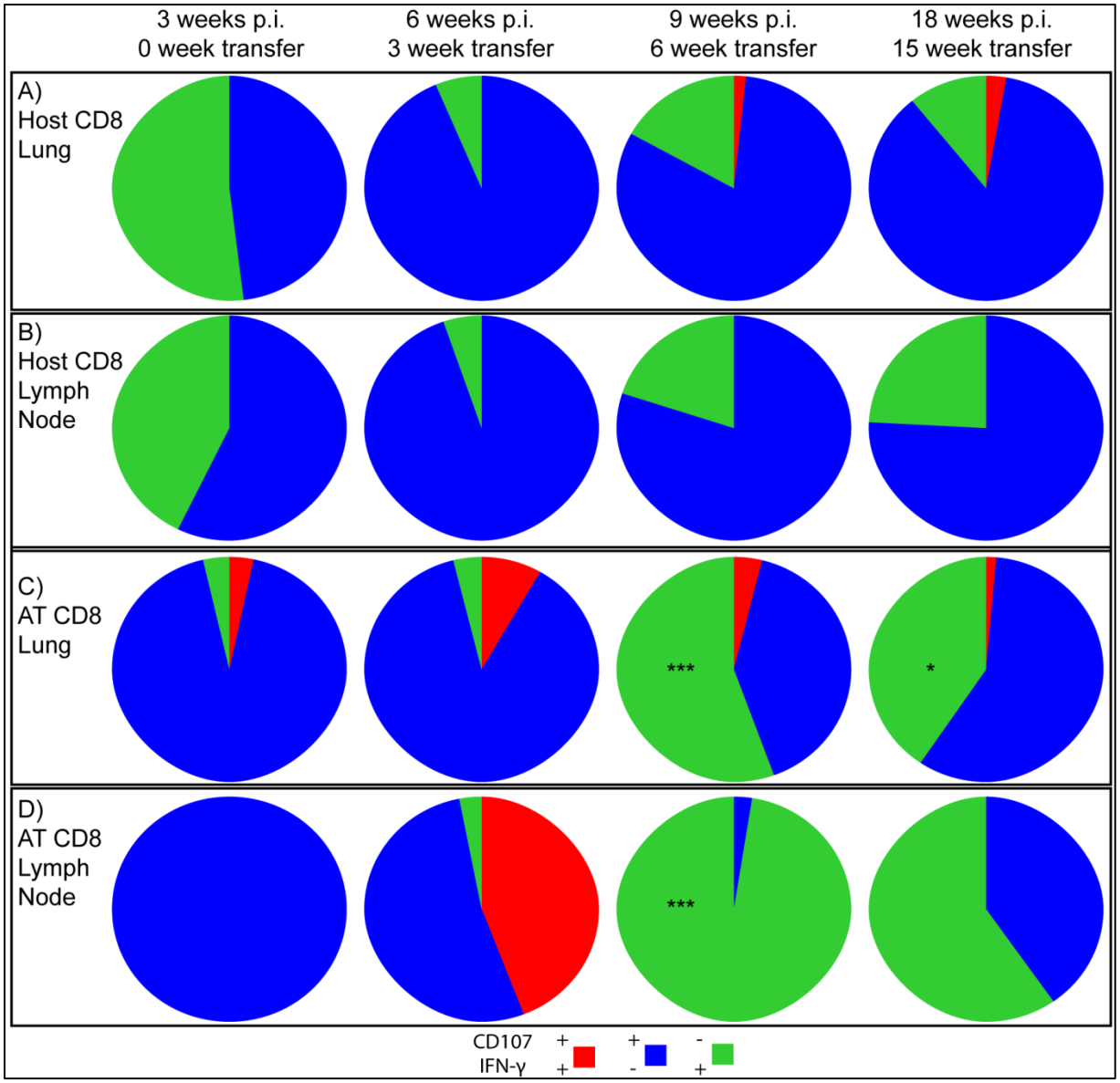


Figure 22 Cytotoxicity and secretion of IFN-γ.
Surface expression of CD107, indicating cytotoxicity, and secretion of IFN-γ by host ova-specific CD8 T cells ("Host CD8) in the lungs (A) and lymph nodes (B), as well as OTI cells ("AT CD8", C and D), adoptively transferred at different times post-infection. $5*10^3$ OTI and OTII cells were adoptively transferred at 0, 3, 6 or 15 weeks post-infection, and were harvested 3 weeks later. Green slices show cells that secreted IFN-γ, blue slices show cells that degranulated, and red slices show cells capable of both functions. OTI cells secreted significantly more IFN-γ than host CD8 responses when the cells were adoptively transferred at six and fifteen weeks post-infection ($P<0.0003$ and $P<0.01$ in the lungs, respectively, and $P<0.0009$ at week 6 in the lymph nodes). Significantly more cells were able to both secrete IFN-γ and degranulate when cells were transferred at three weeks post-infection (lymph node, $P<0.01$ AT vs Host, and $P<0.01$ week three post-infection AT vs other weeks AT). Data are the means from test results for four mice per time-point.

Cells which are able to simultaneously secrete IFN-γ, IL-2 and TNF (multifunctional cells) have been shown to correlate with increased protection in other infection models, and to secrete higher levels of cytokines than cells with fewer functions [82, 227]. As *M. tuberculosis*-specific CD8 T cells can secrete all three cytokines, we examined the multifunctionality of the CD8 T cells (Figure 23). Our results indicate that very few host CD8 T cells are multifunctional (red), while OTI cells adoptively transferred in at three weeks post-infection or later can develop into multifunctional cells. Interestingly, all multifunctional cells transferred in at three weeks post-infection degranulated as well (data not shown).

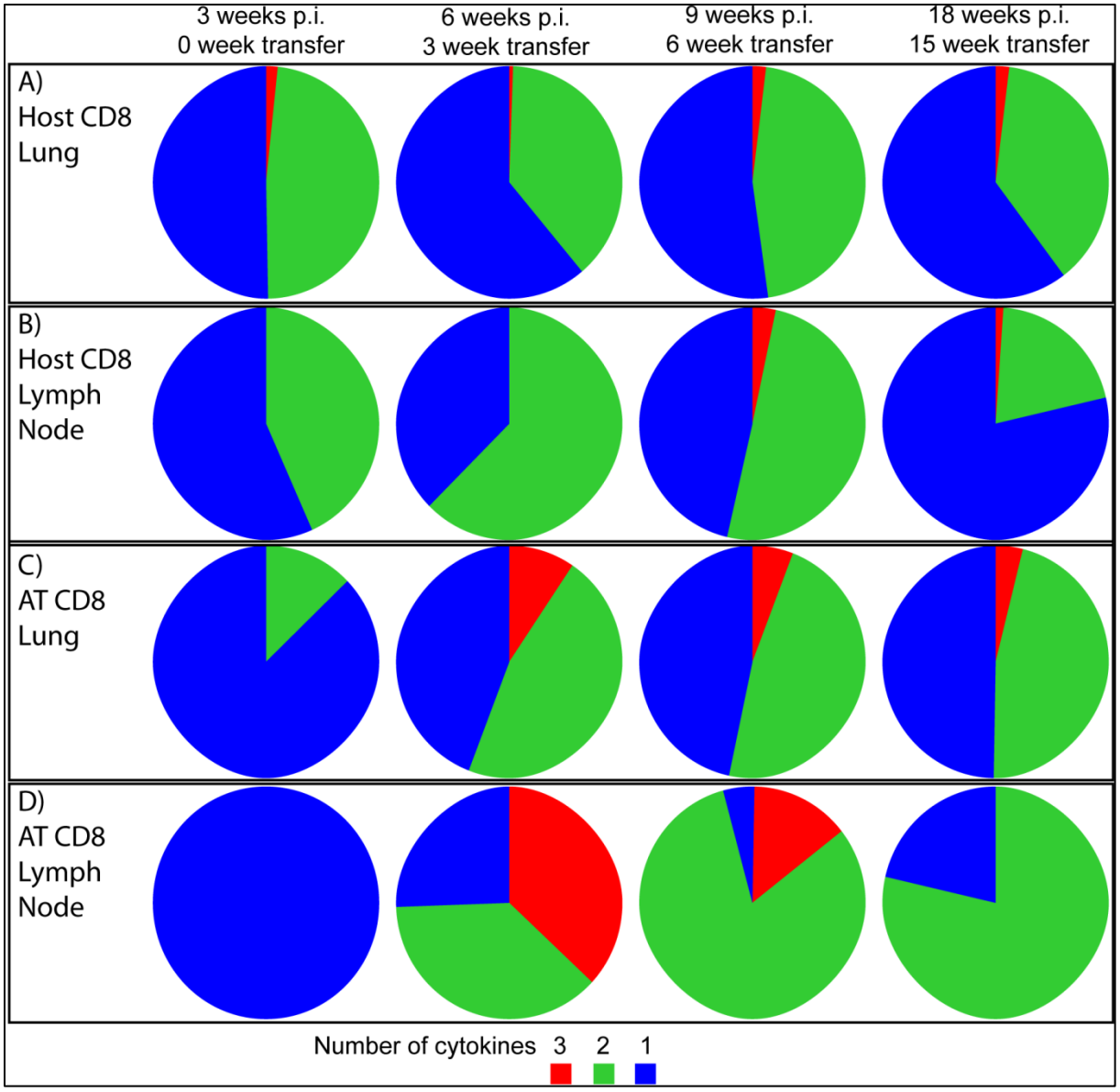


Figure 23 Multifunctional OTI cells.

Number of functions carried out by host CD8 T cells ("Host CD8) in lung (A) and lymph node (B) at different times post-infection, as well as the functions of adoptively transferred OTI cells ("AT CD8", C and D), adoptively transferred at different times post-infection. $5*10^3$ OTI and OTII cells were adoptively transferred at 0, 3, 6 or 15 weeks post-infection, and were harvested 3 weeks later. Blue slices show cells secreting one cytokine (IFN-γ, TNF or IL-2), green slices show cells that secrete two cytokines, and red slices show cells secreting all three cytokines. Cells transferred at week three post-infection were significantly more multifunctional than host cells ($P<0.05$ in lung and $P<0.01$ in lymph node). Data are the means from test results for four mice per time-point.

When we examined the geometric mean fluorescence intensity (GMFI) of cytokines produced by the cells (Figure 24), the multifunctional cells (black bars) expressed equal or higher levels of cytokines compared to other groups of cells. The largest difference was in the lymph nodes, where multifunctional cells expressed significantly more IFN-γ and TNF. Multifunctional cells in the lungs expressed significantly more TNF, but similar amounts of IFN-γ and IL-2 compared to other cell groups.

