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Memory CD8+ T Cell Function during Mycobacterium Tuberculosis Infection: A Dissertation

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MEMORY CD8⁺ T CELL FUNCTION DURING *MYCOBACTERIUM*
TUBERCULOSIS INFECTION

By: Stephen M. Carpenter

MEMORY CD8⁺ T CELL FUNCTION DURING *MYCOBACTERIUM*
TUBERCULOSIS INFECTION

A Dissertation Presented

By

Stephen Matthew Carpenter

Submitted to the Faculty of the University of Massachusetts Graduate
School of Biomedical Sciences, Worcester in partial fulfillment of the
requirements for the degree of

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June 30th, 2016

DEPARTMENT OF MICROBIOLOGY AND PHYSIOLOGIC SYSTEMS

MEMORY CD8⁺ T CELL FUNCTION DURING *MYCOBACTERIUM*
TUBERCULOSIS INFECTION

A Dissertation Presented By

STEPHEN MATTHEW CARPENTER

This work was undertaken in the Graduate School of Biomedical Sciences
Millennium Ph.D. Program

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signifies that the student has met all graduation requirements of the School.

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Dean of the Graduate School of Biomedical Sciences

June 30th, 2016

DEDICATION

This work is dedicated to Jeanne Riz, Brooke, and Lukas. Thank you for your love, support and patience that made all of this work (and career path) possible.

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ABSTRACT

T cell vaccines against *Mycobacterium tuberculosis* (Mtb) and other pathogens are based on the principle that memory T cells rapidly generate effector responses upon challenge, leading to pathogen clearance. Despite eliciting a robust memory CD8⁺ T cell response to the immunodominant Mtb antigen TB10.4 (EsxH), we find the increased frequency of TB10.4-specific CD8⁺ T cells conferred by vaccination to be short-lived after Mtb challenge. To compare memory and naïve CD8⁺ T cell function during their response to Mtb, we track their expansions using TB10.4-specific retrogenic CD8⁺ T cells. We find that the primary (naïve) response outnumbers the secondary (memory) response during Mtb challenge, an effect moderated by increased TCR affinity. To determine whether the expansion of polyclonal memory T cells is restrained following Mtb challenge, we used TCR β deep sequencing to track TB10.4-specific CD8⁺ T cells after vaccination and subsequent challenge in intact mice. Successful memory T cells, defined by their clonal expansion after Mtb challenge, express similar CDR3 β sequences suggesting TCR selection by antigen. Thus, both TCR-dependent and independent factors affect the fitness of memory CD8⁺ responses. The impaired expansion of the majority of memory T cell clonotypes may explain why some TB vaccines have not provided better protection.

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Figure 1.1: TB pathogenesis

Figure 1.2: Paradigms of protective immunity to TB.

Nunes-Alves, C., Booty, M. G., Carpenter, S. M., Jayaraman, P., Rothchild, A. C., & Behar, S. M. (2014). In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology*, 12(4), 289–299.

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List of Abbreviations

- AIDS Acquired Immune Deficiency Syndrome (due to infection with HIV).
- BSL-3 Biosafety level 3
- CDR3..... Complementarity-determining region 3: The region on the α and β TCR chains responsible for the diversity in recognition of peptides presented by MHC molecules.
- CFU Colony-forming units. The growth of a colony attributable to proliferation from a single bacterium.
- CTL..... Cytotoxic T lymphocyte
- ESAT-6 Early secreted antigenic target 6Kd, an immunodominant Mtb antigenic target of CD4⁺ and CD8⁺ T cells (in both humans and animal models of TB).
- gDNA Genomic deoxyribonucleic acid
- HIV..... Human Immunodeficiency Virus
- IFN γ Interferon gamma
- IL-2 Interleukin-2; the same nomenclature applies to other interleukins, such as interleukins 10 (IL-10), 12 (IL-12), 21 (IL-21), or 27 (IL-27)
- Lm Δ ActA-TB10...*Listeria monocytogenes* engineered to secrete a fusion protein containing full-length TB10.4.
- MHC I, II Major Histocompatibility Complex I or II
- mAb Monoclonal antibody
- Mtb..... *Mycobacterium tuberculosis*
- MVA85A Modified vaccinia virus expressing Mtb antigen 85A: A TB vaccine tested in a recent phase 2b clinical trial in South Africa
- NTM..... Non-tuberculous mycobacteria
- PMBC Peripheral blood mononuclear cells
- PPD Purified protein derivative (of *Mycobacterium tuberculosis*)
- Rg Retrogenic; referring to transgene expression in mice that receive bone marrow transplantation containing hematopoietic stem cells

transduced with a retrovirus that express an antigen-specific T cell receptor

RNAseq Ribonucleic acid sequencing: A whole-exome analysis of gene expression by quantitative sequencing the from total RNA (or mRNA).

SPF..... “Specific pathogen free” mouse facility

TB Tuberculosis

TB10.4 (EsxH) An immunodominant Mtb antigen against which humans and mice generate CD8⁺ and CD4⁺ T cells

TB10 / α CD40 / Poly(I:C)...A vaccine injected intravenously that includes 3 components: TB10.44-11 peptide, anti-CD40 monoclonal antibody, and poly(I:C) (TLR3 agonist).

TCR T cell receptor

TNF Tumor necrosis factor alpha

Th T helper subset of CD4⁺ T cells (Th1, Th2, Th17, Treg, etc...)

t-spot TB An ELISpot assay for IFN used to detect TB by exposing a patient’s T cells to 3 Mtb-specific peptide pools

TST..... Tuberculin skin test

Preface to Chapter I

This chapter comprises the introduction to the dissertation which was written by Stephen Carpenter. The introduction includes both new, original text as well as text written by Stephen Carpenter in three recently published manuscripts, including:

Carpenter, S. M., Nunes-Alves, C., Booty, M. G., Way, S. S., & Behar, S. M. (2016). A Higher Activation Threshold of Memory CD8+ T Cells Has a Fitness Cost That Is Modified by TCR Affinity during Tuberculosis. *PLoS Pathogens*, 12(1), e1005380. <http://doi.org/10.1371/journal.ppat.1005380>

Behar, S. M., Carpenter, S. M., Booty, M. G., Barber, D. L., & Jayaraman, P. (2014). Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. *Seminars in Immunology*, 26(6), 559–577. <http://doi.org/10.1016/j.smim.2014.09.003>

Nunes-Alves, C., Booty, M. G., Carpenter, S. M., Jayaraman, P., Rothchild, A. C., & Behar, S. M. (2014). In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology*, 12(4), 289–299. <http://doi.org/10.1038/nrmicro3230>

CHAPTER I

Introduction

***Mycobacterium tuberculosis* infection**

Tuberculosis (TB) is the deadliest infectious disease in human history, recently surpassing HIV in the number of deaths attributable to disease (World Health Organization, 2015). *Mycobacterium tuberculosis* (Mtb), the acid-fast bacterium that causes TB, infects one third of the world's population and kills 1.5 million people per year (World Health Organization, 2015). Mtb is transmitted from person to person through the inhalation of aerosol droplets. Droplets containing the bacteria reach alveoli in the distal lung where they primarily infect resident alveolar macrophages (J. Lee et al., 2009; Leemans et al., 2001) (Fig 1.1). The initial stages of infection are characterized by an innate immune response that includes the recruitment of inflammatory cells to the lung. However, the initiation of adaptive immunity is delayed compared to pathogens that cause acute infection, and occurs only after dissemination of *M. tuberculosis* to draining lymph nodes (Chackerian et al., 2002; Reiley et al., 2008; Wolf et al., 2008).

In the lymph node, presentation of bacterial antigens by dendritic cells leads to priming and expansion of antigen-specific T cells (Chackerian et al., 2002; Reiley et al., 2008; Wolf et al., 2008). During this process, naïve T cells differentiate into effector T cells, which then migrate to the infected lung. The recruitment of

immune T cells, in combination with other leukocytes leads to granuloma formation, a hallmark of tuberculosis. Macrophages in granulomas, with support from other macrophages and T cells, attempt to kill mycobacteria using oxidative damage in phagolysosomes, the induction of apoptosis, autophagy and efferocytosis (Divangahi et al., 2013; Hartman and Kornfeld, 2011; C. J. Martin et al., 2012; Molloy et al., 1994; Moraco and Kornfeld, 2014; Oddo et al., 1998). Overall, granulomas are thought to contain Mtb, restrict its dissemination, and prevent replication. However, some evidence suggests that the local spread of actively dividing mycobacteria is facilitated during early granuloma formation (Cambier et al., 2014; J. M. Davis and Ramakrishnan, 2009; Ramakrishnan, 2012). Infection with Mtb is followed either by immune control of bacterial growth and the development of latency, or progression to active pulmonary tuberculosis (Fig 1.1).

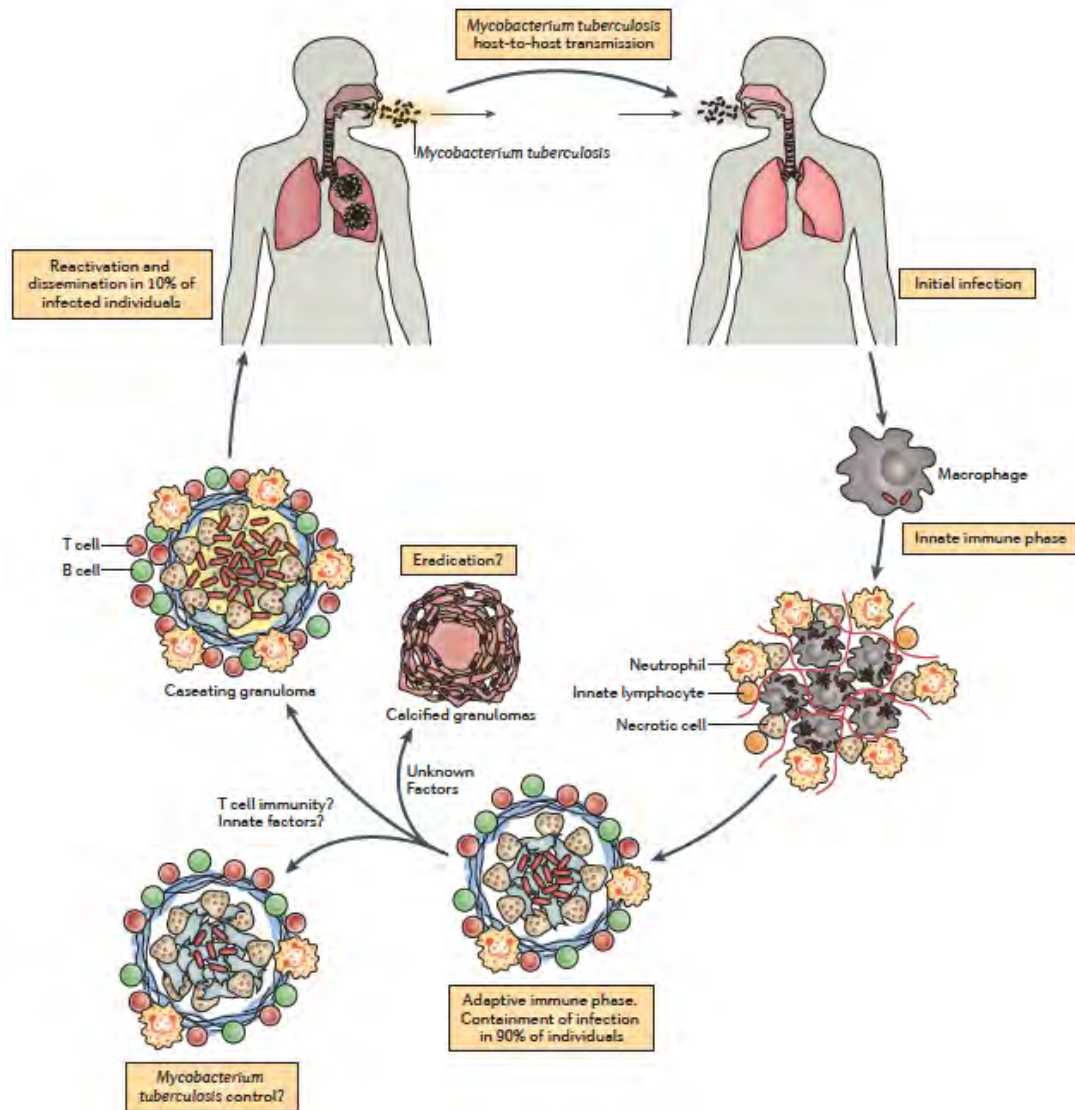


Figure 1.1: TB pathogenesis. Infection is initiated by the inhalation of aerosol droplets that contain bacteria. The initial stages of infection are characterized by innate immune responses that involve the recruitment of inflammatory cells to the lung. Following bacterial dissemination to the draining lymph node, dendritic cell presentation of bacterial antigens leads to T cell priming and triggers an expansion of antigen-specific T cells, which are recruited to the lung. The recruitment of T cells, B cells, activated macrophages and other leukocytes leads to the establishment of granulomas, which can contain *Mycobacterium tuberculosis*. Most infected individuals will remain in a 'latent' state of infection, in which no clinical symptoms are present. A small percentage of these people will eventually progress and develop active disease, which can lead to the release of *M. tuberculosis* from granulomas that have eroded into the airways. When individuals with active tuberculosis cough, they can generate infectious droplets that transmit the infection.

Active pulmonary TB is characterized by the development of a chronic pneumonia that progresses over a period of months to years, accompanied by fevers, night sweats, weight loss, productive cough, and, frequently, hemoptysis. In some cases, Mtb disseminates to other organs via the bloodstream, causing meningitis, enteritis, pyelonephritis, and occasionally infiltrates the bones and marrow (Kato-Maeda et al., 2016). The sequelae of TB dissemination are particularly lethal and disfiguring. However, those with a healthy immune system that control pulmonary TB early after inoculation develop latent disease and have a 5-20% lifetime risk of reactivation (Horsburgh, 2004). The risk of reactivation and the development of active pulmonary tuberculosis is greatest in the first 2 years after infection (Horsburgh, 2004). Patients with newly-diagnosed, latent TB are typically treated with antibiotics for 4-9 months in an attempt to eradicate sequestered mycobacteria and reduce the risk of reactivation disease (Horsburgh and Rubin, 2011).

The treatment of tuberculosis requires the use of multiple antibiotics for at least 6 months once active disease ensues (Zumla et al., 2015). Complications such as drug-resistance or intolerance can limit the use of many effective regimens (Velayati et al., 2009; S. Wu et al., 2013). Antibiotic resistance has made some cases of TB impossible to treat (Velayati et al., 2009), and mainstream international travel is leading to the rapid spread of extensively drug-resistant Mtb strains. The extreme social and economic burden of TB, the difficulty in completion of treatment regimens, and the risk of antibiotic-resistant infection highlight the

need not only for more effective antibiotics, but for the development of an effective TB vaccine.

The delayed onset of adaptive immunity

The delay in initiating T cell responses in the Mtb-infected is the most significant limitation in the ability of T cells to protect the host from TB, and may also be hindering protective vaccination. A delay in the adaptive immune response to Mtb was first observed in early studies of household contacts of TB patients. Tuberculin skin test (TST) responses were tested weekly in the household contacts of patients diagnosed with active pulmonary TB in Scandinavia in the early 1930s (Stewart, 1956; Wallgren, 1948). Those who converted to a positive TST did so 3 – 6 weeks after close contact with an Mtb- infected individual. Although difficult to determine the exact time of inoculation, these data also likely underrepresent the time required to convert their skin test: As patients diagnosed with TB were likely to already be contagious prior to their diagnosis, their delay in the development of adaptive immunity (newly positive TST) is considerable.

Several animal studies have also shown that adaptive immunity in the lung is delayed nearly 2 weeks after inoculation of mice. Three landmark studies showed that T cells are first primed in the lung-draining mediastinal lymph nodes (MLN), where actively-dividing mycobacteria must first be transported, prior to responding to infection in the lung (Chackerian et al., 2002; Reiley et al., 2008; Wolf et al., 2008). Initiation of T cell responses in the MLN (9 - 11 days post-

infection) are followed by T cell trafficking to the lung (d12 – 14). In addition to its relevance to priming naïve T cells that target Mtb-infected cells, Jung et al. have shown that memory T cells responding to aerosol Mtb challenge also experience a delay in their response to infection (Jung et al., 2005). The delay in priming a primary (or secondary) adaptive immune response to Mtb may be one of the most important limitations in our immune system's ability to reliably prevent or eradicate *Mycobacterium tuberculosis* infection. A successful TB vaccine will likely need to drive earlier T cell responses in order to be effective.

Current TB vaccines

Vaccines have proven to be the most cost-effective intervention against infectious diseases in history, yet the development of a vaccine that reliably prevents tuberculosis has eluded scientists for nearly a century. Classic vaccine approaches using heat-killed or live-attenuated pathogens have almost exclusively been used to generate protective antibodies- relying on the immune system to develop memory B cell (and likely CD4⁺ T cell) responses targeting a broad array of antigens (Plotkin, 2010). However, the protection conferred by memory T cells elicited by vaccination with an attenuated pathogen has been disappointing for TB. Natural immunity to TB can be modeled in mice by clearing primary infection with antibiotics (Jung et al., 2005). Upon re-challenge with aerosolized Mtb, a 10-fold CFU reduction is observed but the host remains chronically-infected. Furthermore, protection is short-lived and is accompanied by little or no change in survival (Jung

et al., 2005; Kamath and Behar, 2005). Vaccines tested in animal models have displayed a similar pattern of early control of CFU but rarely achieve prolonged survival after *Mtb* infection, and certainly have failed to provide sterilizing immunity (Henao-Tamayo et al., 2014; Nunes-Alves et al., 2014; Ottenhoff and Kaufmann, 2012).

BCG, an attenuated strain of *Mycobacterium bovis*, is the only approved TB vaccine, used widely outside of the U.S. However, its ability to prevent active pulmonary disease is inconsistent and appears to be particularly ineffective in countries with a high TB incidence (Brewer, 2000; Brewer and Colditz, 1995; Fine, 1995). In a recent, double-blind phase 2b clinical trial, a novel TB vaccine (MVA85A), used as a boost to BCG vaccination in South African children, did not prevent active pulmonary TB (Tameris et al., 2013). Convincing animal and epidemiologic data has shown that both helminth infection (Elias et al., 2008; Fine, 1995) and exposure to environmental non-tuberculous mycobacteria (NTM) (Collins, 1971; Palmer and Long, 1966; Youmans et al., 1961) in areas endemic with TB are potential confounders to the protection attributed to BCG vaccine efficacy (Black et al., 2001; Brandt et al., 2002; Elias et al., 2008). However, such confounders would not explain the overall high rates of tuberculosis in endemic areas despite environmental NTM exposure, BCG vaccination, or prior *Mtb* infection, all of which elicit T cells specific for mycobacterial antigens in the infected host. Furthermore, a large retrospective study recently described that individuals successfully treated for active TB are not protected from reinfection (Millet et al.,

2013). In this study, patients diagnosed with recurrent TB were estimated to have an increased risk of re-exposure to infection. Furthermore, in half of the patients whose Mtb isolates were genotyped, a new isolate was found to be causing re-infection. Although both vaccination and prior infection elicit antigen-specific memory T cells that control or prevent infection by many other pathogens, memory T cells, in general, have not shown the same protective function against Mtb. Therefore, to generate a more effective TB vaccine design, these data indicate the need to better understand what constitutes protective memory T cell responses for TB.

Protective memory T cell responses

The goal of T cell vaccination is to establish pre-existing immunity against pathogens in the form of memory T cells. Two features of memory T cells make them superior to naïve T cells in mediating protection: First, memory T cells have undergone a cycle of expansion and contraction, leading to a greater frequency of pathogen-specific T cells than are present among the naïve T cell repertoire. Second, memory T cells do not classically require “priming” and rapidly express effector activity after antigen recognition, even after presentation by non-professional APCs. These features, together with their long-lived nature and their ability to survey non-lymphoid organs allow memory T cells to quickly respond to acute infection (Ahmed and Gray, 1996; Jameson and Masopust, 2009; Swain et al., 1991; 1990; Vitetta et al., 1991).

Memory T cells are generated after vaccination or prior infection of the host. Interestingly, Mtb vaccines show little evidence of long-lived protection, even in mouse models (Orme, 2006), despite 5–10 fold reductions in bacterial burden early after aerosol infection (Henao-Tamayo et al., 2014). These results imply an early but transient benefit to memory T cells after Mtb challenge. Furthermore, despite the development of memory T cells following treatment of active TB, observations of those cured by antibiotics show that they are not protected from reinfection (Crofts et al., 2010; Millet et al., 2013; Verver et al., 2005). Although we have some understanding of the fate of memory T cell responses against pathogens that cause chronic infections, such as LCMV clone-13, a model pathogen that causes chronic infection in mice (Vezys et al., 2006; West et al., 2011), little is known about the relative contribution of naïve and memory T cells (and the resulting 1° and 2° responses, respectively) to the recall response elicited by the human pathogen *Mycobacterium tuberculosis* (Mtb).

The ability to identify protective memory T cell responses elicited by vaccination would significantly accelerate TB vaccine design. Studies in mice have proven that interferon γ (IFN γ) produced by T cells is critical for attenuating Mtb growth (Flynn et al., 1993; Wolf et al., 2008), however, IFN γ levels have not successfully predicted protection against TB. In fact, prospective studies of BCG vaccination in mice reported that increased numbers of IFN γ -secreting T cells were a better correlate of TB disease burden than of protection (Mittrücker et al., 2007). Similarly, an increase in IFN γ -secreting T cells were found to predict the latent TB

patients *most likely* to progress to active disease (Diel et al., 2011). At least one study associates “polyfunctional” CD4⁺ T cells (secreting 2 or more T-helper type 1 (Th1) cytokines) with protection against TB, by evaluating these T cell functions in patients with active TB over their course of treatment (Sutherland et al., 2010). However, confounders included differences in the activation states of the T cells that were compared, the effect of HIV on T cell function, the use of peptide stimulation in the quantification of cytokines, and the low frequencies of T cells found to secrete cytokines. A well-controlled study in mice comparing T cells elicited by a series of TB vaccines also did not find increased numbers of polyfunctional memory T cells to correlate with the ability to control Mtb growth (Tchilian et al., 2009). Finally, the MVA85A study group suggested that IFN γ secretion (McShane et al., 2004) and polyfunctional T cells (Scriba et al., 2010) in vaccinated individuals were markers that herald a protective response by vaccine-elicited T cells. However, this vaccine was not able to reduce the incidence of active TB in vaccinated individuals (Tameris et al., 2013). A transcriptional signature of host gene expression associated with risk for active TB in latently-infected individuals was recently published (Zak et al., 2016). These hits were determined from RNAseq of PBMCs obtain from the blood of latent TB cases and control patients, followed to determine which would develop active TB. Although these markers negatively correlated with protection, data such as these give us a better understanding for how markers of immune function can reveal a detrimental response or correlate with protection during TB.

The role of CD8⁺ T cells in tuberculosis

The adaptive immune response to Mtb is comprised predominantly of a T helper type 1 (Th1) cytokine response, as most CD4⁺ T cells elicited by infection secrete IFN γ (Fig 1.2a). CD8⁺ T cells have also been shown to be essential for optimal control of Mtb infection (Behar et al., 1999; C. Y. Chen et al., 2009; Flynn et al., 1992; Lin and Flynn, 2015; Moguees et al., 2001), in addition to the impact of CD4⁺ t cells in the host defense against TB. Although the survival impact on mice depleted of CD8⁺ T cells is more modest than CD4⁺ T cell depletion during Mtb infection (Moguees et al., 2001), we do not yet know which T cell subsets or functions are most important for a protective TB vaccine. CD8⁺ T cells are prime vaccine candidates for the prevention of disease since they are already believed to enforce latency in humans (Bruns et al., 2009; van Pinxteren et al., 2000), and play a larger role in protective immunity in non-human primate models (C. Y. Chen et al., 2009) compared with mouse models of infection.

In addition to cytokine production, CD8⁺ T cells, have the capacity to kill cells that they recognize. In contrast to NK cell mediated killing, T cell killing is generally TCR-dependent. CD8⁺ T cells with the capacity to kill target cells are called cytotoxic T lymphocytes (CTLs). CD8⁺ T cells are elicited during Mtb infection in people and animal models and these CD8⁺ T cells behave as CTLs *in vivo* (Kamath et al., 2004). There exist three dominant molecular pathways that mediate CTL activity: 1) cytotoxic granule exocytosis; 2) Fas/FasL (CD95/CD95L);

and 3) TNF secretion (Fig 1.2b). All three of these mechanisms are used in a hierarchical manner to kill target cells in *M. tuberculosis* infected mice (Woodworth et al., 2008). The increased susceptibility of Fas^{-/-}, FasL^{-/-} and perforin^{-/-} mice to *M. tuberculosis* corroborate the importance of these pathways for immunity (Turner et al., 2001; Woodworth et al., 2008). Importantly, perforin is required for protection mediated by CTLs (Woodworth et al., 2008). Human CD8⁺ T cells require perforin to restrict *M. tuberculosis* growth in vitro, with granulysin being an important granule constituent (Stenger et al., 1998). Other than perforin, the crucial effector molecules for murine CD8⁺ T cells are unknown. All three killing mechanisms used by CTLs induce target cell apoptosis, which is associated with reduced bacterial viability (Behar et al., 2010). Following apoptosis, the engulfment of apoptotic, infected cells by uninfected macrophages – a process known as efferocytosis – targets bacteria trapped in the phagocytosed apoptotic cell (the ‘efferosome’) to lysosomes, which leads to killing of *M. tuberculosis* (Hartman and Kornfeld, 2011; C. J. Martin et al., 2012; 2014).

In addition to their ability to directly kill Mtb-infected cells and secrete cytokines in response to antigen presented by class I MHC, CD8⁺ T cells can recognize infected cell types other than professional APCs, such as lung epithelial cells (Harriff et al., 2014). Furthermore, CD8⁺ T cell responses are an important measure of the protective capacity of new vaccines in clinical trials (Kagina et al., 2014; Penn-Nicholson et al., 2015). The recent lack of protection found in clinical trials using MVA85A or *M. bovis* BCG vaccines, primarily eliciting CD4⁺ T cell

responses, highlights our need to consider the importance of alternate T cell subsets and antigens in vaccine design (Lin and Flynn, 2015; Orme, 2006; Tameris et al., 2013).

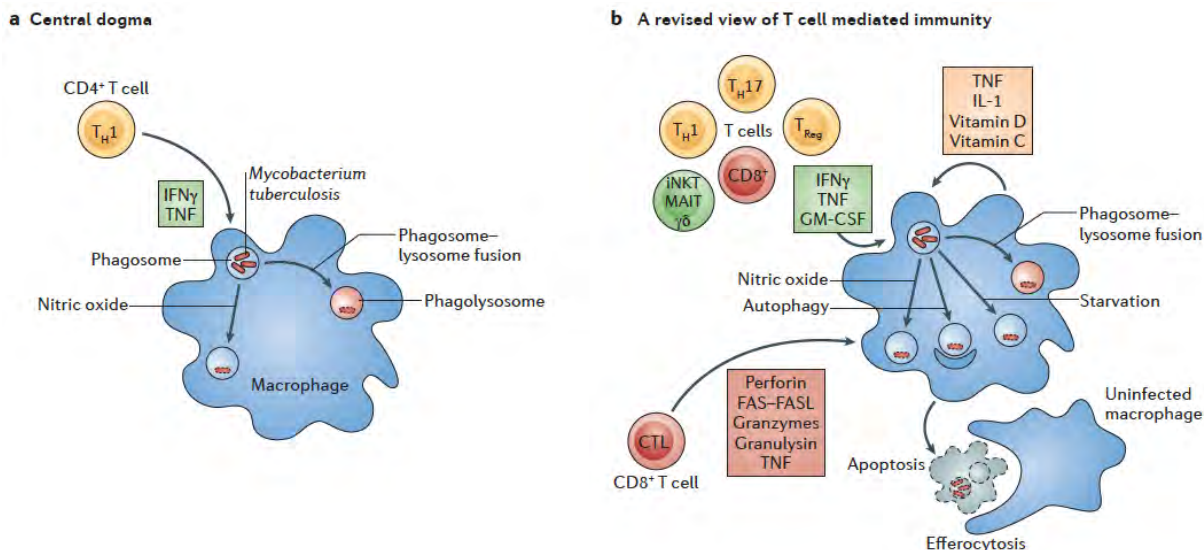


Figure 1.2: Paradigms of protective immunity to TB. (a) The ‘central dogma’ of protective immunity to tuberculosis (TB) is that $CD4^+$ T cells produce interferon- γ (IFN γ) (T helper 1 (TH1) cells), which synergizes with tumor necrosis factor (TNF; produced by the T cell or the macrophage), and together these activate macrophage antimicrobial activity that is capable of restricting the growth of *Mycobacterium tuberculosis*. Two pathways activated by IFN γ that are capable of killing *M. tuberculosis* are nitric oxide production and phagosome-lysosome fusion, which acidifies the bacterial phagosome. (b) ‘A revised view of T cell-mediated immunity’ incorporates additional T cell subsets ($CD4^+$ T cells, $CD8^+$ T cells and unconventional T cells: $\gamma\delta$ T cells, mucosal-associated invariant T (MAIT) cells and CD1-restricted T cells) and includes additional mechanisms by which T cells mediate killing of *M. tuberculosis*. These include additional cytokines (for example, granulocyte–macrophage colony-stimulating factor (GM-CSF)) and cytolysis of infected macrophages. The cytolytic mechanisms vary and can include cytotoxic granules, which can deliver antimicrobial peptides, such as granulysin, but can also deliver granzymes, which can trigger apoptotic cell death. Cytotoxic T lymphocyte (CTL) activity mediated by FAS ligand (FASL)-FAS or TNF can also lead to apoptosis. Apoptosis can have a beneficial effect on the outcome of infection, as infected apoptotic cells can be engulfed by bystander macrophages, which are capable of destroying the apoptotic cells, including any intracellular bacteria. Finally, several components of the innate response, including interleukin-1 (IL-1) and vitamins, can synergize with cytokines that are produced by T cells.

The TB10.4-specific CD8⁺ T cell response

The *Mycobacterium tuberculosis* genome contains hundreds of epitopes that have the potential for recognition by CD8⁺ T cells (Blythe et al., 2007). The CD8⁺ T cell response against Mtb is heavily focused on the secreted protein antigen TB10.4 (EsxH; Rv0288) in people and experimentally infected animals (Axelsson-Robertson et al., 2013; 2010; Billeskov et al., 2007; Hoang et al., 2009; Majlessi et al., 2003; Woodworth et al., 2011). Following aerosol infection of C57BL/6 mice, 30-50% of the responding CD8⁺ T cells in the lungs recognize the H2-K^b-restricted epitope TB10.4₄₋₁₁, defining it as an immunodominant epitope (Billeskov et al., 2007; Carpenter et al., 2016; Nunes-Alves et al., 2015; Woodworth et al., 2008). Although we have found CD8⁺ T cells specific for TB10.4₄₋₁₁ to lower bacterial burdens in T cell deficient mice aerosol-infected with Mtb (Carpenter et al., 2016; Nunes-Alves et al., 2015), vaccination against this epitope alone has not yielded protection (Carpenter et al., 2016; Lindenstrøm et al., 2014). One caveat to the study of CD8⁺ T cells during TB is the difficulty in distinguishing protective from non-protective CD8⁺ T cells specific for individual antigens. The reason for this is that the ability of individual groups of antigen-specific CD8⁺ T cells to reduce Mtb bacterial burden is dwarfed by that of CD4⁺ T cells, and the rest of the primary immune response as a whole, during the first few months after infection. On the other hand, generating T cells in mice (ie. vaccination, adoptive transfer) that elicit a modest reduction in bacterial CFUs four weeks after Mtb challenge does not always correlate with prolonged survival (Jung et al., 2005; Kamath and Behar,

2005), or improvement in disease burden. Therefore, the discovery of new functional markers of protective T cells is critical, and applies equally to CD8⁺ and CD4⁺ T cells during TB.

The antigen-specific T cell receptor (TCR) repertoire

The adaptive immune system has the capacity generate $>10^{15}$ unique TCRs (M. M. Davis and Bjorkman, 1988; Sewell, 2012), enabling the recognition of an enormous set of distinct foreign (and self) peptide antigens (Jenkins et al., 2010; Venturi et al., 2008). The TCR repertoire found in each individual are selected in the thymus from the presentation of peptides on their unique repertoire of major histocompatibility complex (MHC) molecules (M. M. Davis et al., 1998). Of the vast number of possible unique TCRs, only a subset are represented in the approximately 10^7 - 10^8 (mouse) and 10^{11} - 10^{12} (human) total T cells within each individual (Arstila et al., 1999; Casrouge et al., 2000; Jenkins et al., 2010). Of these, approximately 2×10^6 (mouse) (Casrouge et al., 2000; Jenkins et al., 2010) and 1×10^8 (human) (Arstila et al., 1999) T cells are calculated to be unique clones containing distinct complementarity-determining 3 (CDR3) sequences. In the mouse, this estimate indicates an average of ~ 30 copies of each antigen-specific T cell clone, and between 5 - 500 unique naïve precursor clones specific for a particular antigen (Casrouge et al., 2000; Jenkins et al., 2010). As discussed in Chapter 3, this is particularly relevant to tracking the frequency of memory and naïve T cells specific for Mtb antigens, especially CD8⁺ T cells specific for the

TB10.4 antigen. In fact, we have previously estimated the *naïve* precursor frequency in C57BL/6 mice to be ~800 T cells per mouse, which is relatively high at ~1 of every 20,000 CD8⁺ T cells (Nunes-Alves et al., 2015). This frequency is within the range estimated by others of the naïve precursor frequencies in mice for various antigens: B8R (vaccinia virus), ovalbumin, and antigens from HSV-1 and LCMV, range from ~15 – 1100 naïve T cell precursors per mouse (Haluszczak et al., 2009; Jenkins et al., 2010; Kotturi et al., 2008; Obar et al., 2008). Based on work studying the factors that lead to antigenic immunodominance (W. Chen et al., 2001; Kotturi et al., 2008; Moon et al., 2007; Yewdell, 2006; Yewdell and Del Val, 2004), high precursor frequency is likely a major contributor to the immunodominance of TB10.4 among Mtb antigens.