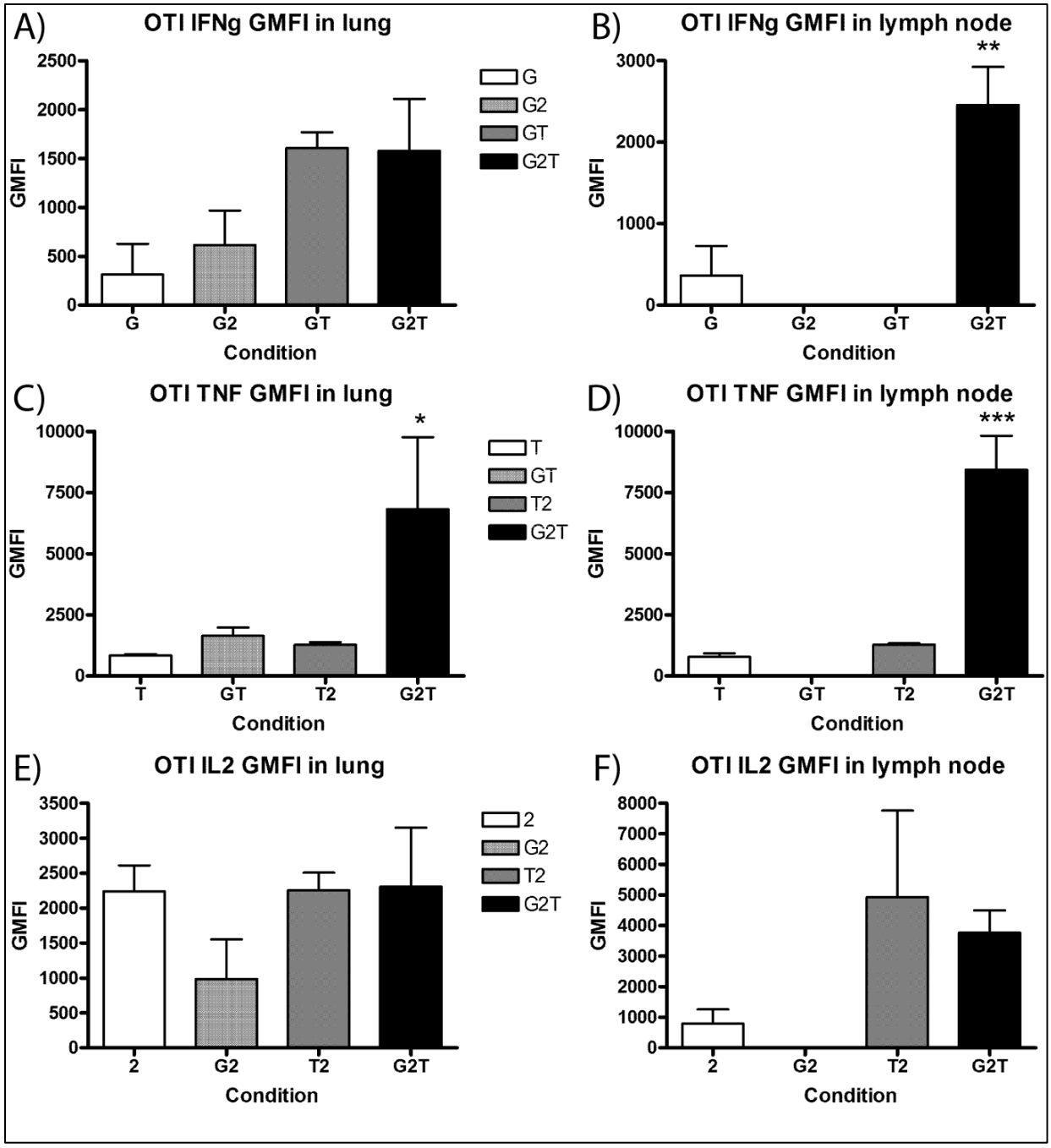


Figure 24 Mean fluorescence intensity of cytokines secreted by OTI cells.
Geometric mean fluorescence intensity (GMFI) of IFN-γ (A and B), TNF (C and D) and IL-2 (E and F) in cells making one cytokine (white bars), two cytokines (light grey and dark grey bars) or three cytokines (black bars) in the lungs (A, C and D) and lymph nodes (B, D and F). G is IFN-γ, 2 is IL-2 and T is TNF. IFN-γ+IL-2+TNF+ ("G2T") OTI cells made significantly more IFN-γ ($P<0.002$ in lymph nodes,) and TNF ($P<0.05$ in lungs and $P<0.0001$ in lymph nodes) than other groups. Data are the means ± standard errors of the means from test results for four mice per time-point.

5.3.4 Function of IFN-γ+CD107+ OTI cells

As transfer of OTII cells influences the expansion of OTI cells (Figure 20G and H), we next examined whether OTII cells influence the function of OTI cells as well. To do this, we adoptively transferred in OTI cells, with or without OTII cells, at three weeks post-infection and examined their ability to be cytotoxic and secrete IFN-γ (Figure 25).

There were significantly more OTI cells capable of being cytotoxic and secreting IFN-γ when OTI and OTII cells were transferred together (Figure 25A and B). As TB is primarily a lung disease, we further examined the function of the cells in the lungs. The amount of IFN-γ produced on a per-cell basis was higher in IFN-γ+CD107+ cells (black bars) than in IFN-γ+ only cells (white bars) in both the lungs (Figure 25C) and lymph nodes (data not shown). Furthermore, the IFN-γ+CD107+ cells that developed when OTI and OTII cells were transferred together secreted more IFN-γ than cells developed when OTI cells were transferred alone. This did not hold true for lymph nodes, where OTI cells transferred without OTII cells made significantly more IFN-γ (data not shown).

To study cytotoxicity of the cells in the lungs, we first examined the amount of CD107 surface expression on a per-cell basis, as an indicator of how much the cells had degranulated during the stimulation period. Surprisingly, IFN-γ+CD107+ cells (black bars) degranulated less than CD107+ only cells (white bars) in the lungs (Figure 25D). The difference was less in lymph nodes, where degranulation and cytotoxicity are minor compared to the lungs (data not shown). Using the *in vivo* cytotoxicity assay, we measured CD8 T cell-mediated killing of target cells in mice that had received OTI cells, OTI and OTII cells, or had received a mock-transfer (PBS). Transfer of OTI cells alone increased cytotoxicity slightly compared to the mock-transferred group, while transfer of OTI and OTII cells, which is the condition that induces IFN-γ+CD107+

cells, decreased cytotoxicity in all organs tested (Figure 25E and data not shown for lymph nodes and spleen). The data indicate that IFN-γ+CD107+ cells secrete more IFN-γ but are less cytotoxic than cells limited to either function. To examine whether this had a significant effect on the control of *M. tuberculosis* in the mice, we compared the bacterial load of the different groups of mice. There was no significant effect on the bacterial load in any examined organ in any of the groups when we examined them three weeks after transfer (Figure 25F, lung data shown) or six weeks after transfer (data not shown).

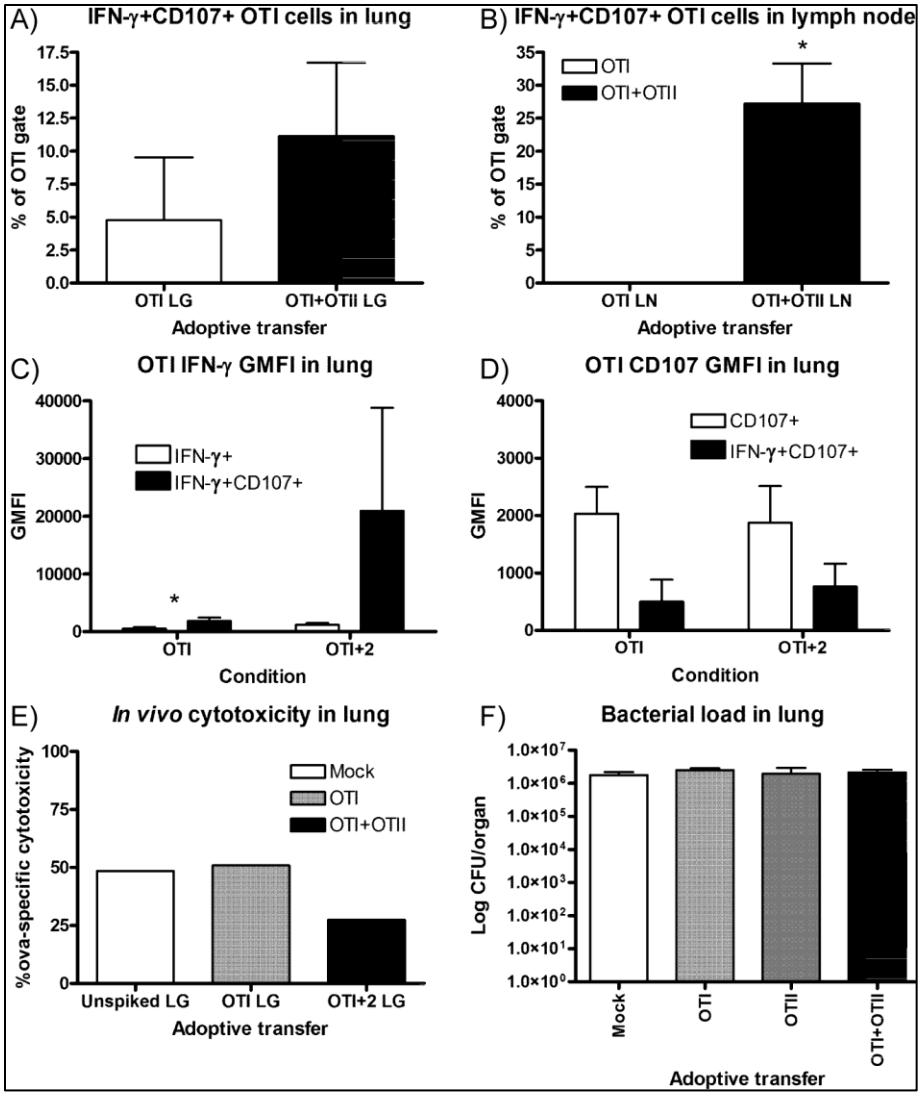


Figure 25 Effect of OTII cells on OTI cell function.
(A and B) Development of IFN-γ+CD107+ OTI cells in the lungs (A) and lymph nodes (B) when OTI cells were transferred with or without OTII cells. There were significantly more IFN-γ+CD107+ OTI cells in the lymph nodes when OTII cells were transferred as well ($P<0.01$). (C and D) Geometric mean fluorescence of cells with one (white bars) or both (black bars) functions in the lungs. (C) is the geometric mean fluorescence intensity of IFN-γ, while (D) is the geometric mean fluorescence of CD107. IFN-γ+CD107+ OTI cells made significantly more IFN-γ+ than cells limited to secretion of IFN-γ+ when OTI cells were transferred alone ($P<0.05$ in lungs). Data are the means ± standard errors of the means from test results for three mice per time-point. (E) Killing of target cells at six weeks post-infection in mice who received OTI cells (grey bars), OTI and OTII cells (black bars) or PBS (white bars) at three weeks post-infection. Data are the means from test results for three mice per group. (F) Bacterial load in the lungs at six weeks post-infection in mice who received OTI (light grey bars), OTII (dark grey bars), OTI and OTII (black bars), or PBS (white bars) at three weeks post-infection. Data are the means ± standard errors of the means from test results for three mice per time-point.

5.3.5 Function of OTI cells primed *in vitro*

To examine whether we could prime IFN-γ+CD107+ OTI cells *in vitro*, we incubated naive OTI cells with tissue homogenates from mice that had been infected for eleven days, three weeks, six weeks, eight weeks or were uninfected. Lung homogenates were able to prime IFN-γ+CD107+ OTI cells, while lymph node homogenates did not (data not shown). No correlation could be found between the cytokine environment and priming of IFN-γ+CD107+ cells, using cytokine data from tissue homogenates *in vivo* (Figure 21) and from *in vitro* culture supernatants (data not shown). Furthermore, priming of IFN-γ+CD107+ OTI cells did not appear to be dependent on CD4 T cell help (data not shown).

5.3.6 Function of OTII cells

We next examined the secretion of IFN-γ, IL-2 and TNF by OTII cells, transferred with OTI cells (Figure 26, white bars) or alone (grey bars), and compared them to host CD4 T cell responses (black bars, mock transferred). As CD4 T cell-mediated IFN-γ secretion is essential for control of *M. tuberculosis* [79], we gated on IFN-γ positive cells and examined the expression of IL-2 and TNF within this gate.

The peak of IFN-γ expression in mice is around three to four weeks post-infection [80, 103]. The cytokine production by OTII cells transferred in before infection was similar to that of host CD4 T cells in both the lungs and lymph nodes when function was examined at three weeks post-infection (Figure 26A and B). However, when cells were transferred in at three weeks post-infection, OTII cells secreted significantly more IFN-γ than the host CD4 T cells at six weeks post-infection, in both the lungs and lymph nodes (Figure 26C and D). The frequency of

multifunctional OTII cells was similar to the host CD4 cells when OTII cells were transferred before infection (Figure 26A and B). Conversely, a higher percent of OTII cells were multifunctional than host CD4 T cells when cells were transferred at three weeks post-infection (Figure 26C). Interestingly, transferring OTII cells with OTI cells caused a significant decrease in multifunctional OTII cells.