Despite significant knowledge of antigen recognition and the genetic and structural basis for TCR diversity, we have a relatively primitive understanding of the forces behind the magnitude and diversity of T cell responses to microbial antigens. The pathogen-specific T cell response usually focuses on a small number of the available antigenic epitopes, and uses a narrow TCR repertoire (Jenkins and Moon, 2012; Kotturi et al., 2008; Moon et al., 2007; Yewdell, 2006). Pathogens have numerous strategies to evade host immunity, hindering our ability to determine how T cell diversity relates to antimicrobial immunity. For pathogens that rapidly mutate, such as the Human Immunodeficiency Virus (HIV), a diverse T cell response could benefit the host by efficiently detecting escape mutants, while a biased response could be detrimental. For slowly replicating pathogens,

that encode numerous antigens, such as Mtb, the relationship between diversity and protection is less clear.

While TCR diversity serves to characterize a population of T cells responding to immunodominant microbial antigens, TCR affinity can be used to attribute function to individual CDR3 amino acid sequences when exposed to similar amounts of antigen presented by MHC molecules. TCR affinity affects both the strength of the TCR : peptide-MHC (pMHC) interaction, as well as the “dwell-time” of peptide-MHC complexes of both CD4⁺ and CD8⁺ T cells (Aleksic et al., 2010; Govern et al., 2010; Tubo et al., 2013). For the purpose of this dissertation, “dwell-time” and “confinement time” will be used interchangeably, defined here as the *cumulative* time the TCR and pMHC are engaged during an interaction that has the potential to activate the T cell. TCR affinity can govern the function of individual T cell clonotypes, and therefore affect protective capacity, not only due to the strength of its interaction with pMHC (measured as K_D), but by its effect on the dwell-time of the TCR : pMHC interaction (Aleksic et al., 2010; Govern et al., 2010; Tubo et al., 2013). TCR affinity and dwell-time appear to play a larger role in governing T cell function under conditions of low or limiting antigen (Vanguri et al., 2013). While there is little definitive data in tuberculosis, we have associated both high- and intermediate-affinity naïve TB10.4-specific CD8⁺ T cell clonotypes with control of bacterial growth, but have found significantly greater expansion of the high-affinity T cells during infection (Nunes-Alves et al., 2015). Gallegos et al., however, report no benefit to higher-affinity CD4⁺ T cells specific for another Mtb

antigen (ESAT-6) (Gallegos et al., 2016). Although the two T cell subsets, and antigens, are different, the almost certainly greater abundance of the latter antigen (Skjøt et al., 2000; 2002) likely plays a prominent role in determining the conditions for which increased TCR affinity and TCR-pMHC dwell-time kinetics play the greatest role (Vanguri et al., 2013).

In addition to TCR structure, other factors appear to regulate the activation of memory CD8⁺ T cells. In addition to TCR-pMHC affinity and kinetics, antigen availability has been recently shown to exert a strong influence over CD8⁺ T cell activation (Mehlhop-Williams and Bevan, 2014). Whether differences in TCR structure and antigen availability affect the ability of memory T cells to control tuberculosis is unknown. We observe differences in the function of *memory* (and secondary effector) CD8⁺ T cells that are dependent on the affinities of their TCRs. Using a combination of adoptive transfer of TCR retrogenic CD8⁺ T cells specific for TB10.4₁₁ (TB10Rg3 and TB10Rg4) (Nunes-Alves et al., 2015) and TCRβ deep sequencing of tetramer⁺CD8⁺ T cells after vaccination, and Mtb-challenge, in intact mice, we track primary (1°, those expanding from naïve T cells) and secondary (2°, from memory precursors) TB10.4-specific CD8⁺ T cells during infection. Compared with naïve CD8⁺ T cells, we observe that memory CD8⁺ T cells exhibit a decreased sensitivity to antigen that impairs their ability to expand in response to antigenic stimulation during tuberculosis. As a result, memory CD8⁺ T cells are unable to efficiently expand, compared with the primary response, making vaccine-elicited T cells unable to substantially contribute to host protection

in the first 2-3 weeks after aerosol Mtb challenge. Based on the data presented in the following chapters, I propose that the TCR affinity of memory T cells correlates with their capacity to protect the host from TB. An inability to compete with the primary immune response to Mtb is a major limitation; I argue that this mechanism limits their contribution to counter Mtb infection. These data imply that generating higher-affinity memory T cells could improve TB vaccine design. Furthermore, the use of memory T cell expansion after Mtb challenge may be a useful benchmark for identifying protective TB vaccine candidates during vaccine development.

Preface to Chapter II

This chapter has been adapted from the recently published manuscript:

Carpenter, S. M., Nunes-Alves, C., Booty, M. G., Way, S. S., & Behar, S. M. (2016). A Higher Activation Threshold of Memory CD8+ T Cells Has a Fitness Cost That Is Modified by TCR Affinity during Tuberculosis. *PLoS Pathogens*, 12(1), e1005380. <http://doi.org/10.1371/journal.ppat.1005380>

With additional data (including Figure 2.10) included from:

Nunes-Alves, C., Booty, M. G., Carpenter, S. M., Rothchild, A. C., Martin, C. J., Desjardins, D., Steblenko K., Kløverpris H.N., Madansein R., Leslie A., Correia-Neves M., Behar S. (2015). Human and Murine Clonal CD8+ T Cell Expansions Arise during Tuberculosis Because of TCR Selection. *PLoS Pathogens*, 11(5), e1004849. <http://doi.org/10.1371/journal.ppat.1004849>

The exact figures and their organization are different from those in the published manuscripts, and additional data are included here. This manuscript represents the first part of the main thesis project of Stephen Carpenter, who designed and performed the experiments, analyzed the data, and wrote the manuscript with contributions from co-authors and mentorship from Dr. Samuel Behar.

CHAPTER II

A Higher Activation Threshold of Memory CD8⁺ T Cells Has a Fitness Cost That Is Modified By TCR Affinity During Tuberculosis

Introduction

A TB vaccine is urgently-needed for the prevention of active tuberculosis. TB10.4 (EsxH, Rv0288) is an immunodominant Mtb antigen recognized by human (Axelsson-Robertson et al., 2010) and murine (Woodworth et al., 2008) CD8⁺ T cells. TB10.4 is already being tested in clinical trials (Geldenhuys et al., 2015) as a TB vaccine candidate since it is an essential gene and a secreted protein antigen of Mtb (Sasseti et al., 2003). TB10.4-specific CD8⁺ T cells account for 30-50% of all CD8⁺ T cells in the lungs of infected C57BL/6 and BALB/c mice. TB10.4-specific CD8⁺ T cells can confer protection against Mtb after adoptive transfer into mice lacking $\alpha\beta$ T cells (Nunes-Alves et al., 2015), indicating that cells of this antigen specificity have the ability to attenuate bacterial growth. Although the protective capacity of antigen-specific CD8⁺ T cells is difficult to distinguish among the dominant CD4⁺ T cell response in intact mice, T cell vaccination that elicited a robust TB10.4-specific CD8⁺ T cell response did not protect mice from tuberculosis (Lindenstrøm et al., 2014). We find that TB10.4-specific memory CD8⁺ T cells elicited by vaccination undergo early and robust expansion after aerosol Mtb challenge, however the number of TB10.4-specific CD8⁺ T cells is similar to that

of unvaccinated mice within four weeks. Using TCR retrogenic CD8⁺ T cells, we compare naïve and memory CD8⁺ T cells expressing the same TCR and observe that both the 1^o (naïve) and the 2^o (memory) CD8⁺ T cell responses are initiated in the draining lymph node at ~d11 post-infection. Following the activation of the TB10.4-specific CD8⁺ T cells, the 2^o effector response does not rapidly expand in response to infection, but initially has the same kinetics as the 1^o response. As the T cells are recruited to the lung, we also observe the 2^o response becomes outnumbered 99:1 by a highly-proliferative 1^o response, indicating that TCR-independent factors cause the memory-recall response to be less fit than the primary response during chronic infection. As we observe 2^o effector CD8⁺ T cells to have a reduced proliferative response, particularly in chronic, low antigen settings, we speculate that effective T cell vaccines for tuberculosis will need to elicit high affinity TCRs and respond earlier than the primary response to infection.

Results

Vaccination elicits TB10-specific memory CD8⁺ T cells that expand after Mtb challenge.

Vaccination with the peptide epitope TB10.4₁₁ (TB10), anti-CD40 mAb and poly(I:C), a vaccination strategy used in multiple infection and tumor models (Ahonen et al., 2004; Cho and Celis, 2009; S. Lee et al., 2012; Phoolcharoen et al., 2011), generates a large number of TB10-specific memory CD8⁺ T cells in C57BL/6 mice. Boosting leads to an additional 10-fold expansion such that TB10-

specific CD8⁺ T cells comprise ~10% of circulating CD8⁺ T cells (Fig 2.1a). Eight weeks after boosting, 1.5-2% of the circulating CD8⁺ T cells are specific for TB10 (Fig 2.1b). As described, this vaccine formulation represents a powerful and simple strategy to elicit high-frequency memory CD8⁺ T cell responses to multiple different tumor and viral epitopes under inflammatory conditions, and the T cells it generates are potent CTLs shown to eradicate melanoma lung metastases, and lower viral loads in both Ebola and RSV model infections (Ahonen et al., 2004; Cho and Celis, 2009; S. Lee et al., 2012; Phoolcharoen et al., 2011). The TB10 tetramer⁺CD8⁺ T cells elicited one week after priming are predominantly KLRG1^oIL-7R^h, the phenotype of memory precursor effector cells (MPECs) (Joshi et al., 2007; Kaech et al., 2003). After boosting, ~50% express KLRG1 but low IL-7R levels, characteristic of terminally-differentiated effectors (Fig 2.1c), with early effector cells (Obar et al., 2011) (EECs, KLRG1^oIL-7R^o) and MPECs each comprising ~20% of TB10 tetramer⁺CD8⁺ T cells. Eight weeks later, TB10-specific CD8⁺ T cells are predominantly IL-7R^h and ~50% express CD62L (Fig 2.1c). The majority of the TB10-specific CD8⁺ T cells express CXCR3, a chemokine receptor associated with CD27/CD70-dependent clonal expansion during priming (Peperzak et al., 2013), as well as trafficking of memory T cells to the airway during inflammation (Slütter et al., 2013) (Fig 2.1c). Thus, the TB10 / α CD40 / poly(I:C) vaccination strategy elicits large numbers of TB10-specific central memory and effector memory CD8⁺ T cells.

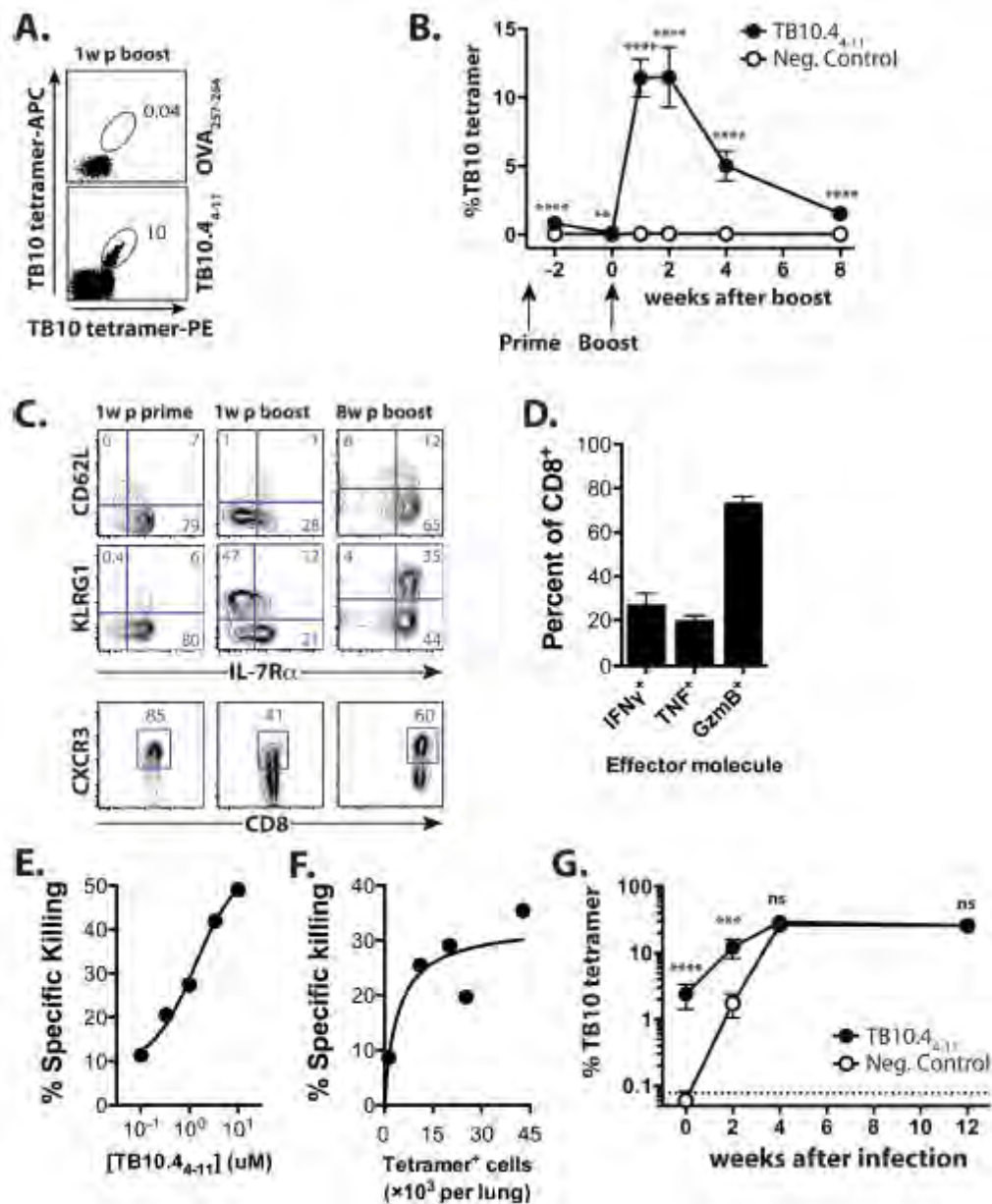


Figure 2.1: TB10 vaccination elicits memory CD8⁺ T cells that generate 2^o effectors during Mtb infection. (a) The TB10 tetramer⁺ response enumerated by dual-tetramer staining in blood 1 week post-boost with TB10₄₋₁₁ or Ova₂₅₇₋₂₆₄ (control) vaccination. Numbers indicate the % of CD8⁺ T cells. (b) TB10 tetramer⁺ responses from blood after TB10₄₋₁₁ or control (B8R₂₀₋₂₇ or Ova₂₅₇₋₂₆₄) vaccination at time points post-boost. (c) Representative plots showing CD62L, KLRG1, IL-7R, and CXCR3 expression by TB10 tetramer⁺ CD8⁺ T cells from blood 1w after prime, and 1w or 8w after boost. (d) Ex vivo TB10₄₋₁₁-stimulated production of IFN γ , TNF, or granzyme B from CD8⁺ T cells isolated from combined lungs, spleens, or LNs of TB10-vaccinated mice. (e) In vivo specific killing

of targets coated with TB10₄₋₁₁ peptide. **(f)** In vivo specific killing of 1 μ M TB10₄₋₁₁-coated targets vs. TB10-specific response. **(g)** TB10 tetramer responses of mice vaccinated with TB10₄₋₁₁ or the control peptide B8R₂₀₋₂₇ immediately prior, or 2w, 4w, or 12w after Mtb infection. **** p < 0.0001, *** p < 0.001, by two-way ANOVA with Sidak's post test. Data are representative of 3-6 independent experiments, each with 4-6 mice per group.

Vaccination with peptide / α CD40 / poly(I:C) elicited TB10-specific memory CD8⁺ T cells that secrete IFN γ and TNF after *ex vivo* restimulation with the TB10 peptide, or express granzyme B (Fig 2.1d). Vaccine-elicited TB10-specific CD8⁺ T cells also efficiently lysed peptide-loaded targets *in vivo* in a dose-dependent manner (Fig 2.1e, f). These data show that the effector functions expressed by vaccine-elicited CD8⁺ T cells are similar to those possessed by CD8⁺ T cells elicited by Mtb infection (Kamath et al., 2006). To determine the function of these memory T cells during infection, we vaccinated with TB10 or an irrelevant peptide (B8R₂₀₋₂₇ from vaccinia (Baur et al., 2010)) and eight weeks after the boost, challenged the mice with Mtb. A discrete population of TB10-specific CD8⁺ T cells was detected in the lungs of the TB10 vaccinated mice even before infection (Fig 2.1g). Two weeks after Mtb challenge, TB10-specific CD8⁺ T cells were more abundant in the lungs of TB10-vaccinated mice compared to the B8R-vaccinated group, although there was no difference in the number of tetramer⁺ CD8⁺ T cells between the two groups by four weeks (Fig 2.1g). Thus, by the peak of adaptive immunity in C57BL/6 mice (4 wpi), there was no difference in the number of TB10-specific CD8⁺ T cells in the lungs of vaccinated and control-vaccinated mice, despite the effectiveness of vaccination with TB10 / CD40 / poly(I:C) in eliciting numerous functional memory CD8⁺ T cells.