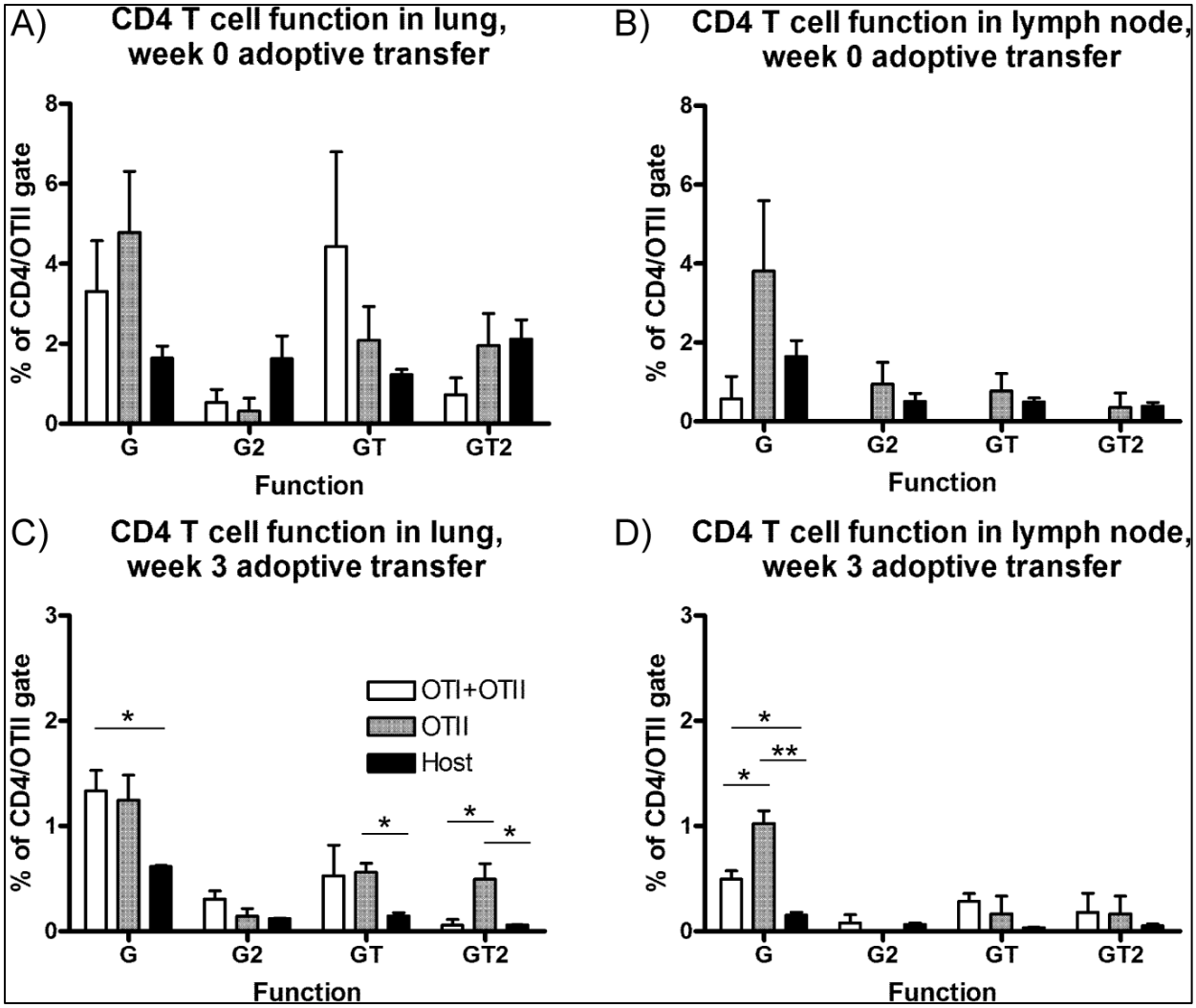


Figure 26 Function of OTII cells.
OTII cells, transferred with (white bars) or without (grey bars) OTI cells. Host CD4 T cells from mock-transferred mice are shown for comparison (black bars). The data show cells who secrete IFN-γ ("G"), with or without IL-2 ("2") and TNF ("T). (A and B) are cells transferred before infection, and harvested at three weeks post-infection while (C and D) are cells transferred three weeks post-infection, and harvested at six weeks post-infection. OTII cells transferred with OTI cells at three weeks post-infection secreted significantly more IFN-γ alone (G) compared to the host CD4 T cell responses ($P<0.02$ in lungs and $P<0.02$ in lymph nodes), but less IFN-γ alone ($P<0.02$ in lymph nodes) and IFN-γ, IL-2 and TNF (G2T, $P<0.05$ in lungs) than cells transferred without OTI cells. OTII cells transferred alone at three weeks post-infection secreted significantly more IFN-γ ($P<0.002$ in lymph nodes), IFN-γ and TNF (GT, $P<0.01$ in lungs) and IFN-γ, IL-2 and TNF ($P<0.04$ in lungs) than host CD4 T cells. Data are the means ± standard errors of the means from test results for three mice per time-point.

When we compared the amount of cytokine produced, OTII cells transferred in at three weeks post-infection secreted higher levels of IFN-γ and TNF than OTII cells transferred in before infection (Figure 27A and B) in the lungs. There was no significant difference in IL-2 secretion between the groups (Figure 27C). OTII cells transferred without OTI cells secreted more cytokines than cells transferred with OTI cells, of which multifunctional cells produced the highest amounts (green fragment). The same did not hold true for the lymph nodes (Figure 27D to F), where cells transferred before infection secreted higher levels of cytokines than cells transferred at three weeks post-infection. Cells transferred without OTI cells made significantly more IFN-γ and IL-2, but the highest amount of cytokines came from cells other than multifunctional cells (red, white and black).

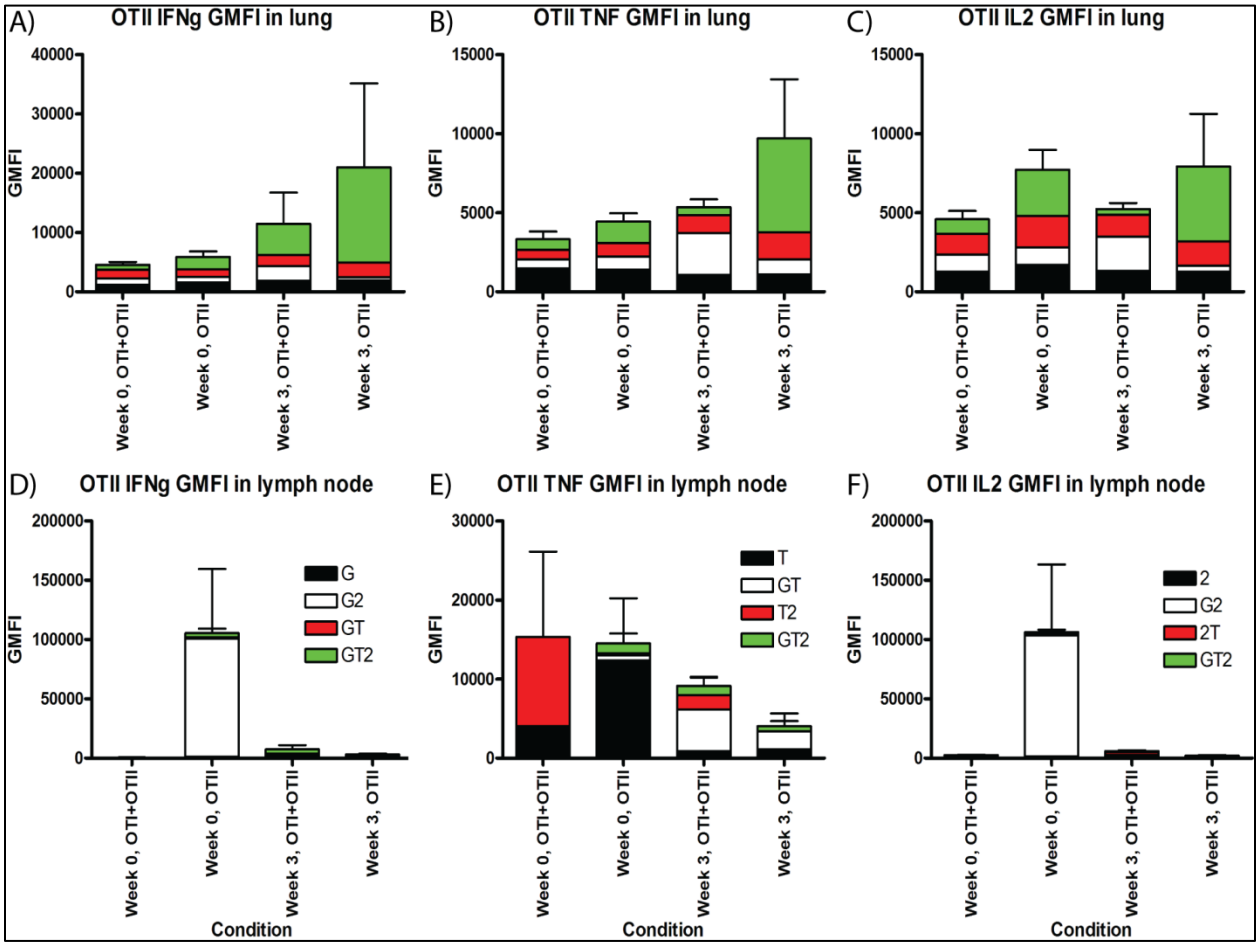


Figure 27 Geometric mean fluorescence intensity of cytokines secreted by OTII cells.
Geometric mean fluorescence intensity (GMFI) of IFN-γ ("G", A and D), TNF ("T", B and E) and IL-2 ("2", C and F) in OTII cells expressing one cytokine (black), two cytokines (white and red) or three cytokines (green) in the lungs (A to C) and lymph nodes (D to F). OTII cells were adoptively transferred, with or without OTI cells, either before infection or at three weeks post-infection, and were harvested three weeks later. Data are the means ± standard errors of the means from test results for four mice per time-point.

5.4 DISCUSSION

This study examined the effect of the environment on the functional phenotype of CD4 and CD8 T cells during *M. tuberculosis* infection in mice. To study this, we infected mice with *M. tuberculosis*-ova, a recombinant strain that expresses ovalbumin epitopes which are recognized by T cell receptor transgenic CD4 (OTII) and CD8 (OTI) T cells, as well as by non-transgenic T cells (host cells) [103]. Adoptive transfer of naive OTI and OTII cells into infected mice allowed us to examine the quantity and quality of the T cell response that developed. By transferring cells during different phases of infection, we show that the environment that the cells develop in can alter the functional profile of the cells.

The role of CD4 T cells in controlling *M. tuberculosis* infection has been firmly established [79], while the role of CD8 T cells is less understood. CD8 T cells play a much smaller role in the mouse model of TB compared to nonhuman primates [104]. There are several possible reasons for this disparity, e.g. the murine lack of granulysin, a cytotoxic molecule that can kill *M. tuberculosis*. Thus it has been difficult using the mouse model of TB to establish which CD8 T cell functions are important in controlling *M. tuberculosis* bacterial growth or disease. However, Woodworth et al. [112] showed that CD8 T cell-mediated cytotoxicity appears to be an important function, as the bacterial load is higher in mice lacking cytotoxic molecules. CD8 T cell mediated IFN-γ secretion is also likely to be important, as CD8 T cells can compensate for the lack of IFN-γ in CD4 deficient mice by increasing their production, although this is not sufficient to save the mice [79]. We showed (chapter 3) that cytotoxicity and secretion of IFN-γ are carried out by distinct CD8 T cells during *M. tuberculosis* infection. The majority of the CD8 T cells are cytotoxic (CD107+), with IFN-γ secretion peaking at three to four weeks post-infection, after which CD8 T cell-mediated IFN-γ secretion is insignificant

[103]. When OTI cells were adoptively transferred prior to infection, the cells developed an almost exclusively cytotoxic phenotype. When cells were transferred into chronic infection, i.e. after bacterial expansion had been contained, significantly more OTI cells secreted IFN-γ than was observed in the host ova-specific CD8 T cell population. Interestingly, OTI cells that were adoptively transferred at the peak of infection, at three weeks post-infection, could both secrete IFN-γ and be cytotoxic. The IFN-γ+CD107+ cells were also multifunctional, i.e. they secreted all three cytokines examined, while very few host CD8 T cells were multifunctional. As has been shown in other infections, the multifunctional cells secreted higher levels of IFN-γ. However, the ability to secrete IFN-γ seems to impair the ability of the cells to be cytotoxic, as the cells degranulated less and were less cytotoxic *in vivo*.

New CD8 T cells are presumably continuously primed during the infection, rather than cells being primed early in infection and expanding as necessary thereafter. This is supported by work published by Hoang et al. [102], who recently described an *M. tuberculosis*-specific CD8 T cell population that does not expand until late in infection. Thus, while both adoptively transferred OTI cells and host ova-specific CD8 T cells were presumably primed in the chronic phase of infection, their functional profiles differed. This disparity may be due to priming in different organs. The *in vitro* data indicate that CD4 and CD8 T cells can be primed in the lungs, as has been suggested by others [247], although this is a very artificial system where cells are in close proximity for extended periods of time and there is no influx of new cells. While lung homogenates were able to prime multifunctional OTI cells, the lymph node homogenates were not. This indicates that adoptively transferred cells may be partly or fully primed in the lungs, while host T cell responses are primed in the lymph nodes. To establish where the cells are primed, it will be important to identify better markers of priming. We have not found CD69, a

commonly used early activation marker [70, 71], to be a reliable marker of priming (unpublished data). Nor have we found proliferation to be useful, as the cells can be primed in one location and expand in another. Simultaneous transfer of OTI and OTII cells improved OTI cell function and expansion, which indicates that one of the limiting factors in CD8 T cell responses to *M. tuberculosis* may be the quality of the CD4 T cell help. CD4 T cell help was not required, however, when OTI cells were primed *in vitro*. This indicates that one function of CD4 T cells during *M. tuberculosis* infection is to recruit CD8 T cells to DC [248]; a function that is not required when cells are primed *in vitro*.

As *M. tuberculosis*-ova induces weak host ova-specific CD4 T cell responses, we compared OTII responses to global CD4 T cell responses induced in the host. Host CD4 T cell responses were stronger at three weeks post-infection than at later time-points. This correlates with a peak in IFN-γ production in infected mice, as CD4 T cells are a major source of IFN-γ. There were no significant differences between OTII cells that were transferred before infection and the host CD4 T cell response. When OTII cells were transferred at three weeks post-infection, significantly more OTII cells secreted IFN-γ than host CD4 T cells, and there were significantly more multifunctional cells in the lungs. OTII cells transferred at three weeks post-infection secreted more cytokines on a per-cell basis in the lungs than cells transferred before infection, the majority of which came from multifunctional cells, and the cells expanded more. This did not hold true for OTII cells in the lymph nodes, where OTII cells transferred at three weeks post-infection expanded less, and more cytokines were made by cells transferred before infection, most of which came from cells with two functions rather than multifunctional cells. Due to the discrepancy in OTII cell behavior between the lungs and lymph nodes, which was not reproduced in OTI cells, it is unclear whether the focus of CD4 T cell priming should be on the

lung or the lymph node, as they both play an important role. The lymph node is a source of long-lived central memory cells which provide long-term protection, while the lung is the source of effector cells and effector memory cells, which are the first cells to respond during infection [249]. Simultaneous transfer of OTI and OTII cells appeared to be detrimental to OTII function. As OTI and OTII epitope expression and presentation is likely to be parallel, OTI cell-mediated cytotoxicity could kill the antigen presenting cells, thus limiting the availability of OTII antigens. Alternatively, the presence of OTI cells may directly suppress OTII function, or skew OTII function towards a function that was not examined in these studies.

Collectively, the data indicate that both the environment and the quality of the CD4 T cell help contribute to priming of CD8 T cells. The conditions for CD8 T cell priming are optimal at the peak of infection, resulting in more cellular expansion, more multifunctional cells and the ability to be both cytotoxic and secrete IFN-γ. Priming of CD4 T cells at the peak of infection can also increase the frequency of multifunctional CD4 T cells compared to host CD4 T cells. However, the presence of CD8 T cells appears to decrease the quality of the CD4 T cell response. The data indicate that the immune response to *M. tuberculosis* can be broadened to induce multifunctional CD4 and CD8 T cells, without manipulation of the bacterium. This experimental model may prove useful for development of TB vaccine strategies, by allowing dissection of how the environment influences the function of the cells.

6.0 QA-1 DOES NOT PLAY A SIGNIFICANT ROLE IN MURINE *M. TUBERCULOSIS* INFECTION.

6.1 INTRODUCTION

As discussed in Chapter 1.2, there are approximately 2 billion people chronically infected with *M. tuberculosis*, in spite of induction of apparently robust immune responses. At present, it is not known why the bacterium is able to persist. It has been hypothesized that the immune response is titrated, so as to contain the bacterial infection while limiting the damage incurred by the immune response [250, 251]. While this may be beneficial to the infected individual, it results in an enormous reservoir of people harboring bacteria who can reactivate and become infectious.

To examine possible mechanisms of *M. tuberculosis* persistence, we looked towards other infections where the immune response allows the microbe to chronically persist. Notably, ocular infection with herpes simplex virus-1 (HSV-1) causes a chronic infection in the peripheral nervous system, characterized by an active CD8 T cell response [252, 253]. Liu et al. showed that CD8 T cells control HSV-1 replication in the trigeminal ganglia by secreting IFN-γ, thus preventing the virus from killing the host cells [254]. Conversely, Suvas et al. showed that Qa-1 expression by infected cells suppressed their killing by CD8 T cells, thus allowing the virally

infected cells to persist. However, high levels of viral antigen can override this suppressive effect [252].

Qa-1 is a nonclassical MHC class I molecule that can present endogenous and exogenous peptides [255, 256]. The leader sequence of classical MHC class I molecules (Qdm) has optimal binding properties to Qa-1 [257, 258]. Qa-1 can be bound by NKG2 molecules on natural killer (NK) cells and CD8 T cells (Figure 28A). [259, 260] Of them, NKG2A is the predominant binding partner [257], sending a suppressive signal and preventing release of cytotoxic granules [261]. Qa-1 can also be bound by the T cell receptor in an antigen-specific manner (Figure 28B) [256, 262].

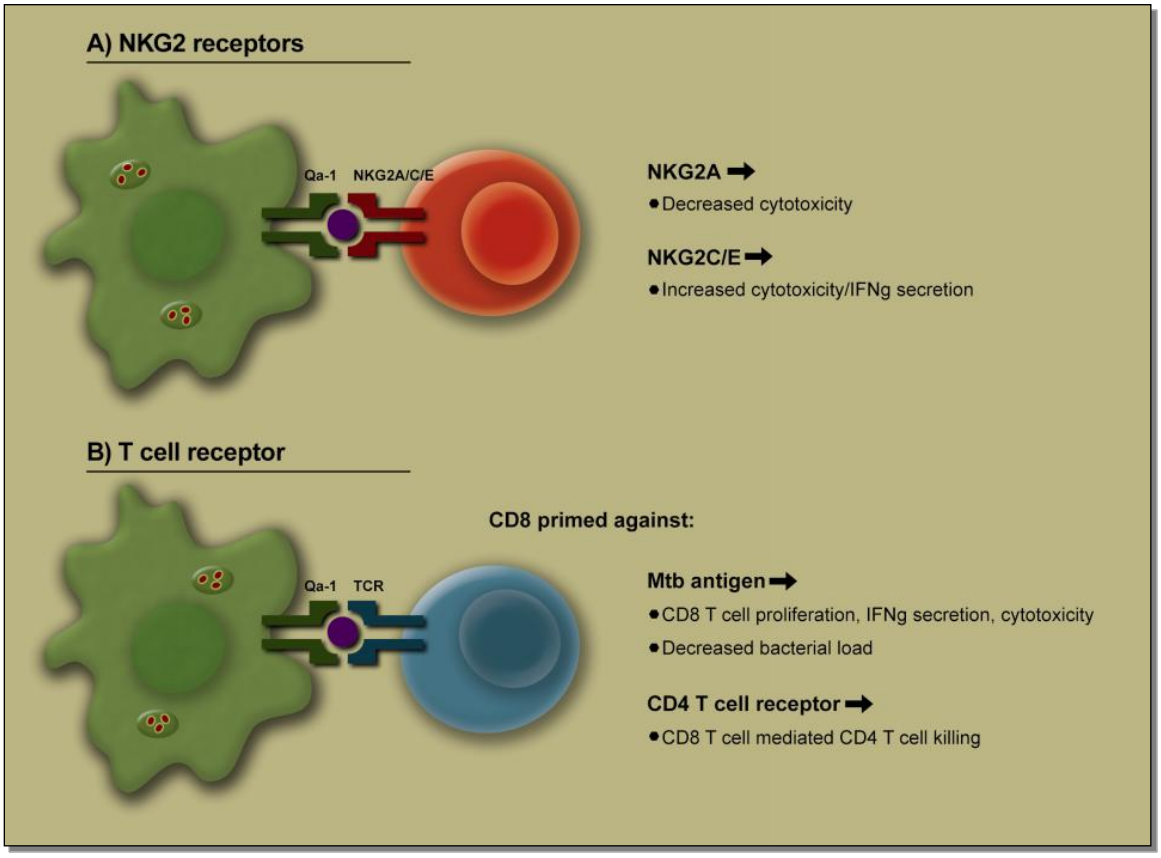


Figure 28 Qa-1 binding partners.
Qa-1 can bind A) NKG2 receptors or B) CD8 T cell receptor. The green cells depict monocyte-derived cells, infected with *M. tuberculosis* (red ovals). The red and blue cells depict CD8 T cells, whose function is suppressed or induced, respectively.

Presentation of peptides from the CD4 T cell receptor can lead to killing of CD4 T cells by CD4 T cell-specific CD8 T cells. This has been exploited in T cell vaccinations, where self-reactive CD4 T cells can be selectively killed by CD8 T cells, resolving diseases like experimental autoimmune encephalomyelitis [263]. Peptides from infectious agents can also be presented in Qa-1 (including *Salmonella typhimurium* [255] and *Listeria monocytogenes* [220]), or in the human homolog, HLA-E (including cytomegalovirus [264] and *M. tuberculosis* [265]). HLA-E-restricted *M. tuberculosis*-specific human CD8 T cell lines have been shown to be cytotoxic and secrete IFN-γ [265]. This indicates that murine Qa-1 may present *M. tuberculosis* antigens to CD8 T cells. This could either result in functional *M. tuberculosis*-specific CD8 T cells or give suppressive CD8 T cells, i.e. CD8 T cells reactive for CD4 T cells or cells receiving suppressive signals through the NKG2A receptor.

In this study, we hypothesized that Qa-1 plays a role in *M. tuberculosis* persistence by suppressing cytotoxicity through NKG2A association. To test this, we compared T cell responses and bacterial loads in WT mice and mice lacking Qa-1 (Qa-1-/-). Our results indicate that despite increased Qa-1 expression by DC and Mϕ following *M. tuberculosis* infection, expression of Qa-1 is neither beneficial nor detrimental to the mice.

6.2 MATERIALS AND METHODS

6.2.1 Mice and bacterial infections

Six to eight week old C57BL/6 mice were obtained from Jackson Laboratories. Robert L. Hendricks kindly provided a Qa-1-/- breeding pair, with the permission of Harvey Cantor. Qa-1-/- mice were bred at the University of Pittsburgh Biotechnology Center and kept in a BSL3 facility, under specific pathogen-free conditions. Mice were aerosol infected using nose-only exposure as in [36], with *M. tuberculosis* (Trudeau Institute). Whole lung homogenate was plated 24 hours post infection to determine the bacterial inoculum, estimated to be 2-10 colony forming units (CFU) per lung. 4 mice were used per group per time-point. Experiments were performed at least twice. The University of Pittsburgh Institutional Animal Care and Use Committee approved all protocols used in this study.

6.2.2 Harvesting cells from organs

Single-cell suspensions were obtained, as in [103]. Serial dilutions of organ homogenates were plated on 7H10 media agar (Difco) and cultured for 3 weeks at 37ºC 5% CO_2 to determine the bacterial load. The samples were processed and live cells counted, as in [103].

6.2.3 ELISpot assay

DC were cultured from bone-marrow [103] and were used to present antigen in ELISpot assays. DC were infected at a multiplicity of infection of 3 for 24 hours (to examine global *M.*

tuberculosis-specific responses), were pulsed with 4μg/ml peptide (GenScript) (to examine single antigen-specific responses) or were left uninfected and unpulsed (to measure background level IFN-γ secretion).

ELISpot plates (MultiScreen HTS 96-well, Millipore) were coated and cells co-cultured for 48 hours with DC, as in [103]. Detection of IFN-γ and counting of spots was performed as in [103].

6.2.4 Flow cytometry

For surface expression, cells were washed in FACS buffer. The cells were then stained in staining buffer (FACS buffer containing 20% mouse serum [Gemini]) with anti-CD11c-PE-Cy7, anti-CD11b-PerCP, anti-Gr-1-APC and anti-Qa-1-biotin followed by anti-strepavidin-APC-Cy7 antibody. Cells were then washed and fixed in 4% paraformaldehyde (Sigma).

For functional characterization, cells were incubated for 30 min at 4ºC with GAP (GAPINSATAM) tetramer-PE (Beckman Coulter) in T cell medium. The cells were then washed and incubated for 5 hours at 37ºC in T cell medium with 3μM monensin (Sigma) and anti-CD107a and b FITC antibody. Cells were nonspecifically stimulated with α-CD28 (clone 37.51, 1μg/ml) and anti-CD3 (clone 145-2C11, 0.1μg/ml) as well as specifically stimulated with 4μg/ml GAP peptide (Genscript). Cells were then washed in FACS buffer and stained in staining buffer with anti-CD8 PerCP for 15 minutes. The cells were then washed and fixed in 2% paraformaldehyde. Permeabilization buffer was used to permeabilize the cells, who were subsequently and stained with anti-IFN-γ-PE-Cy7.

FACS Aria (BD Biosciences) was used to read the samples, and data was analyzed with FlowJo (TreeStar) software. Antibodies were from BD Biosciences, unless otherwise stated.