Although vaccine-elicited TB10-specific CD8⁺ T cells were potent effectors and expanded early during infection, no differences were detected in the bacterial burden of vaccinated versus control mice (Fig 2.2a). We next sought to determine whether the lack of protection was related to insufficient numbers of memory CD8⁺ T cells prior to infection. As lipophilic vaccine adjuvants increase antigenicity (Liu et al., 2014), we modified the TB10 epitope by adding the hydrophobic amino acid residues 'MFVMFVQ' to the N-terminus of the minimal epitope of TB10. Eight weeks after priming and boosting with this amphiphilic peptide, denoted here as amphi-TB10, a greater proportion (20-45%) of circulating CD8⁺ T cells were specific for TB10, the majority of which were MPECs (Fig 2.2b). Despite a more robust response, neither prime-only nor prime-boost vaccination with amphi-TB10 increased antigen-specific CD8⁺ T cell frequency or attenuated bacterial growth compared to sham-vaccinated mice by 4wpi (Fig 2.2c, d).

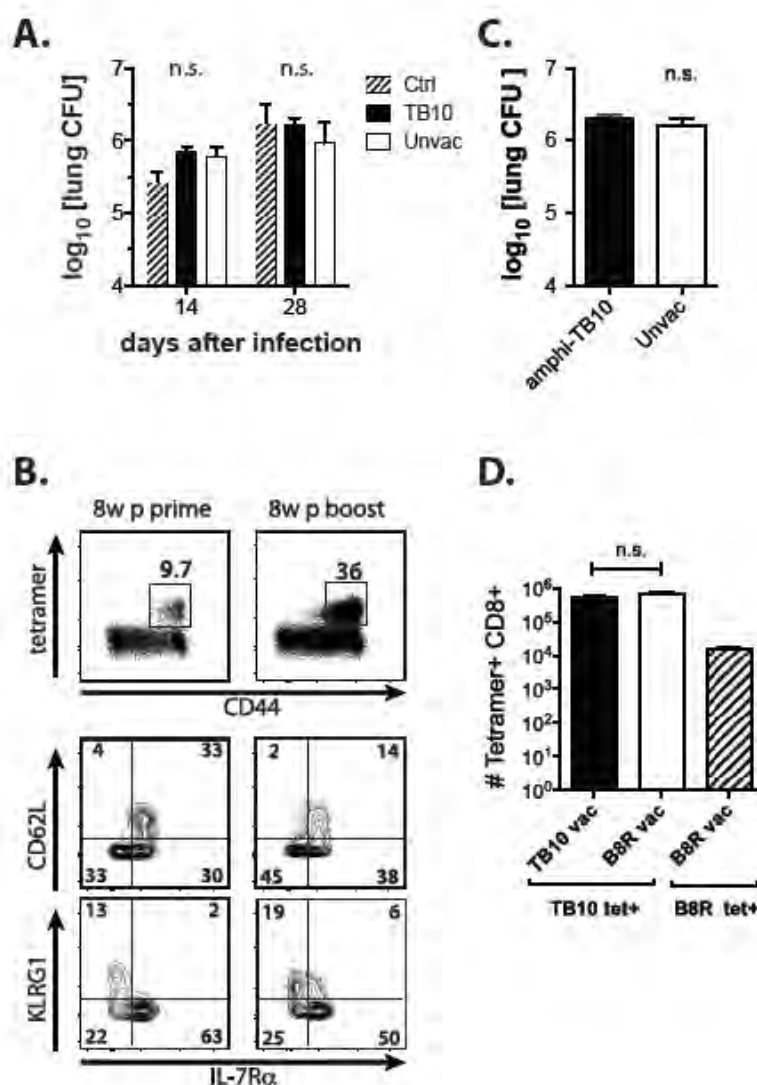


Figure 2.2: Vaccination with TB10₄₋₁₁ does not protect mice against Mtb infection. (a) Lung CFU 14d and 28d after Mtb infection of TB10₄₋₁₁ vaccinated, control vaccinated (B8R₂₀₋₂₇ or Ova₂₅₇₋₂₆₄), or unvaccinated mice. (b) Peripheral blood TB10 tetramer⁺ responses 8w post-prime or 8w post-boost with amphi-TB10 vaccination. CD62L, KLRG1, and IL-7R expression by tetramer⁺ CD8⁺ T cells is shown for each time point. (c) Lung CFU 28d after Mtb infection of amphiphilic-TB10₄₋₁₁ (amphi-TB10) or B8R₂₀₋₂₇ vaccinated mice. Bacterial counts were \log_{10} -transformed and compared using a student's t-test or one-way ANOVA. (d) Lung tetramer⁺ responses from amphi-TB10 prime/boost-vaccinated (3 weeks apart), negative control-vaccinated (B8R), or unvaccinated mice 28d after Mtb infection. n.s., not significant. Data are representative of 3 - 6 independent experiments, each with 4 - 6 mice per group.

Finally, to determine whether the progressive decrease in abundance of memory CD8⁺ T cells during TB, or their inability to control bacterial CFU, are related only to those specific for the TB10.4 antigen, we evaluated the response of a second Mtb antigen. The H2-D^b-restricted epitope GAPINSATAM (Mtb32A₃₀₉₋₃₁₉) (Irwin et al., 2005), referred to hereafter as Mtb32a, is known to elicit CD8⁺ T cells after aerosol Mtb infection. Therefore, mice were vaccinated using Mtb32a peptide together with αCD40 mAb and poly(I:C), which generated a robust response 1 week after boost (Fig 2.3a, left panel) and contracted to a smaller cohort of memory T cells by 8 weeks (Fig 2.3a, right panel), at which point they were aerosol-infected with Mtb. Four weeks after Mtb aerosol challenge, the numbers of Mtb32 tetramer⁺ CD8⁺ T cells in vaccinated and unvaccinated mice were again the same (Fig2.3b), and no difference in bacterial growth in the lungs was observed (Fig 2.3c). Thus, despite being highly functional, as measured by their expression of IFN γ , TNF, and granzyme B after stimulation, their CTL activity, their abundance 8-12 weeks after the boost, and their response to Mtb aerosol challenge, memory CD8⁺ T cells elicited by peptide / αCD40 mAb / poly (I:C) vaccination are unable to maintain their abundance or control bacterial CFU in intact mice after aerosol Mtb infection. Together, these data raise the possibility that the inability of 2^o effector CD8⁺ T cells to predominate in response to Mtb is related to a failure of memory CD8⁺ T cells to optimally expand rather than insufficient numbers prior to infection.

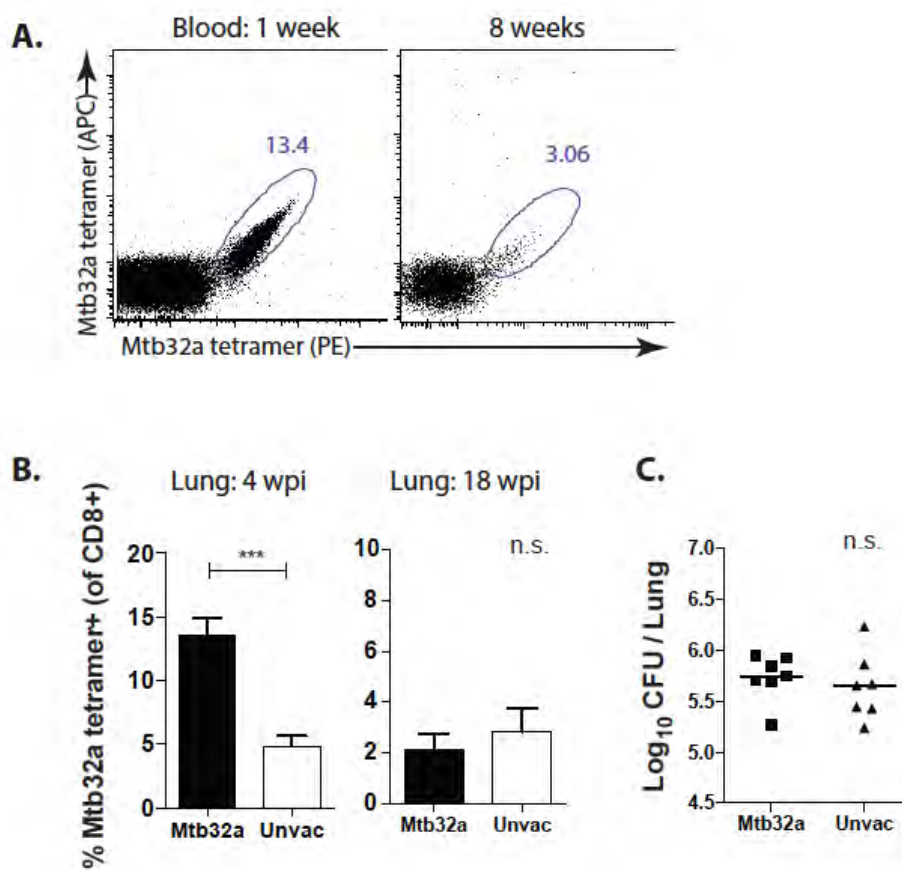


Figure 2.3: Vaccination against the Mtb32a antigen does not lead to protection or a greater frequency of memory-derived T cells. (a) Representative flow cytometry plots of the proportion of TB10 tetramer+ CD8⁺ T cells (gated on CD8⁺) from the blood of mice 1 week (left panel) or 8 weeks (right panel) after prime / boost vaccination. (b) Bar graphs of the proportion of Mtb32a-specific CD8⁺ T cells (mean ± SEM) in the lungs of Mtb32a-vaccinated vs. unvaccinated mice 4 weeks post aerosol Mtb infection (left), or 18 weeks post-infection (right panel). (c) Lung CFU (mean ± SEM) in Mtb32a-vaccinated vs. unvaccinated mice 4 weeks post-infection. *** $p < 0.001$, n.s. not significant by the student's t-test. Data are representative of 3 independent experiments, each with 4-7 mice per group.

Direct comparison of memory and naïve T cells using TCR retrogenic TB10-specific CD8⁺ T cells

To directly compare how memory and naïve TB10-specific CD8⁺ T cells behave during Mtb challenge, we used TCR retrogenic (Rg) mice producing CD8⁺ T cells specific for TB10 (TB10Rg3) (Nunes-Alves et al., 2015). Vaccination increased the frequency of TB10Rg3 CD8⁺ T cells (GFP⁺V α 2⁺), and 60-70% of expressed CD44 compared with \leq 5% in unvaccinated mice (Fig 2.4a). After 8 weeks, TB10Rg3 cells contracted into a uniform population of central memory T cells (CD62L^hIL-7R^h) (Fig 2.4b).

We previously found that the adoptive transfer of activated TB10Rg3 CD8⁺ T cells reduced bacterial CFU and prolonged the survival of susceptible mice (Nunes-Alves et al., 2015). Here we compared the protective capacity of flow-sorted naïve (GFP⁺V α 2⁺CD44^o cells) or memory (GFP⁺V α 2⁺CD44^H) TB10Rg3 CD8⁺ T cells by transferring 10⁵ of each into TCR α ^{-/-} mice and challenging with Mtb. Both naïve and memory TB10Rg3 CD8⁺ T cells expanded and differentiated into terminally-differentiated effectors (KLRG1^HIL-7R^o) and EECs (KLRG1^{Lo}IL-7R^o), with a small population of MPECs (KLRG1^{Lo}IL7R^H), and produced IFN γ after restimulation in vitro (Fig 2.4c, d). Naïve, effector, and memory TB10Rg3 CD8⁺ T cells transferred protection to immunodeficient mice (Fig 2.4e, f), indicating that these cells have the potential to independently function as effector T cells and attenuate infection.

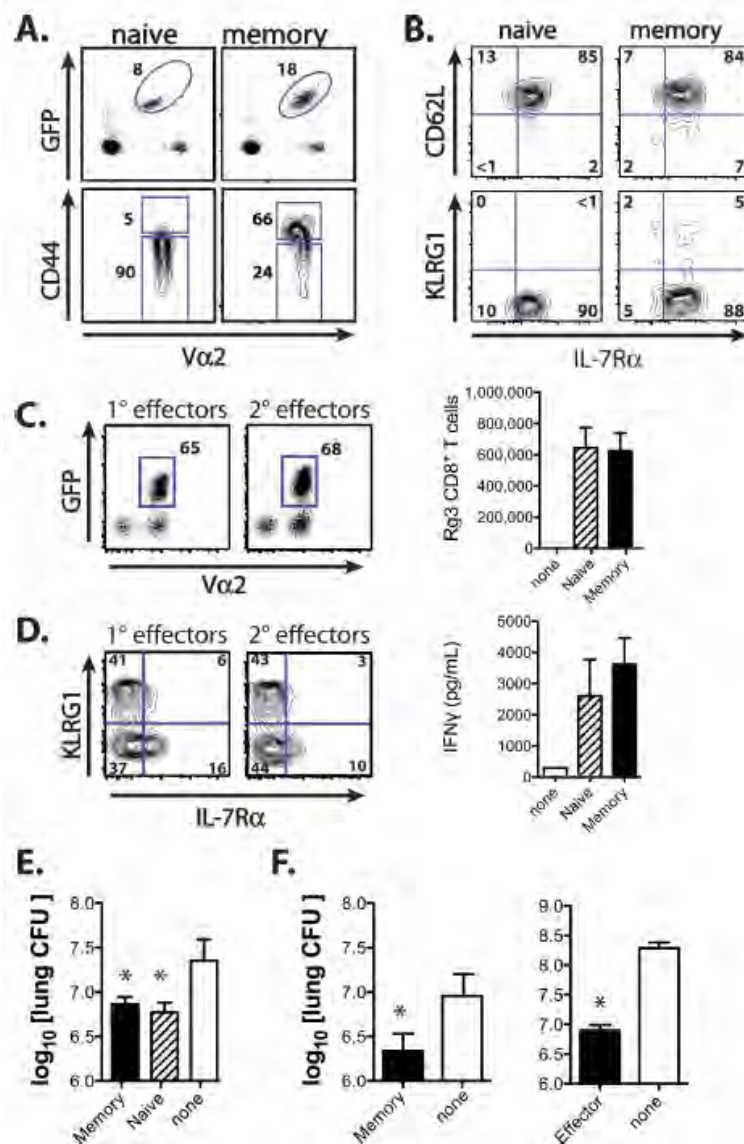


Figure 2.4: TCR retrogenic TB10-specific CD8⁺ T cells allow direct comparison of the 1° and 2° responses during infection. (a) The proportion of TB10Rg3 cells (%GFP⁺Vα2⁺) among CD8⁺ T cells and their CD44 expression, 8w after vaccination of TCR Rg mice (memory) or age-matched unvaccinated TCR Rg mice (naïve). (b) CD62L, KLRG1, and IL-7R expression of naïve TB10Rg3 cells, and 8w after a single immunization with TB10₄₋₁₁. (c) Proportion (left) and absolute numbers (right) (mean ± SEM) of TB10Rg3 cells in the lungs of TCRα^{-/-} mice 28d after adoptive transfer of 10⁵ naïve or memory TB10Rg3 CD8⁺ T cells and Mtb infection. (d) *Ex vivo* KLRG1 and IL-7R expression by TB10Rg3 cells (left) and IFN γ production after *ex vivo* stimulation of lung cells [from (c)] with TB10₄₋₁₁ peptide (right). (e) Lung CFU (mean ± SEM) of TCRα^{-/-} mice 28d after transfer of naïve or memory TB10Rg3 CD8⁺ T cells and Mtb infection. (f) Lung CFU of sub-lethally irradiated C57BL/6 mice 21d after Mtb infection and transfer of *memory*

TB10Rg3 (8w post-vaccination; left) or *effector* TB10Rg3 (1w post-vaccination; right). Numbers in quadrants or gated regions represent percent events. CFU were \log_{10} -transformed before a student's t-test or one-way ANOVA with a Bonferroni post-test. Data are representative of 2–4 independent experiments, each with 5 mice per group. * $p < 0.05$. n.s., not significant.

Although memory TB10Rg3 CD8⁺ T cells expanded, differentiated, and attenuated bacterial growth after adoptive transfer, the inability to distinguish the memory-recall response in intact vaccinated mice from the primary response in unvaccinated mice by 4wpi (Fig 2.1g), as well as the inability of TB10 vaccination to confer additional protection to the endogenous primary immune response (Lindenstrøm et al., 2014) (S1 Fig), led us to hypothesize that memory CD8⁺ T cells were not optimally responding in vivo. In our comparison of TB10-specific CD8⁺ T cell responses of vaccinated and unvaccinated mice, the primary response (e.g., in unvaccinated mice) appears to undergo more rapid expansion than the recall response (e.g., in vaccinated mice) (Fig 2.1g). Therefore, we developed an adoptive co-transfer model to study the 1° and 2° effector responses generated from naïve and memory TB10Rg3 CD8⁺ T cells, respectively, during Mtb challenge.