6.2.5 *In vivo* cytotoxicity assay

Uninfected splenocytes were labeled with CFSE (Molecular Probes, Eugene, OR) and pulsed with peptide (GAP for antigen-specific killing, LCMV gp33-41 for controls), as in [103]. $CFSE^{high}$ and $CFSE^{low}$ cells were mixed at a ratio of 1:1 and approximately $5*10^7$ cells injected into mouse tail veins (4 mice/group/time-point). 18 hours later, mice were sacrificed and lung and spleen homogenates harvested. Cells were washed in FACS buffer and fixed in 4% paraformaldehyde. FACSCalibur (BD Biosciences) was used to read samples, and FlowJo software used to analyze the data.

$$\%\text{GAP-specific lysis} = 100 - 100*[(\%CFSE^{GAPlow}/\%CFSE^{high})/(\%CFSE^{LCMVlow}/\%CFSE^{high})]$$

6.2.6 Statistical analysis

Graphing and statistical analysis were performed using GraphPad Prism 4.01 (GraphPad Software). Unpaired two-sided t tests were used for two-group analysis. P values of <0.05 were considered significant (*).

6.2.7 Histology

Lung lobes were fixed in 10% normal buffered formalin and then embedded in paraffin. 5-6 µm sections were cut and stained with Harris' hematoxylin and eosin.

6.3 RESULTS

6.3.1 Qa-1 expression

Qa-1 expression increased on both DC (DC, CD11c+) and Mϕ (CD11c-CD11b+Gr-1-) in the lungs of WT mice following *M. tuberculosis* infection (Figure 29). This increase is likely due to increased availability of peptide suitable for presentation by Qa-1 [258]. However, it is unknown which peptides are presented following *M. tuberculosis* infection, and whether there are changes in peptide presentation over time.

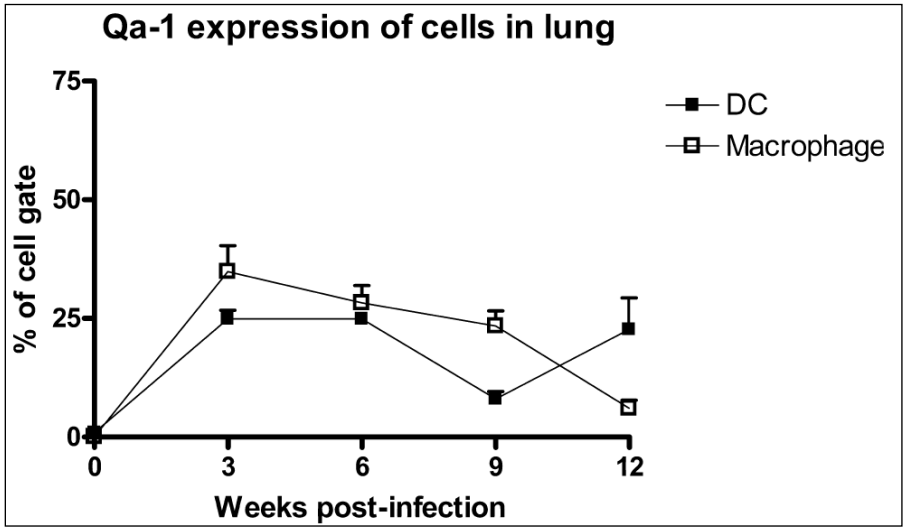


Figure 29 Qa-1 expression on DC and Mϕ following *M. tuberculosis* infection.
Data are mean ± standard error of mean (SEM) of 4 mice per time-point.

6.3.2 Role of Qa-1 in priming of CD8 T cells

T cells can be primed in the lymph node and then migrate to the lungs to fight the infection [251]. There was no significant difference in the number of cells recovered from WT and Qa-1-/- mice, except a single time-point in two of three experiments. In these experiments, there were significantly more cells in WT lymph node 6 weeks post-infection (Figure 30) or 9 weeks post-infection (data not shown), but in both cases this difference did not carry over to the lungs.

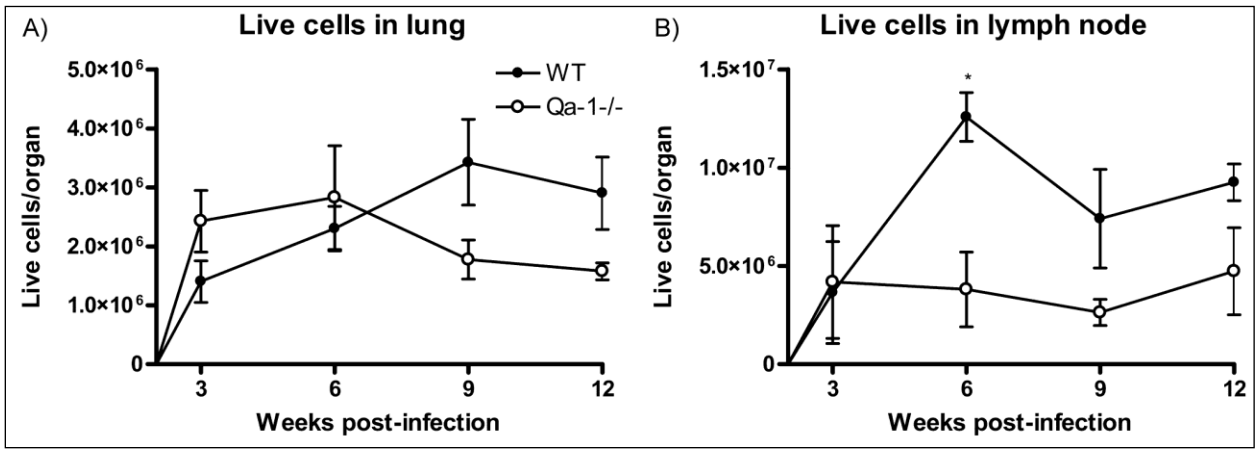


Figure 30 Live cells recovered from lung and lymph nodes of *M. tuberculosis* infected mice. There was no significant difference in the number of cells between WT and Qa-1-/- mice in A) lung, while there was a significant difference in B) lymph node at 6 weeks post-infection. Data are mean ± SEM of 4 mice per time-point.

There was no significant difference in the ratio of CD4 and CD8 T cells in the lymphocyte gate between WT and Qa-1-/- mice (Figure 31), except for CD4 T cells at 9 weeks post-infection and CD8 T cells at 3 weeks post-infection in the lungs. Additionally, upon repetition there was no significant difference in the lungs between the two mouse strains (data not shown). While a decreased CD4 T cell frequency and increased frequency of CD8 T cells in the lymphocyte gate could indicate that Qa-1 expression led to priming of CD8 T cells (either

CD4 T cell-specific or *M. tuberculosis* specific) in the WT mice, the brief duration of the difference would argue against a significant biological impact.

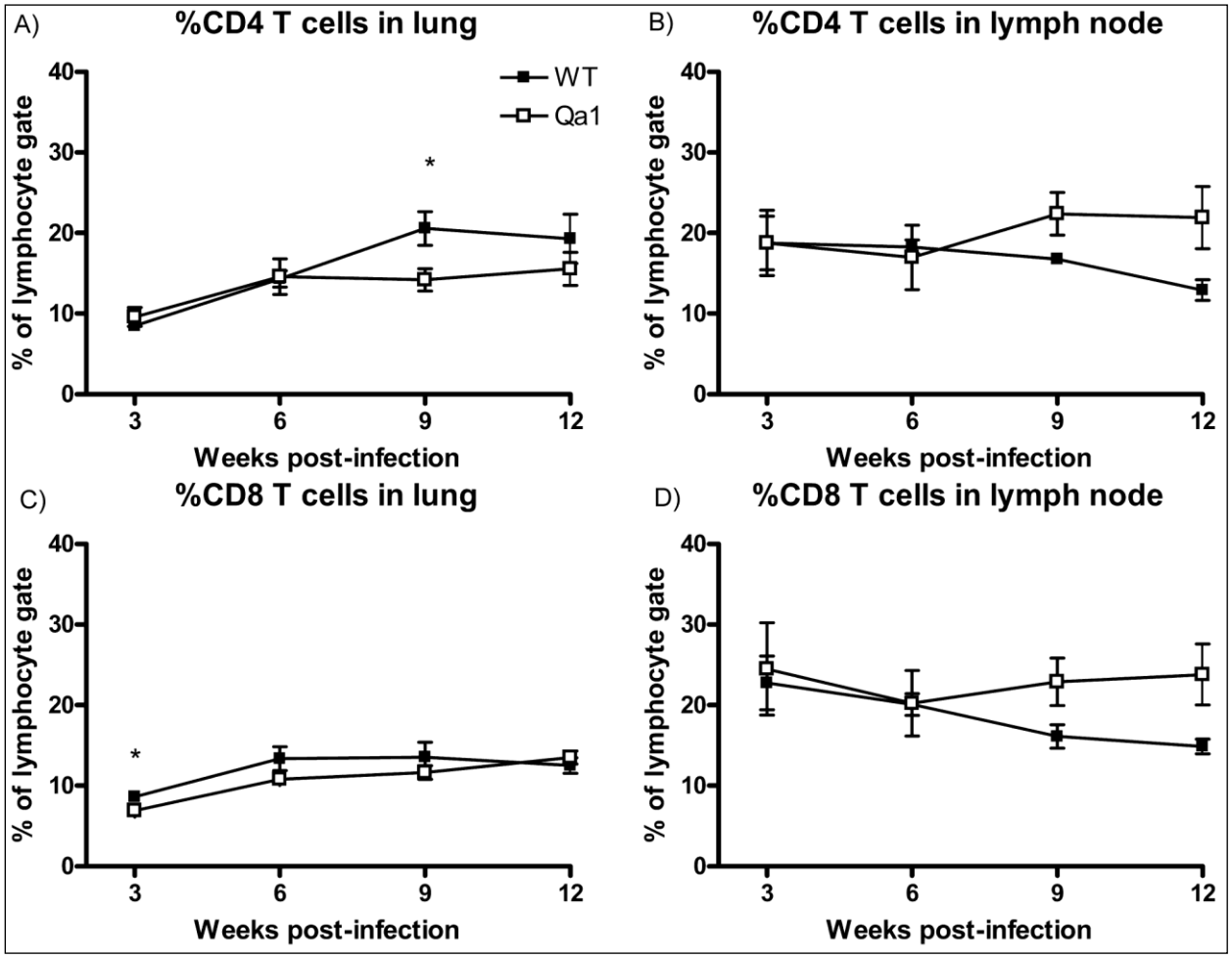


Figure 31 Percent CD4 and CD8 T cells in lymphocyte gate.
There was no significant difference in the frequency of A and B) CD4 T cells and C and D) CD8 T cells in the lung A, C) and lymph node B, D), except for CD4 T cells at 9 weeks post-infection and CD8 T cells at 3 weeks post-infection in the lung. Data are mean ± SEM of 4 mice per time-point.

When GAP-specific CD8 T cells were examined using tetramers, there was no significant difference in percent GAP cells between the two strains (Figure 32). Collectively, these data indicate that Qa-1 did not play a significant role in inducing CD8 T cell priming, whether it was against *M. tuberculosis* antigens or against CD4 T cell receptor.

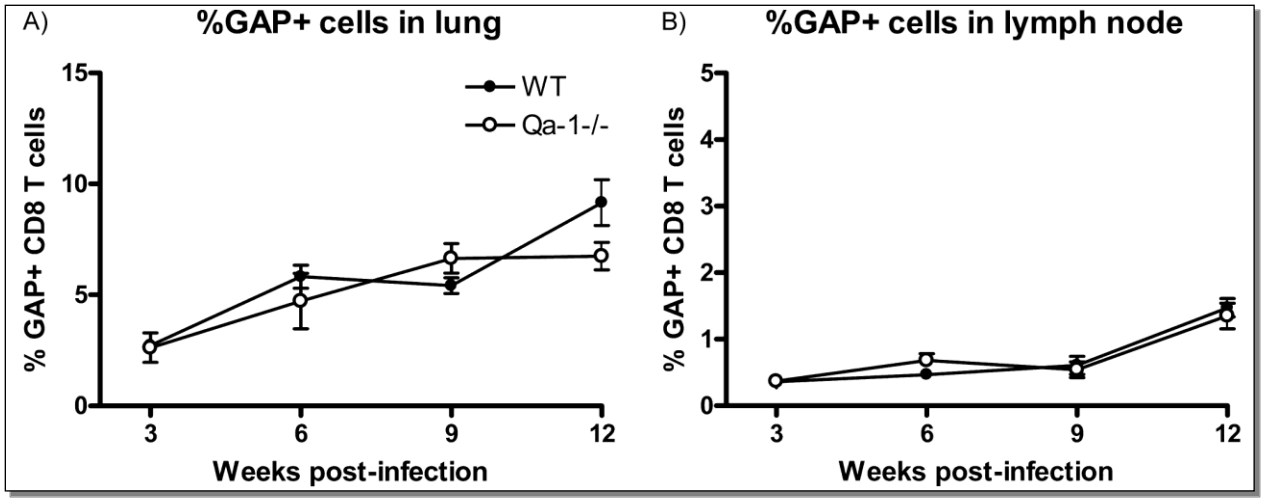


Figure 32 Percent GAP-specific CD8 T cells.
There was no significant difference between WT and Qa-1-/- mice in either A) lung or B) lymph node. Data are mean ± SEM of 4 mice per time-point.