Primary effectors progressively outnumber secondary effector CD8⁺ T cells during Mtb infection

Thy1.1⁺ memory and Thy1.2⁺ naïve TB10Rg3 CD8⁺ T cells were transferred (1:1 ratio, 10⁴ cells each) into CD45.1⁺ recipient mice infected with Mtb seven days earlier, before the onset of adaptive immunity (Fig 2.5a,b). Nearly all (>80%) of the

memory TB10Rg3 CD8⁺ T cells had a central memory phenotype (CD62L^h KLRG1^oIL-7R^h), suggestive of a high proliferative potential (Fig 2.5b). As a control, the memory and naïve TB10Rg3 CD8⁺ T cells were adoptively transferred into uninfected or Mtb-infected mice and analyzed the next day. Analysis of these cells showed that they maintained their phenotype, 1:1 ratio, and based on results with the eFluor450 proliferation dye, neither group had begun to proliferate (Fig 2.5c).

While naïve T cells require priming in the lung-draining mediastinal lymph nodes (MLN) before responding to infection in the lung (Wolf et al., 2008), whether memory T cell activation occurs in the MLN or lung is unknown. By loading naïve and memory TB10Rg3 CD8⁺ T cells with eFluor450, we determined that both memory and naïve TB10Rg3 CD8⁺ T cells begin to proliferate and downregulate CD62L first in the MLN by d11 post-infection (Fig 2.5d), during which time they maintain their 1:1 ratio despite significant proliferation (see below). This timing correlates with priming of the endogenous CD8⁺ T cell response to TB10 (Nunes-Alves et al., 2015).

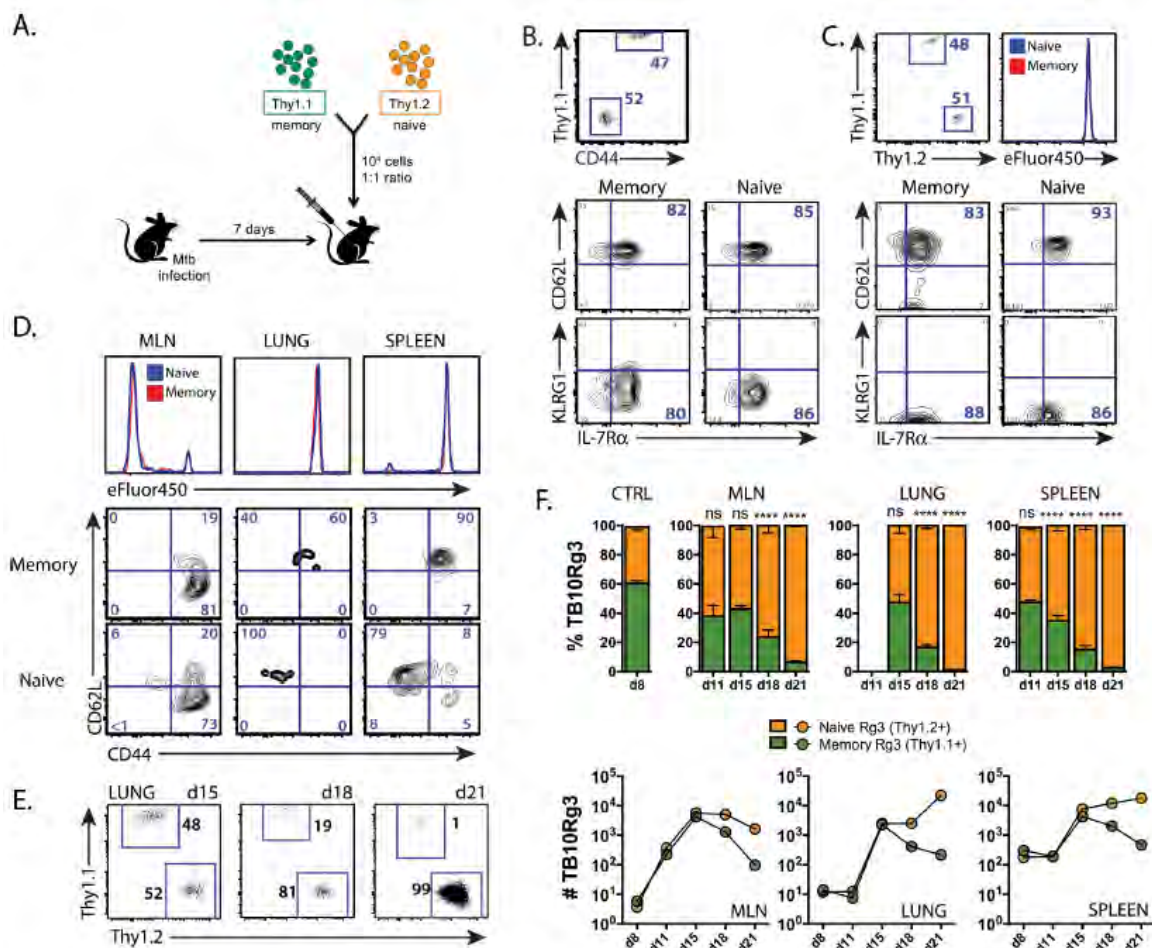


Figure 2.5: The primary response outnumbers the memory-derived secondary response during Mtb infection. (a) Experimental strategy for adoptive co-transfer experiments. Relative proportion of naïve and memory TB10Rg3 CD8⁺ T cells and their expression of CD62L, KLRG1, and IL-7R before transfer (b) and in the spleen 1d after transfer into uninfected mice (c). Baseline labeling with the eFluor450 proliferation dye is shown. (d) Concatenated histograms of eFluor450 staining of naïve and memory-derived TB10Rg3 cells in the MLN, lung, and spleen (top) and their CD62L and CD44 expression (bottom) from a representative experiment on d11 post-infection. (e) Proportion of adoptively-transferred memory (Thy1.1⁺) and naïve (Thy1.2⁺)-derived TB10Rg3 CD8⁺ T cells in the lung 15, 18, or 21d after Mtb infection. (f) The relative proportion of memory (Thy1.1) and naïve (Thy1.2)-derived TB10Rg3 CD8⁺ T cells in the MLN, lung, and spleen after infection, compared to spleens from uninfected mice 1 day after transfer (CTRL) (top). Cell numbers of memory (Thy1.1⁺) and naïve (Thy1.2⁺)-derived TB10Rg3 CD8⁺ T cells from the same mice (bottom). **** p < 0.0001, n.s. not significant, n.d. < 10 cells detected. Data are representative of 2–10 independent experiments, each with 3–4 mice per group.

Following activation in the MLN, massive expansion of naïve and memory TB10Rg3 CD8⁺ T cells occurs in the MLN, lung and spleen through day 15, still maintaining an equal ratio (Fig 2.5e,f). After day 15, the 1^o effectors (derived from naïve T cells) become dominant in all three tissues, and by day 21, the 1^o effectors outnumber the 2^o effectors (derived from memory T cells) by a ratio of 99:1 in the lung (Fig 2.5e,f). The accumulation of the 1^o effectors is driven by their ongoing proliferation and dropout of 2^o effector cells, particularly in the lung (Fig 2.5f, bottom row). Thus, the 1^o CD8⁺ T cell response expands more efficiently than the 2^o response during Mtb challenge.

To determine whether the observed phenomenon of memory TB10Rg3 CD8⁺ T cells becoming outnumbered by naïve TB10Rg3 cells during TB was specific to the method of generating memory, we compared two other vaccination strategies. In the previous experiments, we generated memory CD8⁺ T cells by direct vaccination of intact TB10Rg3 mice containing a low frequency of TB10Rg3 cells due to the concern that adoptively transferring low numbers of T cells followed by stimulation by vaccination would impair their function (Fraser et al., 2013). However, to evaluate the function of memory TB10Rg3 cells generated by a standard approach, we used the “transfer-then-vaccinate” strategy. One day after transferring 1x10⁴ naïve CD45.1⁺ TB10Rg3 CD8⁺ T cells, recipient mice were vaccinated with TB10 / αCD40 / Poly (I:C), rested for 12 weeks, and the memory TB10Rg3 CD8⁺ T cells were flow-sorted (CD8⁺ Vα2⁺ GFP⁺ CD45.1⁺ KLRG1^{Lo} CD44^h) and adoptively co-transferred (together with CD45.2⁺ naïve TB10Rg3

cells) into Thy1.1⁺ hosts 6d after aerosol infection with Mtb as in Fig 2.5a. Memory and naïve TB10Rg3 CD8⁺ T cells were injected at a ~1:1 ratio, however by d22 the naïve CD8⁺ T cells outnumbered memory CD8⁺ T cells >90:1 (Fig 2.6a). Thus, memory CD8⁺ T cells specific for TB10.4 become outnumbered even when generated using the standard transfer-then-vaccinate approach. Finally, to determine whether memory CD8⁺ T cells generated by a different vaccination strategy suffer the same fate, we adoptively transferred 1x10⁴ naïve CD45.1⁺ TB10Rg3 CD8⁺ T cells and, one day later, vaccinated recipient mice with recombinant *Listeria monocytogenes* engineered to secrete a fusion protein containing full-length TB10.4 (LmΔActA-TB10) (Curtis et al., 2010; Shafiani et al., 2013). 12 weeks later, memory TB10Rg3 CD8⁺ T cells were again flow-sorted and adoptively co-transferred (1:1 with CD45.2⁺ naïve TB10Rg3 cells) into Thy1.1⁺ hosts 6d after aerosol infection with Mtb. 22 days post-infection, the naïve CD8⁺ T cells again outnumbered memory CD8⁺ T cells >95:1 (Fig 2.6b). Thus, the observed phenomenon of naïve CD8⁺ T cells outnumbering memory CD8⁺ T cells specific for TB10.4 during TB occurs with memory T cells generated by other vaccine strategies.

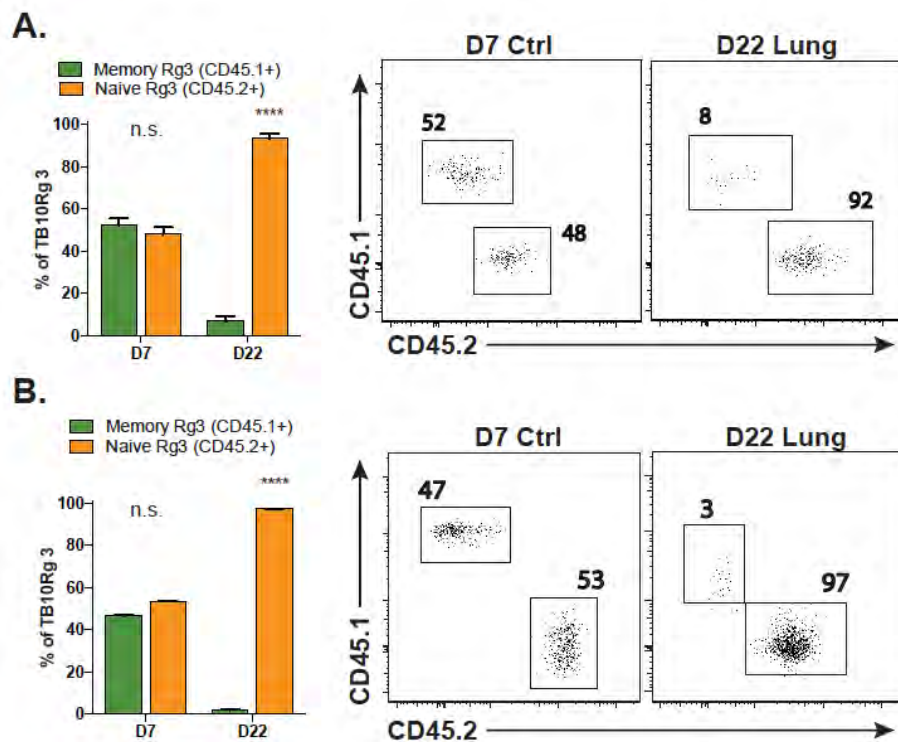


Figure 2.6: Memory TB10Rg3 CD8⁺ T cells generated by alternative methods also become outnumbered by naïve CD8⁺ T cells during Mtb infection. Bar graphs (Left) and flow cytometry plots (right) of the relative proportion of memory (CD45.1) and naïve (CD45.2)-derived TB10Rg3 CD8⁺ T cells (mean \pm SEM) in the lung 22d after infection (D22 Lung), compared to spleens from uninfected mice 1 day after transfer (D7 Ctrl) for each strategy: **(a)** Memory TB10Rg3 CD8⁺ T cells were generated by adoptive transfer of 10^4 naïve TB10Rg3 cells followed by vaccination with TB10 / α CD40 mAb / Poly(I:C) one day later, rested for 12 weeks, followed by co-transfer with naïve TB10Rg3 into Mtb-infected hosts. **(b)** Memory TB10Rg3 CD8⁺ T cells generated by adoptive transfer of 10^4 naïve TB10Rg3 cells followed by vaccination with Lm Δ ActA-TB10 one day later, rested for 12 weeks, followed by co-transfer with naïve TB10Rg3 into Mtb-infected hosts. **** $p < 0.0001$, n.s. not significant. Data are representative of 2 independent experiments, each with 4 mice per group.

Sustained proliferation of primary effectors during infection leads to their dominance

Joshi et al. find that during infection, a subset of effector CD8⁺ T cells differentiate into cells that can no longer proliferate in response to antigen and express the inhibitory receptor KLRG1, now identified as a marker of terminal differentiation (Joshi et al., 2007). We examined whether the attrition of the secondary effectors correlated with terminal differentiation. Both 1^o and 2^o effector CD8⁺ T cells were predominantly EECs (KLRG1^{Lo} IL-7R^{Lo}) at all time points (day 15-21), with slightly more terminally-differentiated effectors (KLRG1^H IL-7R^{Lo}) in 1^o effectors, rather than in 2^o effectors (Fig 2.7a), arguing against terminal differentiation as an explanation for their observed decreased rate of expansion. The attrition of secondary effector CD8⁺ T cells during infection is also independent of TCR affinity, since TCR retrogenic TB10Rg3 cells were the source of both the naïve and memory precursors. Furthermore, we found TCR expression to be equivalent between both memory and naïve-derived TB10Rg3 cells during infection (Fig 2.7b). Finally, since Mtb-specific T cells can differ in their ability to traffic to the lung (Moguche et al., 2015; Sakai et al., 2014a), we considered whether naïve and memory-derived TB10Rg3 cells might differentially home to the lung. Equal proportions of 1^o and 2^o effector CD8⁺ T cells were found in the “intravascular” or “parenchymal” compartments, as defined by intravenous administration of anti-Vα2 mAb (Fig2.7c). Thus, the 1^o and 2^o effector CD8⁺ T cell responses were able to traffic similarly to the lungs of Mtb-infected hosts.

Interestingly, we found the cohort of CD8⁺ T cells (from both groups) to have a higher “intravascular” proportion at the early time point (d14), compared with later time points (d18, d21) (Fig 2.7c). Sakai et al. show that “intravascular” T cells exhibit more terminal differentiation, a decreased ability to control Mtb growth, and are less likely to traffick to the lung parenchyma after adoptive transfer into Mtb-infected hosts. (Sakai et al., 2014a). Therefore, the sequential increase in proportion of “parenchymal” CD8⁺ T cells specific for TB10.4 (and decrease in “intravascular” T cells) as infection progresses implies appropriate trafficking, and the potential for both the 1^o (naïve-derived) and 2^o (memory-derived) TB10.4-specific CD8⁺ T cell responses to control Mtb growth in the lungs.

Next, we determined whether 1^o and 2^o effector CD8⁺ T cells proliferate differently during infection. On d18 post-infection, 1^o effector TB10Rg3 CD8⁺ T cells had ~40% more EdU uptake than 2^o effectors (Fig 2.7d). In contrast, no differences in the frequency of apoptotic cells, measured using an activated caspase-3 antibody or with a viability dye, were detected (Fig 2.7e). These data suggest 2^o effectors become outnumbered due to a decreased rate of 2^o effector CD8⁺ T cell proliferation after d15, while a greater rate of 1^o effector proliferation leads to continued exponential expansion.

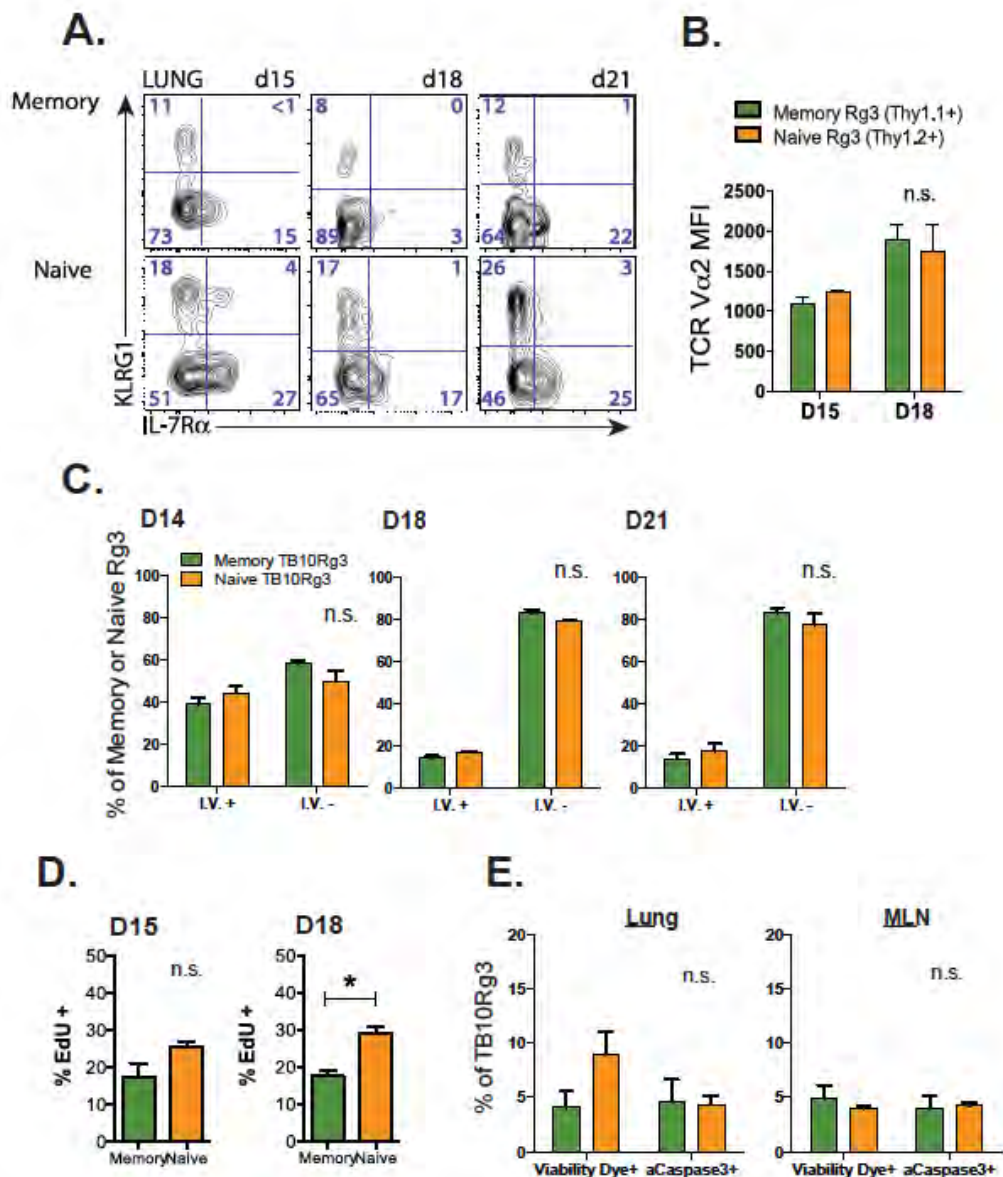


Figure 2.7: Increased proliferation accounts for differences in 1° and 2° TB10Rg3 CD8⁺ T cell abundance during TB. (a) KLRG1 and IL-7R α expression by memory and naïve-derived TB10Rg3 cells recovered from lung at each time point. (b) TCR V α 2 median fluorescence intensity (MFI) (median \pm SEM) in memory and naïve-derived TB10Rg3 CD8⁺ T cells from the same mice at d15 and d18 post-infection. (c) Proportion (mean \pm SEM) of memory or naïve-derived TB10Rg3 cells in lung homogenate that stained with anti-TCR V α 2 mAb that was injected i.v. immediately prior to euthanasia as well as anti-TCR V β 11 staining of the lung homogenate (i.v.+ “intravascular”), vs. those that only stained with anti-TCR V β 11 (i.v.-, “parenchymal”) at d14, d18, and d21 post-infection. (d) EdU uptake (mean \pm SEM) by memory and naïve-derived TB10Rg3 cells recovered from

lung. **(e)** Bar graphs of the frequency of activated caspase-3 expression and viability dye (Zombie Aqua, Biolegend) expression on TB10Rg3 cells derived from naïve (1°) and memory (2°) precursors in the lungs of Mtb-infected mice 15d post aerosol Mtb challenge. * p<0.05, n.s. not significant. Data are representative of 3 independent experiments, each with 3-4 mice per group.

Memory CD8⁺ T cells have a higher activation threshold than naïve CD8⁺ T cells

To determine whether the observed reduced proliferation was an intrinsic property of memory CD8⁺ T cells, or was precipitated by extrinsic signals in the inflammatory environment of the infected lung, we studied T cell expansion in a model of acute infection and two non-infectious models. First, naïve and memory TB10Rg3 CD8⁺ T cells were co-transferred into mice challenged with amphi-TB10 peptide together with anti-CD40 mAb and poly(I:C) one day earlier. One week after transfer into amphi-TB10 challenged mice, significant expansion had occurred in both groups but the ratio of 1° and 2° effectors remained ~1:1, with a predominance of 2° effectors late during expansion (Fig 2.8a). One day after 1:1 co-transfer of memory and naïve TB10Rg3 CD8⁺ T cells, mice were also challenged intravenously with the recombinant *Listeria monocytogenes* strain engineered to secrete TB10.4 (LmΔActA-TB10). Four days after LmΔActA-TB10 challenge, during a period of robust expansion, both groups expanded equally to TB10.4 antigen. During the contraction of the response (d7), the TB10Rg3 CD8⁺ T cells derived from memory were more abundant than those derived from naïve TB10Rg3 CD8⁺ T cells resulting in an 80:20 ratio favoring the 2° effectors (Fig 2.8b). Finally, homeostatic proliferation of naïve and memory TB10Rg3 CD8⁺ T

cells was also measured three weeks after their transfer into TCR α ^{-/-} mice, and the dividing cells also maintained an equal ratio (Fig 2.8c). Thus, 2^o effector CD8⁺ T cells have the potential to proliferate as well as 1^o effectors during acute infection or after non-infectious antigenic stimuli.

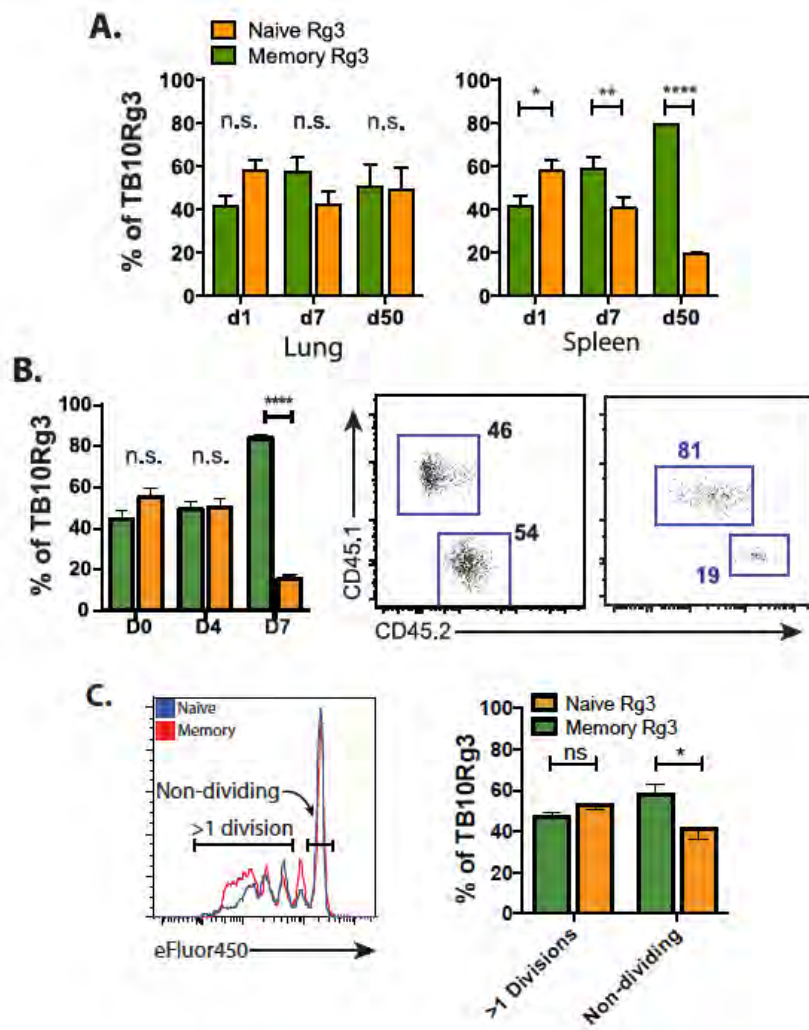


Figure 2.8: Memory and naïve CD8⁺ T cells both exhibit strong proliferation potential
(a) The proportions of naïve and memory-derived TB10Rg3 cells in the lung (left) or spleen (right) 1, 7, or 50 d after adoptive co-transfer into mice administered amphi-TB10/poly(I:C)/CD40 1d prior to transfer (mean \pm SEM). **(b)** Bar graphs (left) and representative flow plots (right) of the proportion of splenic TB10Rg3 CD8⁺ T cells (mean \pm SEM) derived from memory (CD45.1⁺) or naïve (CD45.2⁺) TB10Rg3 0, 4, or 7 days after i.v. Lm Δ ActA-TB10 challenge of Thy1.1⁺ hosts, in which 10⁴ memory and naïve TB10Rg3 cells were co-transferred at a 1:1 ratio 1 day prior to challenge. **(c)** Histogram of proportions of memory (red) and naïve (blue) TB10Rg3 cells that have diluted proliferation dye eFluor450, recovered from the spleens 21d after adoptive-co-transfer into TCR α ^{-/-} mice. Bar graph comparing the ratios of naïve and memory TB10Rg3 cells (mean \pm SEM) among those undergoing >1 division during homeostatic proliferation. Ratios were compared using student's t-tests. * p < 0.05, ** p < 0.01, **** p < 0.0001, n.s. not significant. Data are representative of 2-3 independent experiments, each with 3-4 mice per time point.

Mehlhop-Williams and Bevan find that memory CD8⁺ T cells require more antigen for activation than naïve CD8⁺ T cells, which results in less proliferation of secondary effectors when antigen is limiting (Mehlhop-Williams and Bevan, 2014). Both the antigen-challenge and the listeria models are scenarios in which TB10.4₁₁ is likely to be present in abundance. To formally determine whether the proliferation of memory TB10Rg3 CD8⁺ T cells is affected by antigen availability, mice were challenged with amphi-TB10/ α CD40/poly(I:C) i.v. 1d or 21d prior to co-transfer of naïve and memory TB10Rg3 CD8⁺ T cells to simulate high or low antigen conditions, respectively. Both naïve and memory CD8⁺ T cells proliferated similarly 3d after exposure to high antigen conditions (Fig 2.9a, b). However, 1^o effectors underwent more cell divisions than 2^o effectors when exposed to low antigen conditions (Fig 2.9c), and increased in number relative to the 2^o effectors. Finally, naïve and memory TB10Rg3 CD8⁺ T cells differed in their sensitivity to peptide concentration in vitro. Memory T cells required 32-fold more peptide to trigger proliferation than naïve T cells (Fig 2.9c). Thus, a low antigen environment recapitulates the bias towards primary effectors that we identified during Mtb infection.

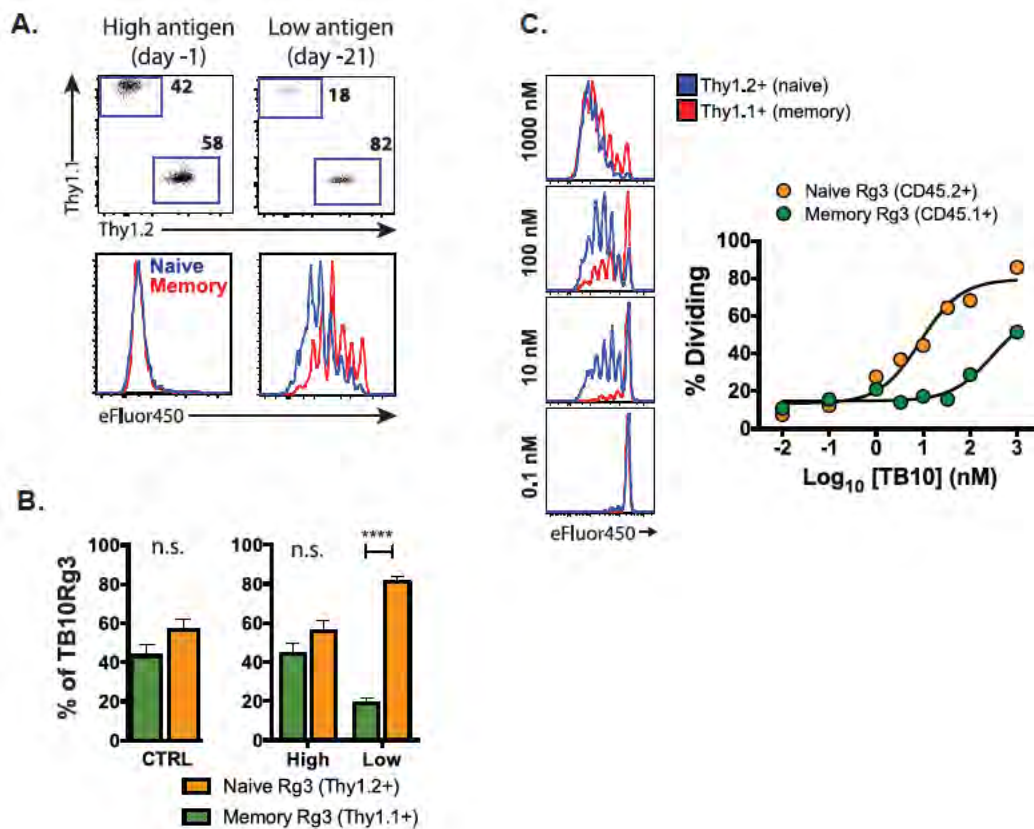


Figure 2.9: Memory CD8⁺ T cells have a higher activation threshold than naïve CD8⁺ T cells. (a, b) The proportion of splenic TB10Rg3 cells (mean ± SEM) derived from naïve or memory TB10Rg3 3d after their co-transfer into mice administered amphi-TB10/poly(I:C)/CD40 1d (high antigen) or 21d (low antigen) prior to transfer. Bar graphs of their ratios (a) and dilution of proliferation dye (b) are shown. (c) eFluor450 dilution by naïve or memory TB10Rg3 cells 64h after culture with peptide-coated splenocytes (left) and summary of dose-response data (right). **** p < 0.0001, n.s. not significant. Data are representative of 2 independent experiments, each with 3-4 mice per group.

Higher TCR affinity offsets memory CD8⁺ T cell loss during tuberculosis.

Since greater amounts of peptide-MHC complexes (pMHC) are required to trigger memory CD8⁺ T cell entry into the cell cycle (Mehlhof-Williams and Bevan, 2014) (Fig 2.9), we sought to determine whether TCR affinity modulated the fitness of memory CD8⁺ T cells during the response to Mtb challenge. We recently generated TCR retrogenic mice with TB10-specific CD8⁺ T cells that contain a TCR with a higher-affinity for TB10.4₄₋₁₁ (TB10Rg4, TCR4), in addition to mice containing the intermediate-affinity TCR (TB10Rg3, TCR3) (Fig 2.10) (Nunes-Alves et al., 2015). The increased affinity of TCR4 in naïve TB10Rg4 cells was determined in naïve TB10-specific CD8⁺ T cells by tetramer staining using limiting dilutions of tetramer (Fig 2.10a). Furthermore, the relative expansion and abundance of TB10Rg4 vs. TB10Rg3 CD8⁺ T cells was measured in lung and draining lymph nodes during priming of the adaptive immune response after aerosol infection with Mtb (Fig 2.10 b,c), exhibiting the preferential expansion of antigen-specific T cells with the higher TCR affinity.