6.3.3 Role of Qa-1 on CD8 T cell function

Multicolor flow cytometry was used to examine CD8 T cell function on a single cell basis. Our results indicate that Qa-1 expression did not have a significant effect on function in the lungs (Figure 33 and Figure 34) or lymph nodes (data not shown). There was no significant difference in degranulation (measured by surface exposure of CD107 molecules) or IFN-γ production between the two mouse strains, whether we examined total CD8 T cell responses (Figure 33) or a population of *M. tuberculosis*-specific cells (GAP-specific CD8 T cells, data not

shown).

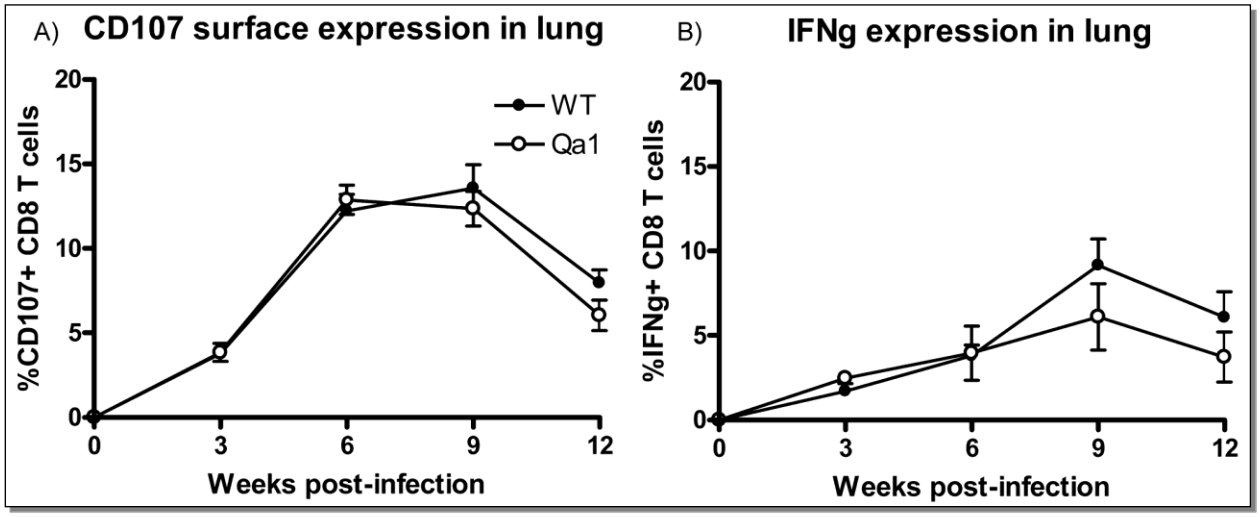


Figure 33 Degranulation and IFN-γ secretion.
A) CD107 surface expression and B) IFN-γ secretion, measured by flow cytometry. There was no significant difference between WT and Qa-1-/- mice. Data are mean ± SEM of 4 mice per time-point.

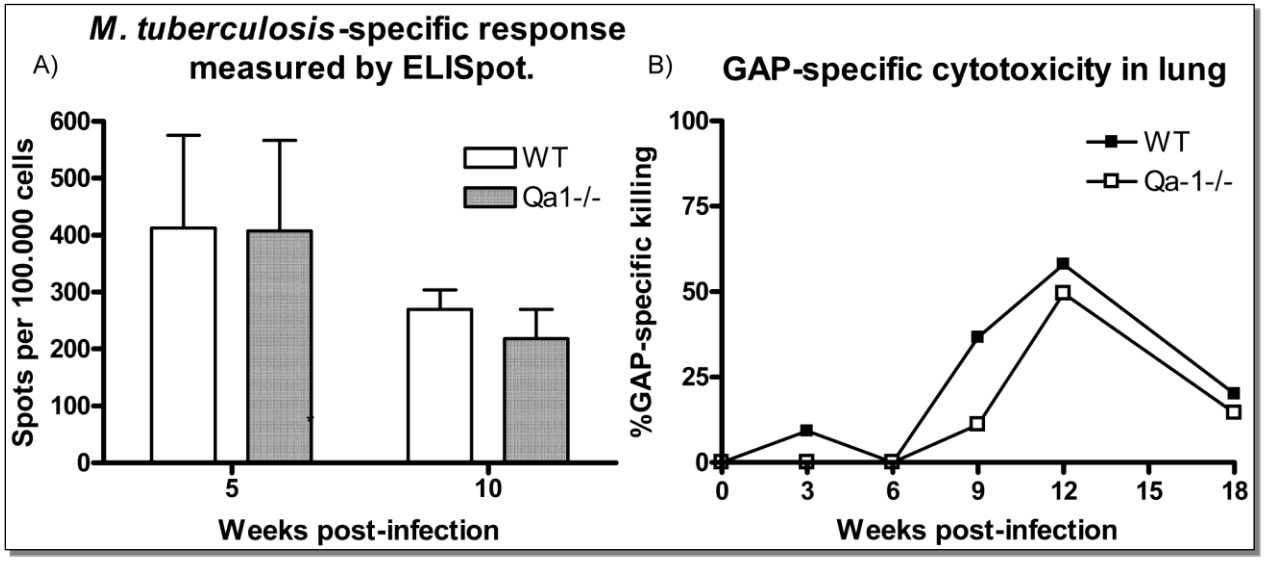


Figure 34 Functional assays.
A) IFN-γ ELISpot assay, measuring IFN-γ secretion in lung in response to *M. tuberculosis*. Data are mean ± SEM of 4 mice per time-point. B) GAP-specific cytotoxicity in lung. Data is an average of 4 mice per group per time-point.

6.3.4 Bacterial load and pathology

There was no significant difference in bacterial load between the two strains (Figure 35), indicating that if Qa-1 primed *M. tuberculosis*-specific cells, these cells did not contribute significantly to control of the bacterium. Histological analysis of the lung indicated that there was not a significant difference in inflammation and granuloma formation between the two mouse strains (Figure 36).

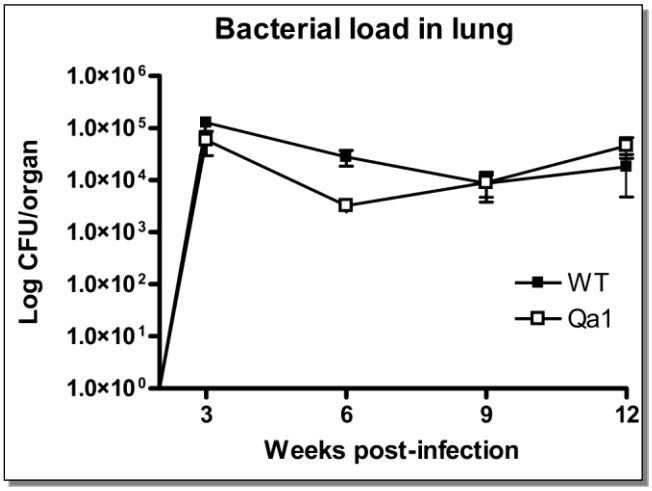


Figure 35 Bacterial load in lung.
There was no significant difference between the two mouse strains. Data are mean ± SEM of 4 mice per time-point.

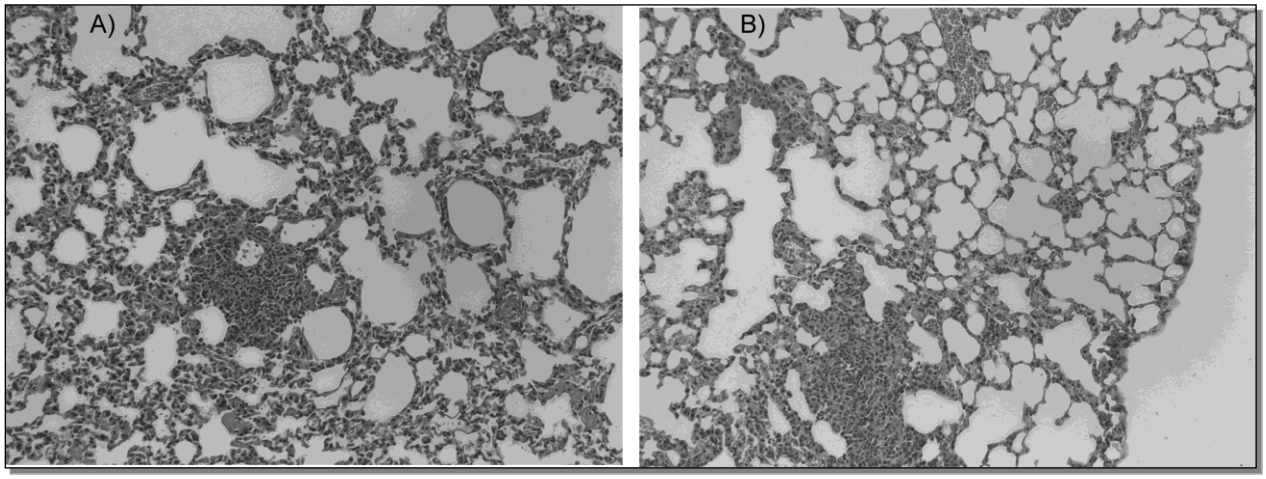


Figure 36 Lung pathology.
5 weeks post-infection, a lobe of lung from A) WT and B) Qa-1-/- mice was fixed in 10% formalin and then embedded, sectioned and stained with hematoxylin and eosin. 10x magnification.

6.4 DISCUSSION

In this study, we examined whether Qa-1 plays a role in the immune response during murine *M. tuberculosis* infection. Qa-1 has been shown to contribute to viral persistence in chronic viral infections, e.g. HSV-1, where Qa-1 was shown to suppress cytotoxicity while allowing CD8 T cells to secrete IFN-γ [252]. Studying Qa-1 is complicated by the fact that it can be bound by both T cell receptor and NKG2 molecules, with both immunostimulatory and -suppressive effects, depending on the binding partner and the peptide [266].

We hypothesized that Qa-1 would bind NKG2A and suppress cytotoxicity during *M. tuberculosis* infection in mice. However, there was no significant difference in degranulation or secretion of IFN-γ between WT mice and mice deficient in Qa-1. The *in vivo* cytotoxicity assay indicated that Qa-1 did not suppress cytotoxicity, as cytotoxicity in WT mice was equal or

greater. However, for technical reasons we used GAP peptide pulsed targets instead of *M. tuberculosis* infected cells for this assay, which could affect the results as target cells may not express Qa-1. Taken together, our results indicate that Qa-1 did not have a significant suppressive effect on CD8 T cell function or affect bacterial survival.

An alternative hypothesis was that Qa-1 would bind T cell receptor and induce CD8 T cell responses. This was based on data showing that HLA-E, the human homolog of Qa-1, can present *M. tuberculosis* antigen to CD8 T cells [265]. The outcome of antigen presentation by Qa-1 depends on which antigen is presented. Jiang et al. have shown that presentation of peptides from CD4 T cell receptor can lead to CD8 T cell mediated killing of CD4 T cells [267]. This does not appear to be the case in murine *M. tuberculosis* infection, as we see normal numbers of CD4 T cells. Alternatively, presentation of bacterial antigens could lead to priming of functional *M. tuberculosis*-specific CD8 T cells. Without knowledge of the antigen, we do not have tetramers to identify these cells. For this reason, we examined total CD8 T cell responses, or GAP-specific cells. Neither total CD8 T cell responses nor GAP-specific responses were significantly different between the mouse strains, indicating that if Qa-1 primes *M. tuberculosis*–specific CD8 T cells in mice, then this population is not immunodominant and does not have a significant effect on the course of the infection.

In conclusion, our study indicates that Qa-1 does not have a significant effect on the course of *M. tuberculosis* infection in mice.

7.0 SUMMARY OF THE THESIS

Tuberculosis (TB) is, for the most part, a treatable disease. Despite this, approximately two million people die of TB every year, the majority of whom are in developing countries. Control of the TB epidemic in more affluent countries gave the misperception that TB no longer posed a serious health threat to humans. However, immunosuppression due to HIV infection and immunosuppressive therapies, e.g. in patients with autoimmune diseases, have caused a resurgence of TB in wealthy places such as New York city [268, 269] and have brought TB back to our attention. The impact of TB on human health in less developed nations remains as serious as ever.

While *M. tuberculosis*-specific T cells are essential for controlling the bacterium, it appears that they are unable to prevent the establishment of infection and clear the bacteria [103, 270]. Similarly, protection conferred by memory cells is limited, as persons vaccinated with BCG or treated for TB with antimycobacterial drugs can be infected and re-infected with *M. tuberculosis* and develop disease [219]. A major hurdle in improving TB vaccines has been the lack of understanding of what constitutes an effective immune response to the bacterium, and how such an immune response can be achieved. In this thesis, we have focused our studies on the function and regulation of CD8 T cells during murine *M. tuberculosis* infection.

Relatively little was known about the function of *M. tuberculosis*-specific CD8 T cells when we started this work five years ago. One reason for this was the belief that CD8 T cells do

not contribute significantly to the control of *M. tuberculosis* [81, 113], which has since been conclusively disproven [104]. Another reason was the dearth of known bacterial epitopes recognized by CD8 T cells [100]. Several epitopes have since been identified, some of which induce CD8 T cell responses early in infection [101, 271], while others induce responses later in infection [102, 272]. Identification of these epitopes have allowed characterization of CD8 T cell responses to single antigens and utilization of many immunological techniques, such as the *in vivo* cytotoxicity assay, that were previously unavailable. In our studies, we made extensive use of one of the CD8 T cell epitopes, referred to as GAP [273], which appears to be expressed throughout infection. Development of a recombinant *M. tuberculosis* strain that expresses ovalbumin epitopes [103] further expanded the available tools for studying *M. tuberculosis*-specific CD8 T cells, and proved to be a great boon to our research. Using the recombinant bacteria, we were able to study the effect of various factors, such as interactions with pre-existing memory cells, the quality of CD4 T cell help and the environment, on the function of CD8 T cells *in vivo*.

We first characterized the *M. tuberculosis*-specific CD8 T cell response during primary and secondary infections using multicolor flow cytometry. We found that cytotoxicity and secretion of IFN-γ were carried out by distinct CD8 T cells, with the majority of the cells being cytotoxic. This contrasted with data published on CD8 T cells specific for other infectious agents, such as HIV [227], where cells could simultaneously carry out both functions. This held true for both primary and secondary infections, and indicated that there was a functional defect in CD8 T cells induced during *M. tuberculosis* infection. Also, it indicated that detection of IFN-γ secretion is an insufficient means of studying CD8 T cell responses to *M. tuberculosis* as only a minority of the cells secrete IFN-γ, even though this is the easier and most common method for

studying human CD8 T cell responses in TB and other infections. When we compared primary and memory CD8 T cell responses, we found that the primary cells secreted higher levels of IFN-γ. Additionally, even though the frequency of degranulating cells and the amount of degranulation on a per-cell basis was higher in the memory cells, the primary cells were more cytotoxic. This indicates that there is a defect in cytotoxicity in the memory cells, although expression of cytotoxic molecules that are stored in the vesicles was comparable to expression in primary CD8 T cells. Collectively, this indicated that although memory cells respond faster than primary cells, the primary cells are more functional than memory cells.

We hypothesized that IFN-γ secretion and cytotoxicity being carried out by distinct CD8 T cells was the consequence of either cellular exhaustion or the regulation by an inhibitory molecule. CD8 T cells can become exhausted during chronic viral infections, in that they lose effector functions such as secretion of IFN-γ [274]. One marker of exhaustion is chronic high expression of PD-1, although other markers exist as well [275]. However, PD-1 expression was not chronically high on *M. tuberculosis*-specific CD8 T cells, and blocking interactions between PD-1 and its ligands had no significant effect on the function of CD8 T cells or the bacterial burden of the mice. The same held true for Qa-1, a nonclassical MHC class I molecule, which inhibits CD8 T cell-mediated cytotoxicity in herpes simplex virus-1 (HSV-1) infected mice while allowing secretion of IFN-γ [252, 254]. When we infected mice deficient in Qa-1 with *M. tuberculosis*, we found no significant effect on CD8 T cell responses or bacterial load compared to WT mice. Thus, cytotoxicity and secretion of IFN-γ being carried out by distinct CD8 T cells during *M. tuberculosis* infection does not appear to stem from exhaustion or inhibition by Qa-1.

To further examine the regulation of CD8 T cells, we examined what effect the presence of pre-existing memory cells had on the quality and quantity of newly primed CD8 T cells.

Infants in TB endemic areas are vaccinated with BCG, which induces memory responses that are cross-reactive with *M. tuberculosis*. As the children progress to adolescence and young adulthood, the likelihood of progressing to TB increases precipitously. Much effort is being put into developing post-exposure vaccines to boost the immune responses induced by BCG, in the hope that adolescents and young adults may be protected. As boosting with BCG has been unsuccessful [159], some new vaccine candidates contain *M. tuberculosis* antigens that are not present in the BCG vaccine [175] in addition to antigens that are cross-reactive between the bacteria. If successful, the immune response would consist of a combination of primary T cells and memory T cells. However, it has been shown in other infections that memory cells can influence the quality and quantity of primary cells, e.g. by providing CD4 T cell help or by inhibiting priming of new CD8 T cells [220-225].

Thus, in chapter four, we examined the effect of memory cells (GAP-specific cells) on the quantity and quality of primary cells (ova-specific cells). While the mice induced ova-specific CD8 T cell responses in the lymph nodes, we found that a sufficient bacterial burden was required for ova-specific responses to appear in the lungs, the primary site of infection. This indicates that memory cells contained the bacterial infection before new CD8 T cells had migrated to the lungs in the mice with the lowest bacterial load. This has implications for human vaccinations, as BCG-specific memory cells may prevent induction of strong primary *M. tuberculosis*-specific CD8 T cell responses in the lungs by the new vaccine candidates.

On the other hand, memory cells also influenced the quality of the primary response, in that the primary cells secreted higher levels of IL-2, a cytokine associated with proliferation and enhanced cytotoxicity [238]. They also secreted lower levels of TNF, a cytokine that is essential for control of *M. tuberculosis*, but high levels can cause tissue pathology [240]. While memory

cells did not affect cytotoxicity or the frequency of cells secreting IFN-γ in the primary response, they did enhance the multifunctionality of the cells secreting IFN-γ, which appears to correlate with increased protection in other infection models [82-84]. This indicates that memory cells can influence both the magnitute and quality of primary CD8 T cell responses.

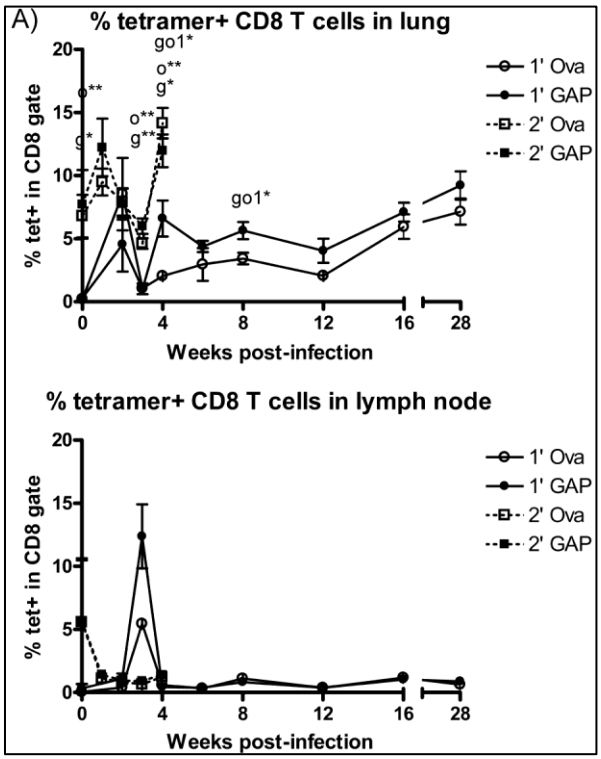
In chapter five, we used adoptive transfer of naive ova-specific CD4 and CD8 T cells to examine what effect the environment had on the quality of the CD8 T cell response. We showed that CD8 T cells primed after mice were infected with *M. tuberculosis* secreted significantly more IFN-γ than CD8 T cells that were present before the infection. Furthermore, CD8 T cells primed at the peak of infection (three weeks post-infection) were significantly more multifunctional than cells primed at other times. Additionally, the multifunctional cells could simultaneously secrete IFN-γ and be cytotoxic. Thus the functional phenotype of CD8 T cells can be dramatically altered by the environment that cells are primed in, although the specific factors that determine cellular function have not been identified. CD4 T cell help appeared to be important for the development of multifunctional CD8 T cells, as CD8 T cells transferred without CD4 T cells had significantly fewer functions and secreted lower amounts of cytokines.

Transfer of CD4 T cells during the peak of infection also resulted in more multifunctional cells, compared to host CD4 T cell responses in the lungs. However, simultaneous transfer of CD8 T cells appeared to be detrimental to priming of multifunctional CD4 T cells. This indicates that there is significant bidirectional interaction between CD4 and CD8 T cells during *M. tuberculosis* infection that needs to be further examined.

Collectively, the data presented in this thesis indicate that *M. tuberculosis*-specific CD8 T cell responses are complex, and that the response is influenced by various factors, such as the priming environment and the quality of CD4 T cell help. The data also indicate that the immune

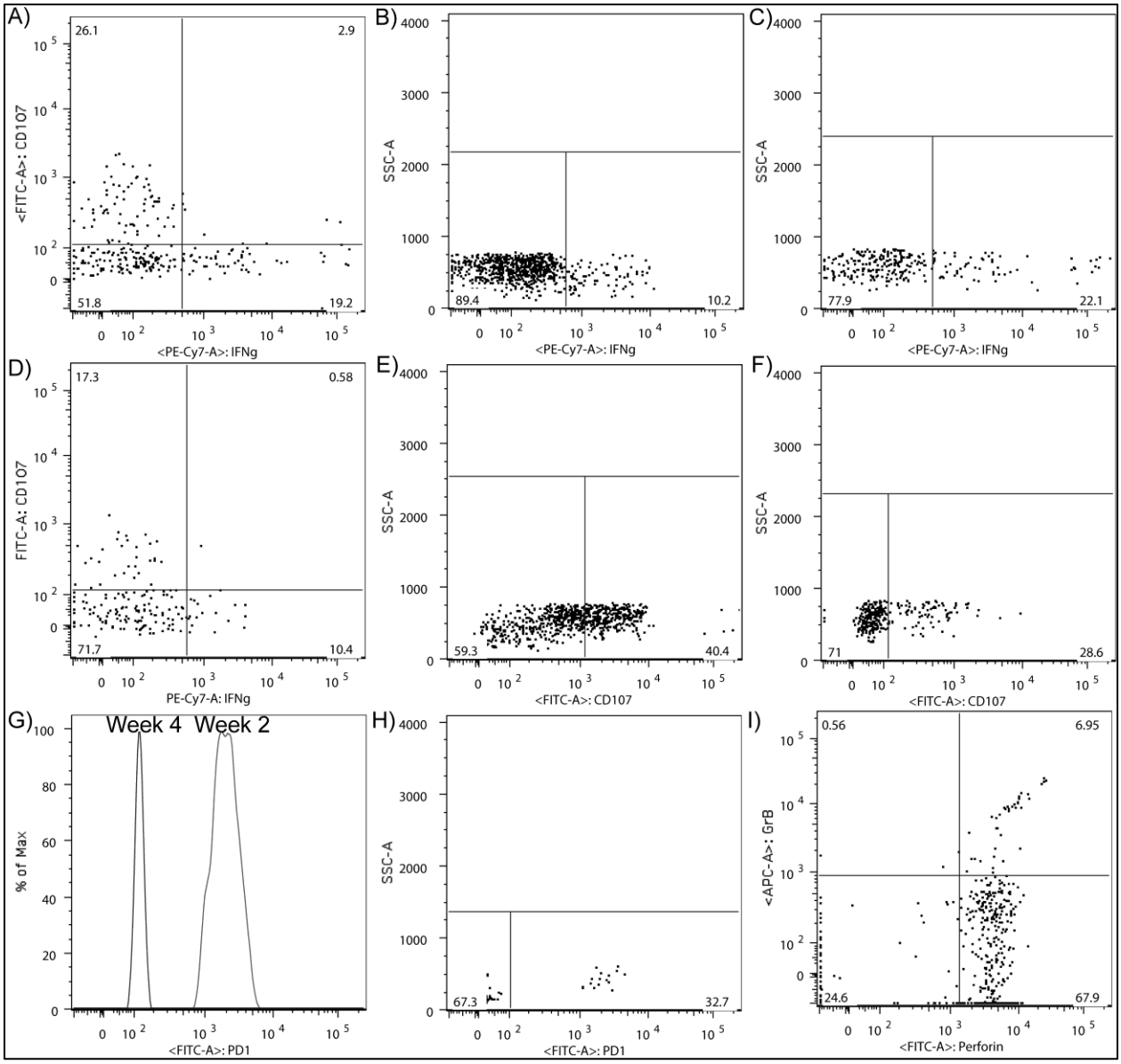
response to the bacterium can be manipulated to generate more functional T cells, without altering the bacterium, by altering the environment in which the cells are primed. This has important implications for vaccination against the bacterium, as the current vaccination protocols only provide partial protection. While it has still not been established what constitutes a protective immune response to the bacterium, it is our hope that the data presented here may lead to advances in development of new TB vaccines, as well as an improvement in efficacy of the existing vaccine, and a better understanding of what shapes the immune response to *M. tuberculosis*.