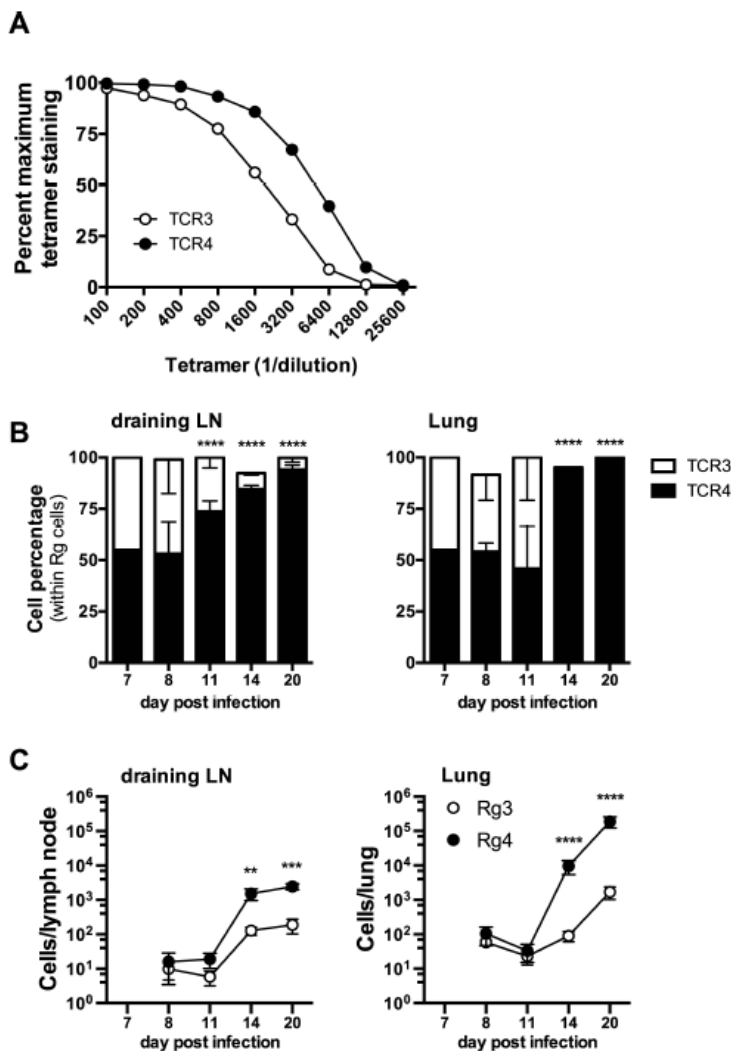


Figure 2.10: Differences in TCR affinity can lead to clonotypic dominance during infection. (a) Flow-cytometry analysis of affinity of TB10Rg3 (TCR3, open circles) and TB10Rg4 (TCR4, filled circles) CD8⁺ T cells from uninfected TCR retrogenic mice. Affinity is based on the frequency of tetramer staining of Rg cells across multiple tetramer concentrations. (b, c) Kinetic analysis of frequency (b) and number (c) of TB10Rg3 (open symbols) or TB10Rg4 (filled symbols) CD8⁺ T cells in the draining LN (left panels) and lung (right panels) following adoptive co-transfer into mice infected with *M. tuberculosis* (mean \pm SEM). Data are representative from two (b, c) or three (a) independent experiments, each with 5 mice per group. (b, c) Two way ANOVA with Holm-Sidak's multiple comparison test; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Using these TCR retrogenic mice, we vaccinated mice containing congenically-marked TB10Rg4 CD8⁺ T cells and rested age-matched, naive TB10Rg4 mice for an equivalent period of time (8-12 weeks) to compare the expansions of higher-affinity memory CD8⁺ T cells with their naïve counterparts. To determine whether the affinity of the TCR affects the relative ability of memory and naïve CD8⁺ T cells to expand, we co-transferred memory and naïve TB10Rg4 CD8⁺ T cells at a 1:1 ratio into Mtb-infected mice. Using the same methodology (Fig 2.5a), we tracked the relative expansions of 1^o and 2^o effector CD8⁺ T cells. Similar to our previous results, the TB10Rg4 naïve CD8⁺ T cells expanded more than TB10Rg4 memory CD8⁺ T cells (Fig 2.11a). Although those derived from TB10Rg4 memory were again outnumbered by d21 post-infection, the effect was less extreme, resulting in a ratio of ~4:1 favoring the 1^o effector CD8⁺ T cells in MLN, lung, and spleen (Fig 2.11a). Differences in the expansion of memory and naïve TB10Rg4 CD8⁺ T cells were again independent of surface TCR levels as TCR V α 2 MFI were equivalent (Fig 2.12). Thus, for a second TB10.4₁₁-specific TCR, we see a similar predilection for the 1^o effectors to outnumber the 2^o effector CD8⁺ T cells. Although these higher-affinity memory CD8⁺ T cells did not begin responding to Mtb earlier than the lower affinity (TB10Rg3) memory response, they displayed improved fitness.

Finally, we sought to determine whether higher affinity memory T cells were more fit than lower affinity naïve T cells. Memory TB10Rg4 and naïve TB10Rg3 CD8⁺ T cells were co-transferred at a 1:1 ratio into mice infected 6-7 days earlier,

and their expansion and ratio tracked through d21 post-infection. Memory TB10Rg4 CD8⁺ T cells successfully competed, significantly outnumbering naïve-derived TB10Rg3 CD8⁺ T cells by d14 post-infection in MLN, lung, and spleen (Fig 2.11b). By d21, the 2° TB10Rg4 effectors dominated the 1° TB10Rg3 effectors by a ratio of 50:1. Although memory CD8⁺ T cells have a higher antigen threshold for their activation, a higher TCR affinity for pMHC helps memory-derived CD8⁺ T cells to compete with those derived from naïve CD8⁺ T cells during tuberculosis. We infer that affinity plays an important role in the success of memory-derived effector CD8⁺ T cells during TB.

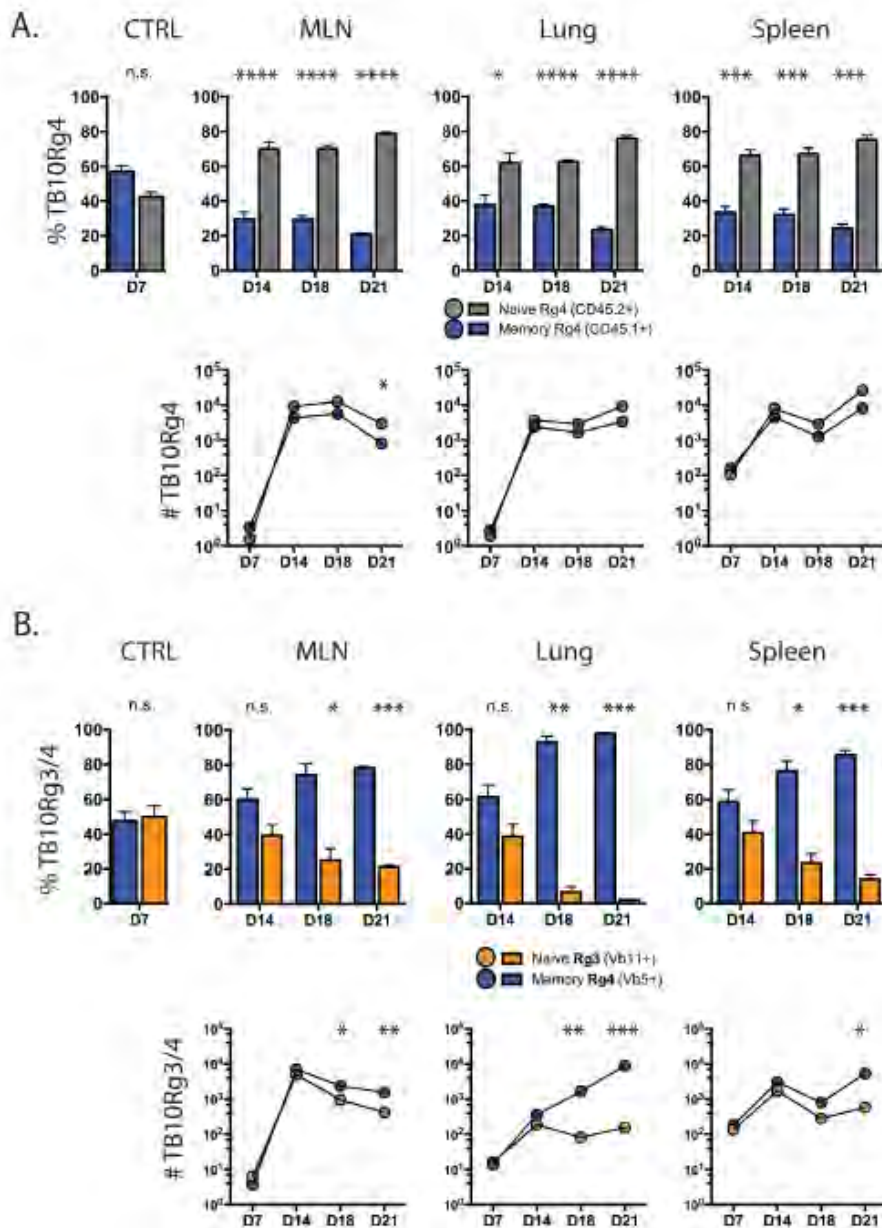


Figure 2.11: Memory CD8⁺ T cells with a higher affinity TCR can display improved responses during tuberculosis. (a) Proportion (mean \pm SEM) of adoptively-transferred memory (CD45.1⁺) and naïve (CD45.2⁺)-derived TB10Rg4 CD8⁺ T cells in the MLN, lung, and spleen 14, 18, or 21d after Mtb infection, compared to spleens from uninfected mice 1 day after adoptive transfer (CTRL) (top). Cell numbers of memory and naïve-derived TB10Rg4 CD8⁺ T cells from the same mice (bottom). **(b)** The relative proportion (mean \pm SEM) of adoptively-transferred, memory-derived TB10Rg4 (V β 5⁺) and naïve-derived TB10Rg3 (V β 11⁺) CD8⁺ T cells in the MLN, lung, and spleen 14, 18, or 21d after Mtb infection, compared to those in the spleens of uninfected mice 1 day after adoptive transfer

(CTRL) (top). Cell numbers of memory-derived (TB10Rg4) and naïve-derived (TB10Rg3) CD8⁺ T cells from the same mice at each time point during infection (bottom). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. not significant. Data are representative of 2 independent experiments, each with 4 mice per group.

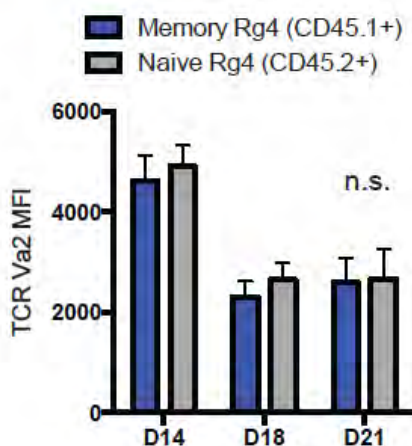


Figure 2.12: 1° and 2° TB10Rg4 CD8⁺ T cells contain equal TCR expression. Bar graphs of median fluorescence intensity (MFI) of TCR V α 2 expression on TB10Rg4 cells derived from naïve (1°) and memory (2°) precursors in the lungs of Mtb-infected mice 14, 18, and 21d post aerosol Mtb challenge. V α 2 MFIs were compared using student's t-tests. n.s. not significant. Data are representative of 2 independent experiments, each with 4 mice per group (time point).

Discussion

In this study, we discovered unexpected limitations in the expansion of memory-derived CD8⁺ T cells specific for an immunodominant Mtb antigen, providing one explanation for why T cell vaccines may be ineffective in preventing TB. By directly comparing naïve and memory CD8⁺ T cells using our adoptive co-transfer model, we show that both memory and naïve T cell responses are initiated in the MLN with similar kinetics. Thus, both primary and secondary responses are subject to significant delay before T cell expansion and recruitment to the lung occurs following Mtb challenge. Furthermore, once T cells traffic to the lungs, 2° effectors derived from memory precursors become rapidly outnumbered as their expansion plateaus after d15 post-infection, making them difficult to detect by d21. While this effect is modulated by TCR affinity, these kinetics may explain why the superiority of natural memory T cell responses are limited to a narrow window early after infection (Jung et al., 2005; Kamath and Behar, 2005). Furthermore, the phenotype of the memory T cells we generated by vaccination is that of central memory (CD62L^H IL-7R^H). As central memory T cells reside mostly in the draining lymph nodes, it may not be surprising that their activation occurs in the MLN and requires trafficking of antigen and/or antigen-laden APCs. Although central memory T cells are speculated to have superior proliferative capacity and mediate robust protection in adoptive transfer models (Sallusto et al., 1999), the requirement for priming in the LN could delay their response to Mtb, hindering the oft-cited benefit of a memory response: rapid recall.

The data presented here indicate that an important characteristic of memory T cells responding to Mtb is their ability to respond to antigen present in low-abundance and proliferate rapidly. We argue that the efficiency of memory T cell proliferation after exposure to low amounts of antigen can be tested during vaccine development as a benchmark for viable TB vaccine candidates. Although sensitivity to low antigen is not the only characteristic sufficient for a successful TB vaccine, it is likely a necessary component. The ability of a TB vaccine candidate to generate memory T cells that respond to limiting amounts of antigen in the lung soon after inoculation, and offer consistent control of bacterial growth in animal models, would represent a significant advance in TB prevention by vaccination. Such a vaccine candidate would be worthy of the rigor and expense of human clinical trials.

Materials and Methods

Ethics Statement. The animal studies were approved by the Institutional Animal Care and Use Committee at the Dana Farber Cancer Institute or the University of Massachusetts Medical School (Animal Welfare Assurance no. A3023-01 [DFCI] or A3306-01 [UMMS]), using the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Office of Laboratory Animal Welfare.

Mice. C57BL/6J (WT; CD45.2⁺Thy1.2⁺), CD45.1 (B6.SJL-Ptprc^aPepc^b/BoyJ; CD45.1⁺Thy1.2⁺), CD90.1 (B6.PL-Thy1^a/CyJ; CD45.2⁺Thy1.1⁺), TCR α KO (B6.129S2-Tcra^{tm1Mom}/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions at Dana Farber Cancer Institute or University of Massachusetts Medical School animal facilities. Mice were 7 to 10 weeks old at the start of all experiments. Infected mice were housed in biosafety level 3 facilities under specific pathogen-free conditions at DFCI or UMMS.

Generation of TCR retrogenic mice. TCR retroviral constructs were generated and retrogenic mice produced using protocols developed by the Vignali lab (Holst et al., 2006). Details of the TCRs, cloning strategies and primer sequences have been recently published (Nunes-Alves et al., 2015). Retroviral-mediated stem cell gene transfer was performed using bone marrow from CD45.2⁺Thy1.2⁺,

CD45.1⁺Thy1.2⁺, or CD45.2⁺Thy1.1⁺ mice, which was transferred into C57BL/6 recipients that were lethally-irradiated one day earlier with a split dose of 1200 Rads administered using a GammaCell 40 Cs¹³⁷ Irradiator (Theratronics, Ottawa, ON, Canada). Reconstitution was measured 6 weeks later.

Vaccination and assessment of immune responses. TB10.4_{4 11} (IMYNYNPAM), B8R_{20 27} (TSYKFESV), “amphi-TB10” (MFVMFVQIMYNYNPAM), and ovalbumin_{257 264} (SIINFEKL) peptides were purchased from New England Peptides (Gardner, MA, USA) and reconstituted in DMSO (10mM). High molecular weight polyinosinic:polycytidylic acid [poly(I:C)] was obtained from InvivoGen (San Diego, CA). Anti-CD40 mAb (clone FGK4.5) was purchased from BioXCell (West Lebanon, NH). Vaccines were prepared by mixing 100 micromoles of peptide, 50 µg poly(I:C), and 50 µg αCD40 mAb, in a total volume of 200 µL sterile PBS and administered intravenously. Where indicated, mice were boosted with the same vaccine 3 weeks later. In some experiments, peripheral blood T cell responses were monitored by flow cytometry. Mice were rested 8–12 weeks after the last vaccination to allow for the development of memory. Memory cells were generated using two different strategies. Since serial adoptive transfers of memory T cells can decrease their protective and proliferative capacities (Fraser et al., 2013), TB10Rg3 or TB10Rg4 mice having a low frequency of peripheral blood retrogenic cells (3-17%), were directly vaccinated with TB10/CD40/poly(I:C). In other experiments, 20,000 naïve TB10Rg3 CD8⁺ T cells were adoptively transferred into

C57BL/6 mice (resulting in 200 - 2,000 naïve precursor T cells after a 1-10% “take”). Those mice were then vaccinated as described above. In both cases, mice were rested for 8-12 weeks after vaccination to allow the development of memory. A comparison of memory TB10Rg3 CD8⁺ T cells elicited by vaccination after adoptive transfer of TB10Rg3 cells into B6 mice to those generated by vaccination of intact retrogenic mice showed similar results. Naïve TB10-specific CD8⁺ T cells were obtained from unvaccinated, age-matched TB10Rg3 or TB10Rg4 mice rested for an equivalent period of time.

Experimental infection and bacterial quantification. Mtb (strain Erdman) infections were performed via the aerosol route as described previously (Chackerian et al., 2002). 1mL Mtb Erdman culture was thawed from a frozen stock of broth culture in Middlebrooke 7H9 medium frozen at -80°C at mid-log phase growth. The same frozen stock was used for all experiments. Mice received an inoculation dose of 100-150 CFU/mouse, as measured within 24 hours of infection. The inoculum was quantified in control mice 1 day after aerosol inoculation by plating neat lung homogenate onto 7H11 agar plates. Infections at UMMS were performed using a Glas-Col full body inhalation exposure system (Terre Haute, IN). Mice received an inoculation dose of 30-190 CFU/mouse, as measured within 24 hours of infection. At different times post-infection, mice were euthanized, organs were aseptically removed, individually homogenized, and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto

7H11 agar plates.