APPENDIX A- SUPPLEMENTARY FIGURES



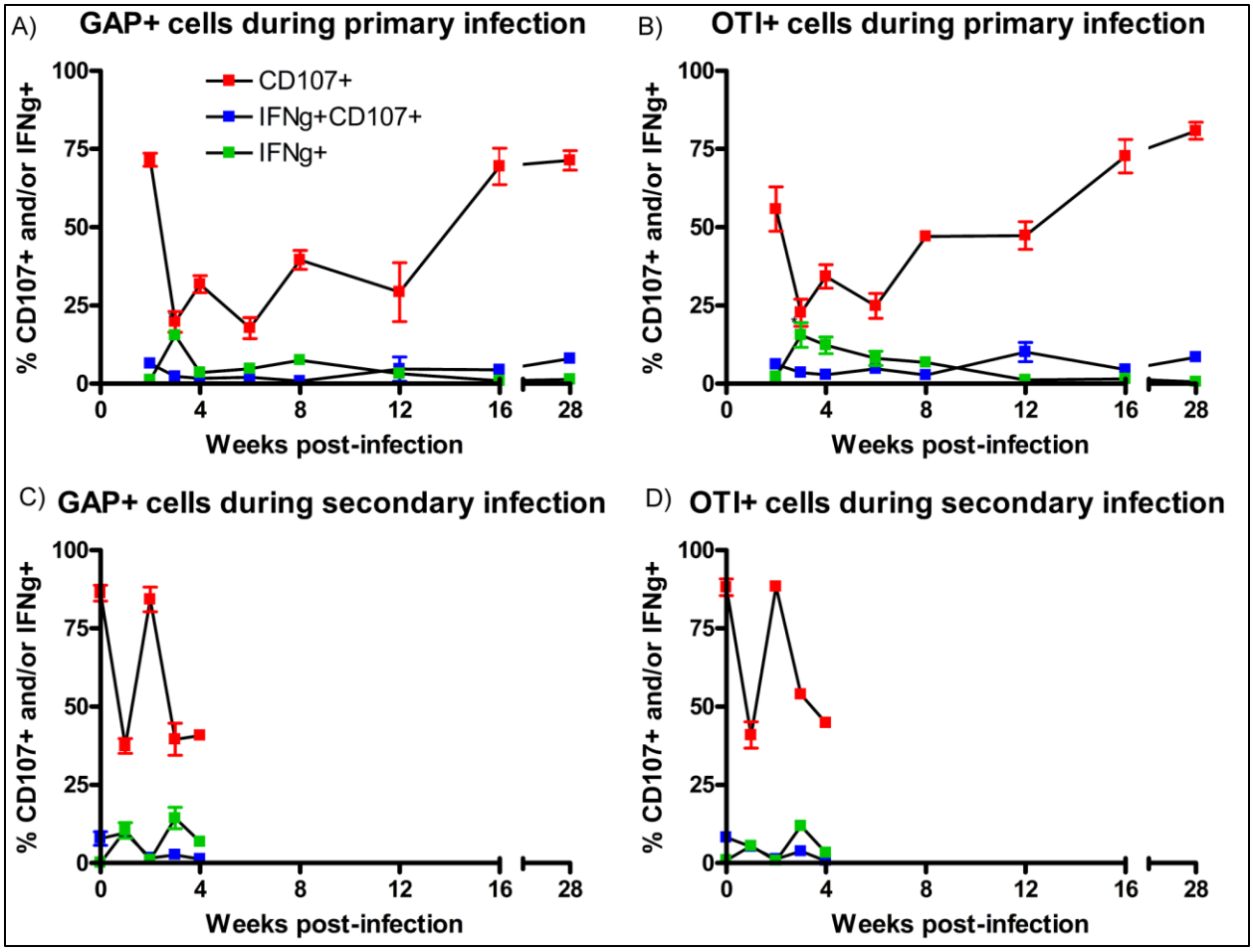
Supplementary Figure 1 *M. tuberculosis-ova* induces similar GAP- and ova-specific responses.

Percent tetramer positive CD8 T cells in A) lung and B) lymph node during primary (1') and secondary (2') infections were significantly different 0, 3 and4 (lung), 2 and 3 (lymph node) weeks post-infection for ova-specific cells (o*) and 0, 3 and 4 (lung), 3 and 4 (lymph node) weeks post-infection for GAP-specific cells (g*). The frequency of ova and GAP-specific cells was significantly different 4 and 8 (lung), 3 and 28 (lymph node) weeks post-primary infection (go1*). Data are mean ± SEM of 4 mice per time-point.



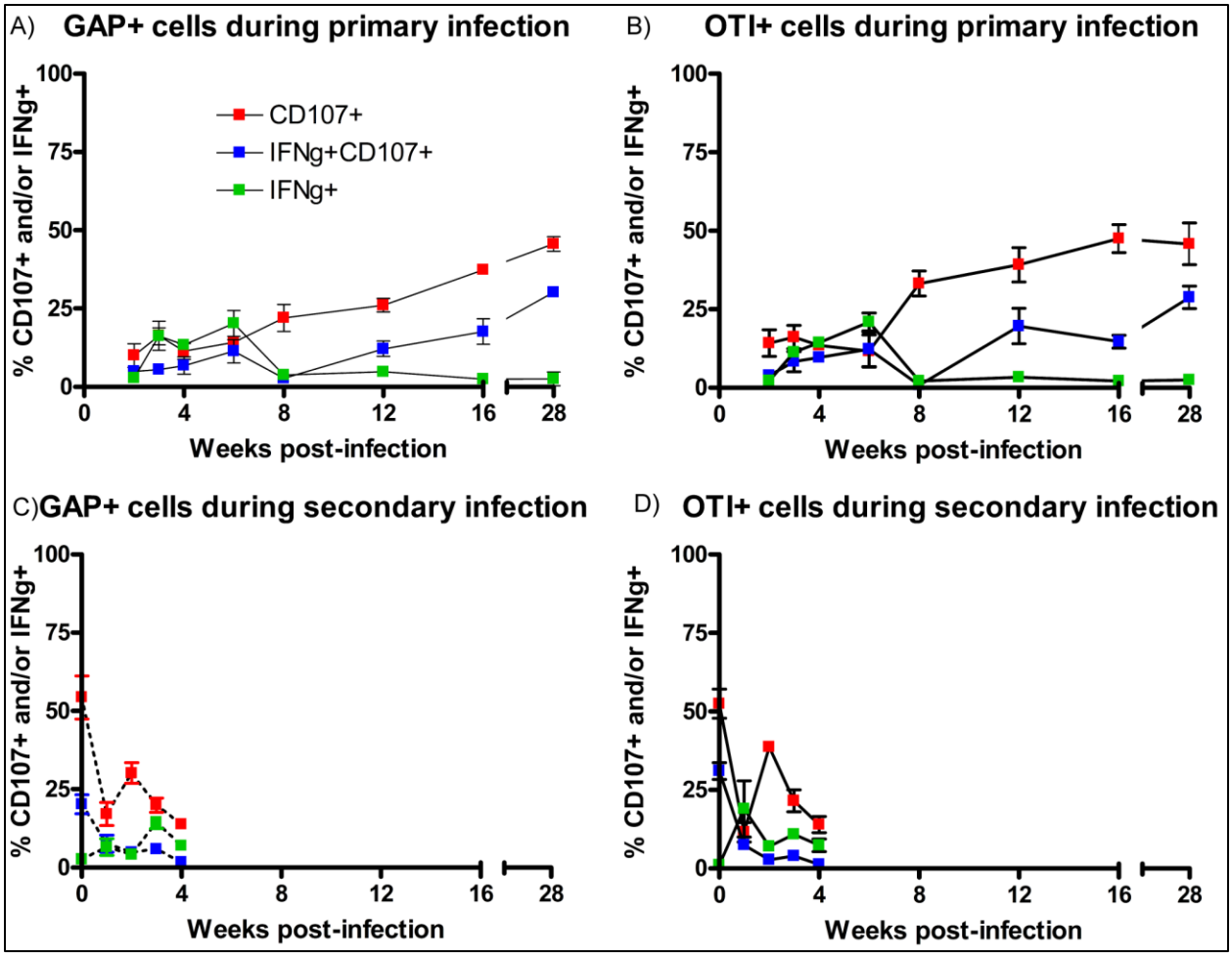
Supplementary Figure 2 Representative dot plots and geometric mean fluorescence intensity.

A) and D) are CD107 versus IFN-γ staining. D) is from a mouse that was treated with anti-PD-1 and anti-PD-L1 antibodies for 4 weeks, while A) was untreated. B) and C) show IFN-γ staining of GAP+ CD8 T cells, 4 weeks post-primary (B) and secondary (C) infection in lung. (E and F) show CD107 staining of GAP+ CD8 T cells, 4 weeks post-primary E) and secondary F) infection in lung. G) shows geometric mean fluorescence intensity of PD-1 staining on OTI cells in lung, 2 and 4 weeks post-infection while H) shows a representative dot plot of PD-1 staining at 2 weeks post-infection. I) shows perforin and granzyme B staining in an unstimulated lung sample at 4 weeks post-infection.



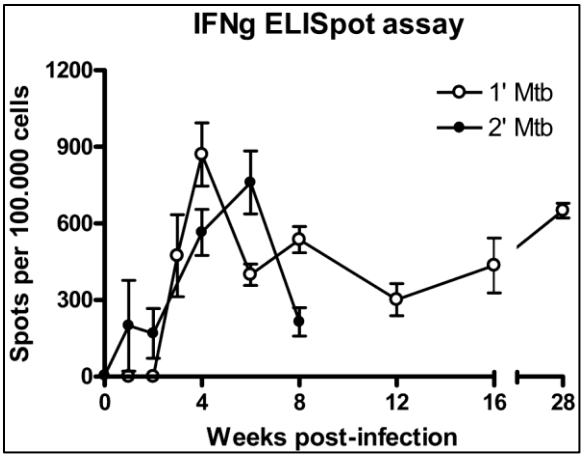
Supplementary Figure 3 IFN-γ secretion and degranulation are carried out by distinct populations of cells in lung.

Percent IFN-γ+ and CD107+ cells during primary infection (A and B) and secondary (C and D) infection. A) and C) are GAP, B) and D) are ova. The data are mean ± SEM of 4 mice per time-point.



Supplementary Figure 4 IFN-γ secretion and degranulation are carried out by distinct populations of cells in lymph node.

Percent IFN-γ+ and CD107+ cells during primary infection (A and B) and secondary (C and D) infection. A) and C) are GAP, B) and D) are ova. The data are mean ± SEM of 4 mice per time-point.



Supplementary Figure 5 ELISpot assay measuring *M. tuberculosis*-specific secretion of IFN-γ by T cells in the lungs.

Cells were incubated with *M. tuberculosis*-infected DC for 48 hours. There was no background level of IFN-γ secretion, measured by incubating cells with either T cell media or unpulsed, uninfected DC (data not shown). Data are mean ± SEM of 4 mice per time-point.

APPENDIX B - A LIST OF MANUSCRIPTS IN PREPARATION

1. Einarsdottir, T. and Flynn, J. L. Priming of Multifunctional CD4 and CD8 T Cells During *M. tuberculosis* Infection in Mice. (in preparation).

2. Einarsdottir, T. and Flynn, J. L. Memory Cells Influence the Number and Quality of New CD8 T Cells During *Mycobacterium tuberculosis*-ova Infection. (in preparation).

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