Recombinant *Listeria monocytogenes* expressing the full-length TB10.4 coding sequence (Lm Δ ActA-TB10) was generated by amplifying the full-length coding sequence of TB10.4 from Mtb genomic DNA and cloning into the gram positive expression vector pAM401 behind the promoter and signal sequence for *Listeria monocytogenes hly* encoding Listeriolysin-O. This construct was electroporated into attenuated Δ ActA *Listeria monocytogenes* that retains access to the cytoplasmic compartment of infected cells as described previously for ESAT-6 and Ag85b constructs (Curtis et al., 2010; Shafiani et al., 2013). Bacteria containing the plasmid were grown to mid-log phase (OD₆₀₀ 0.4-0.8) in brain-heart infusion media (BHI) (Sigma) supplemented with chloramphenicol (10 μ g/mL) (Sigma) and aliquots were frozen at -80°C. Experiments using Lm Δ ActA-TB10 were performed by injecting host mice with 10⁷ bacteria 1 day after 1:1 co-transfer of 10⁴ naïve and memory TB10Rg3 cells. Bacterial titers were enumerated by plating 10-fold serial dilutions of inoculum onto BHI agarose supplemented with chloramphenicol (10 μ g/mL).

Flow cytometric analysis. Lungs, spleen, and LNs were removed after lung perfusion with 10mL of cold RPMI1640. Lung cell suspensions were prepared by coarse dissociation using the GentleMACS tissue dissociator (Miltenyi Biotec, Germany). Tissue was digested for 30-60 min at 37°C with 250 U/mL collagenase

(Sigma) in complete RPMI1640 [10% heat inactivated FCS (Sigma), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 10mM β -mercaptoethanol, 50 mg/ml streptomycin and 50 U/ml penicillin (all from Invitrogen)] followed by homogenization in the GentleMACS dissociator and sequential straining through 70 μ m and 40 μ m nylon cell strainers (Falcon). Spleen and LN cell suspensions were prepared using gentle disruption of the organs through a 70 μ m nylon strainer, followed by a 40 μ m nylon cell strainer. For some experiments, erythrocytes were lysed in using a hemolytic solution. For co-transfer experiments using naïve and memory TCR Rg CD8⁺ T cells, CD8⁺ T cells were enriched prior to surface antibody staining using either positive or negative selection (Mouse CD8 T cell isolation kit or CD8 T cell isolation kit II, Miltenyi Biotec). Surface staining was performed with antibodies specific for mouse CD3 ϵ (clone 145-2C11), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD19 (clone 6D5), CD44 (clone IM7), CD62L (clone MEL-14), CD127 (clone A7R34), KLRG1 (clone 2F1/KLRG1), CXCR3 (clone CXCR3-173), CD45.1 (clone A20), CD45.2 (clone 104), CD90.1 (clone OX-7), CD90.2 (clone 53-2.1), V α 2 (clone B20.1), V β 11 (clone KT11) (all from Biolegend, CA, USA). TB10.4₄ 11-loaded and B8R₂₀ 27-loaded H-2K^b tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA, USA). For most experiments, dual-tetramer staining was performed as described (Moon et al., 2009) using PE- and APC-conjugated TB10.4₄ 11-loaded tetramers together to accurately enumerate low-frequency events and minimize false-positive signal. For adoptive co-transfer

experiments, an amine-reactive viability dye, Zombie Aqua (Biolegend) was used to exclude necrotic cells. The active caspase-3 apoptosis antibody kit was used to measure apoptosis (clone C92-605, Beckton Dickinson, CA). All samples from Mtb-infected mice were fixed with 1% paraformaldehyde before analysis. Data were acquired using a FACS Canto II (Becton Dickinson) or a MACSQuant flow cytometer (Miltenyi Biotec). Data were analyzed using FlowJo Software V9 (Tree Star, OR). For both analysis and cell sorting, single-lymphocyte events were gated by forward scatter area and height versus side scatter area for size and granularity.

Adoptive T cell transfer of CD8⁺ T cells. Single cell suspensions of homogenized spleens and LNs (inguinal, cervical, axillary, mediastinal, and mesenteric) were prepared from vaccinated retrogenic mice (8-12 weeks after the last vaccination) or age-matched unvaccinated mice. CD8⁺ T cells were purified by negative selection using the CD8⁺ T cell isolation kit II (Miltenyi Biotec) or the EasySep™ mouse CD8 T cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) followed by magnetic separation. After purification, cells were stained with eFluor 450 proliferation dye (eBiosciences), antibody-stained and sorted by flow cytometry to achieve uniform populations of naïve or memory CD8⁺ T cells. For TB10Rg3 naïve/memory co-transfer experiments, 1×10^4 cells of each population were mixed at a 1:1 ratio (confirmed by flow cytometry) and were transferred IV into congenic recipients (CD90.1 or CD45.1), which had been infected 0-7 d earlier with Mtb. TB10Rg3 CD8⁺ T cells used for the memory group

were generated on the Thy1.1⁺, CD45.1⁺, and Thy1.2⁺CD45.2⁺ backgrounds to ensure that none of the observed effects were specific to congenic backgrounds of the mice. For protection experiments, 1×10^5 TB10Rg3 Thy1.2⁺CD45.2⁺ memory or naïve cells were transferred into TCR α ^{-/-} mice or sub-lethally irradiated (600 Rads) C57BL/6 mice, and challenged with Mtb the same day.

Cell sorting. Fluorescent antibody-stained cells were sorted using a FACS Aria II (Becton Dickinson) flow cytometer. For adoptive transfer experiments, CD8⁺CD4⁺GFP⁺V α 2⁺ KLRG1^{Lo} CD44^h memory (from vaccinated Rg mice) or CD44^{Lo} naïve TB10Rg3 cells (from age-matched unvaccinated Rg mice) were sorted from pre-enriched CD8⁺ T cells. For TCR β repertoire analysis, we used dual-tetramer staining to identify and sort CD8⁺CD4⁺tetramer⁺⁺CD44^H T cells from blood, one week after vaccination with amphi-TB10/CD40/poly(I:C). Twelve weeks after vaccination, the same mice were infected with Mtb, and five weeks later, CD8⁺CD4⁺tetramer⁺⁺CD44^H T cells were again sorted from the lungs.

Intracellular cytokine staining and ELISAs. Lung cells cultured with rhIL-2 (100 U/mL; Peprotech) were stimulated in the presence or absence of TB10.4₄₋₁₁ peptide (10 μ M; New England Peptides) or α CD3/ α CD28 mAbs (1 mg/mL, BioLegend) as positive control. After 1 h at 37°C, GolgiStop (BD Pharmingen, CA, USA) was added for 4 h. Cells were then stained with antibodies, permeabilized (BD Permashield Kit; BD Pharmingen), and stained for IFN γ (clone XMG1.2;

Biolegend), TNF (clone MP6-XT22; Biolegend), or Granzyme B (clone GB11; Biolegend). For ELISAs, IFN γ , TNF, and IL-2 production were measured in supernatants after 48h stimulation with peptide, using ELISA Max standard kits (Biolegend).

In vivo CTL assay. Cytotoxicity was determined using peptide-coated splenocytes from congenic CD45.1⁺ B6 mice as target cells, differentially-labeled with the cell proliferation dyes eFluor 450 (eBiosciences) and/or CFSE (eBiosciences) as previously described (Woodworth et al., 2008). Briefly, target cells were pulsed with 10 μ M, 1 μ M, 0.33 μ M, 0.1 μ M, 0.033 μ M, or 0.01 μ M of TB10.4_{4 11} peptide, or left unpulsed (as control). Six of the target cell populations were then labeled with 5 μ M, 1.25 μ M, or 0.3125 μ M eFluor 450 dye (two populations for each dye concentration). Prior to washing, three of the six populations (one for each of the eFluor 450 dye concentrations) were also labeled with 5 μ M CFSE. The 7th population of peptide-coated targets was stained only with 5 μ M CFSE. After extensive washing, labeled populations were mixed at equal cell ratios and 3-5 x 10⁶ cells per population (21-35 x 10⁶ total cells) were injected into TB10/CD40/poly(I:C)-vaccinated or unvaccinated control CD45.2⁺ B6 mice. After 20h, the relative proportions of each populations in the lung and spleen were determined by flow cytometry and compared unvaccinated control mice as described (Woodworth et al., 2008).

Measurement of cell proliferation. Analysis TB10Rg3 cell proliferation was measured after adoptive transfer into Mtb-infected, vaccinated, or TCR α ^{-/-} mice, or *in vitro* after stimulation by labeling purified TB10Rg3 CD8⁺ T cells with 5 μ M of the cell proliferation dye eFluor 450 (eBiosciences). Proliferation, as measured by dye dilution, was measured by flow cytometry *in vivo* 11d after aerosol Mtb infection, or *in vitro* 64h after co-culture with APCs coated with a serial dilution of TB10.4₄₋₁₁ peptide. Cell proliferation at later time points (d15 or d18) *in vivo* was assayed by the incorporation of the synthetic thymidine analogue 5-Ethynyl-2'-deoxyuridine (EdU, Life Technologies). Briefly, 1mg EdU diluted in 100 μ L PBS was injected i.p. into each mouse 12h prior to analysis. After antibody staining, single cells suspensions were assayed for EdU incorporation using the Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay kit (Life Technologies).

Statistical analysis. Data are represented as mean \pm SEM. A two-tailed student's t-test was used for normally-distributed data to compare two groups. One-way or Two way ANOVA were used to compare more than two groups, followed by Bonferroni or Sidak post-tests. A p value < 0.05 was considered to be statistically significant. Statistical analyses were performed using Prism V6 (GraphPad Software, San Diego, CA).

Preface to Chapter III

This chapter has been adapted from the recently published manuscript by Carpenter et al in *PLoS Pathogens*:

Carpenter, S. M., Nunes-Alves, C., Booty, M. G., Way, S. S., & Behar, S. M. (2016). A Higher Activation Threshold of Memory CD8⁺ T Cells Has a Fitness Cost That Is Modified by TCR Affinity during Tuberculosis. *PLoS Pathogens*, 12(1), e1005380. <http://doi.org/10.1371/journal.ppat.1005380>

Additional figures have been including, and the figure numbers are different from those in these published manuscripts. This manuscript represents the second part of the thesis project of Stephen Carpenter, who designed and performed the experiments, analyzed the data, and wrote the manuscript with contributions from co-authors and mentorship from Dr. Samuel Behar.

CHAPTER III

Deep sequencing of memory CD8⁺ T cell receptors reveals selection of specific TCR motifs during TB

Introduction

Memory T cells are thought to provide a faster response that is armed to exert a potent effector response against infected cells (Jameson and Masopust, 2009). However, the ability to distinguish the memory-derived effectors (2^o response) from naïve-derived effector T cells (1^o response) during their response to infection has been nearly impossible by conventional methods. While the *magnitude* of the 1^o and 2^o responses can be estimated by comparing the frequency of antigen-specific T cells in vaccinated (or unvaccinated) test subjects, the origin of the individual effector T cells in vaccinated individuals remains obscure. Surface markers are largely unhelpful in distinguishing the two groups, as both 1^o and 2^o responses become activated after challenge. Adoptive transfer of congenically-marked cells into infected animals has been the most common method used to study memory T cells during infection (Jabbari and Harty, 2006). We use this technique heavily in Chapter 2. However, this system has 2 main drawbacks for studying natural responses to infection: First, it is difficult to study polyclonal populations of T cells due to concern for altering the function of antigen-specific T cells with the use of tetramers during sorting. Second, the composition

of the memory T cell cohort may not be physiologic, as the ratios of various memory T cell subsets depends on the organs from which they were isolated (e.g. central vs. effector vs. tissue-resident memory) prior to adoptive transfer (Steinert et al., 2015), and only 1-10% are estimated to “take” after injection (Plumlee et al., 2013). The adoptive transfer method is not a fair direct comparison of memory and naïve T cells, unless both are derived from TCR transgenic (or retrogenic) mice and co-transferred. However, if T cells can be sampled both after vaccination and during infection in the same individual, TCR sequencing can be used to track the expansion and abundance of clonotypes derived from both memory or naïve T cells.

In this chapter, we use next generation sequencing of T cell receptors to both quantify and track individual T cell clonotypes to estimate the roles and behavior of the TB10.4-specific 1^o and 2^o CD8⁺ T cell responses in the lungs of vaccinated mice after Mtb challenge. At the peak of the CD8⁺ T cell response to amphi-TB10 / α CD40 / poly(I:C) vaccination (1 week), we use tetramers to flow-sort antigen-specific T cells from the blood of vaccinated mice (Fig 3.1). After the development of memory (12 weeks), we challenge vaccinated mice with aerosol Mtb, and again isolate TB10.4-specific CD8⁺ T cells from the lungs of vaccinated mice, followed by sequencing of the T cell receptors in all groups (Fig 3.1). Using TCR β deep sequencing, we find enormous clonotypic diversity in the TB10.4-specific CD8⁺ T cell response to vaccination, but after Mtb challenge the 2^o response undergoes selection for a specific TCR motif that we attribute to higher

affinity. By comparing the response of two different TCRs that differ in their affinity for the same epitope, we show that memory-derived CD8⁺ T cells with an increased affinity for antigen have greater fitness, demonstrating that TCR-dependent factors promote successful continued expansion of 2^o effector CD8⁺ T cell responses during chronic infection in the lung.

By adoptively transferring well-characterized naïve and memory TB10Rg3 CD8⁺ T cells at a 1:1 ratio, we showed that factors other than TCR affinity or abundance determined the increased fitness of the naïve T cell response. On the other hand, our experiments using TB10Rg4 CD8⁺ T cells, which recognize the same epitope as TB10Rg3, but with a higher affinity, indicate that increased affinity can offset the disadvantage in the expansion rate of memory CD8⁺ T cells leading to their dominance over lower-affinity naïve CD8⁺ T cells during Mtb challenge. In reality, there exists considerable variation in frequency and TCR affinity in the T cell repertoire, which could affect the success of individual clonotypes. Although impossible to track the fates of memory T cells during infection using flow cytometry alone, we use deep sequencing of the TCRs to study memory CD8⁺ T cells specific for TB10.4 from vaccinated mice during tuberculosis. Using TCR β sequencing, we evaluate the clonality, relative abundance, and TCR β repertoire of memory-derived CD8⁺ T cells during Mtb challenge.

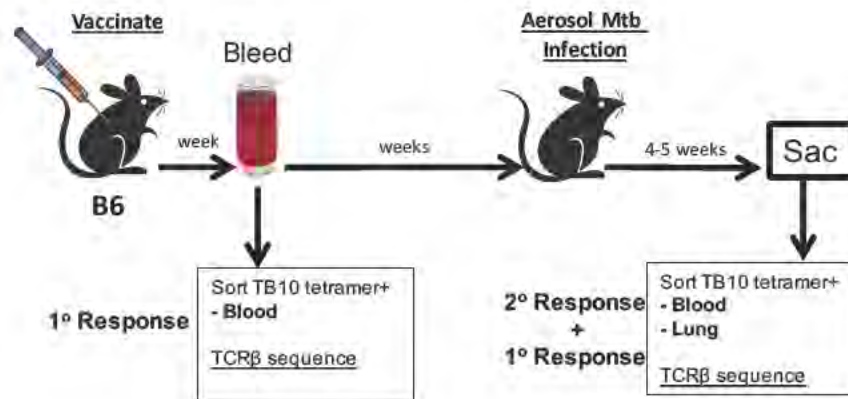


Figure 3.1: Layout of TCR sequencing experiments. C57BL/6j mice were vaccinated with the amphi-TB10 peptide, together with α CD40 mAb and Poly I:C. One week later, TB10 tetramer⁺ CD8⁺ T cells were sorted from blood and genomic DNA was isolated from individual mice. 12 weeks after vaccination (11 weeks later), mice were aerosol-infected with Mtb. 4-5 weeks later, antigen-specific T cells were again sorted from blood and lungs of each mouse, and genomic DNA isolated. TCR β sequencing was performed on consecutive DNA samples isolated from the T cells of each mouse and CDR3 β sequences were compared.

Results

TCR β deep sequencing distinguishes primary and secondary T cell responses

To determine how Mtb infection affects the ability of TB10-specific memory CD8⁺ T cells to expand in mice with an intact and diverse immune repertoire, we used NexGen TCR β sequencing to track the polyclonal TB10-specific CD8⁺ T cell response to vaccination, and the subsequent recall response after Mtb challenge in individual mice. We reasoned that TCR β clonotypes elicited by vaccination that were also detected after challenge represented 2^o effectors. On the other hand, clonotypes detected only after challenge were more likely to be part of a new 1^o response. We purified TB10-specific CD8⁺ T cells using tetramers by flow sorting after vaccination, and again after Mtb challenge in the same individual, and sequenced their TCR β repertoire (Fig 3.1). We find that after vaccination, the clonality of TB10-specific CD8⁺ T cells was not statistically different than that of total T cells from the peripheral blood of uninfected B6 mice (Fig 3.2a). However, TB10-specific CD8⁺ T cells were significantly more diverse after vaccination than after Mtb challenge (Fig 3.2a). Thus, the post-challenge TCR β repertoire was more similar to what we observed following primary Mtb infection (Nunes-Alves et al., 2015). Interestingly, the TB10-specific CD8⁺ T cells appeared to be less clonal after Mtb infection in mice that were previously vaccinated [compare ‘challenged’ vs. ‘primary Mtb’; ‘primary Mtb’ data from (Nunes-Alves et al., 2015)], raising the possibility that vaccination leads to a more diverse T cell response during infection.

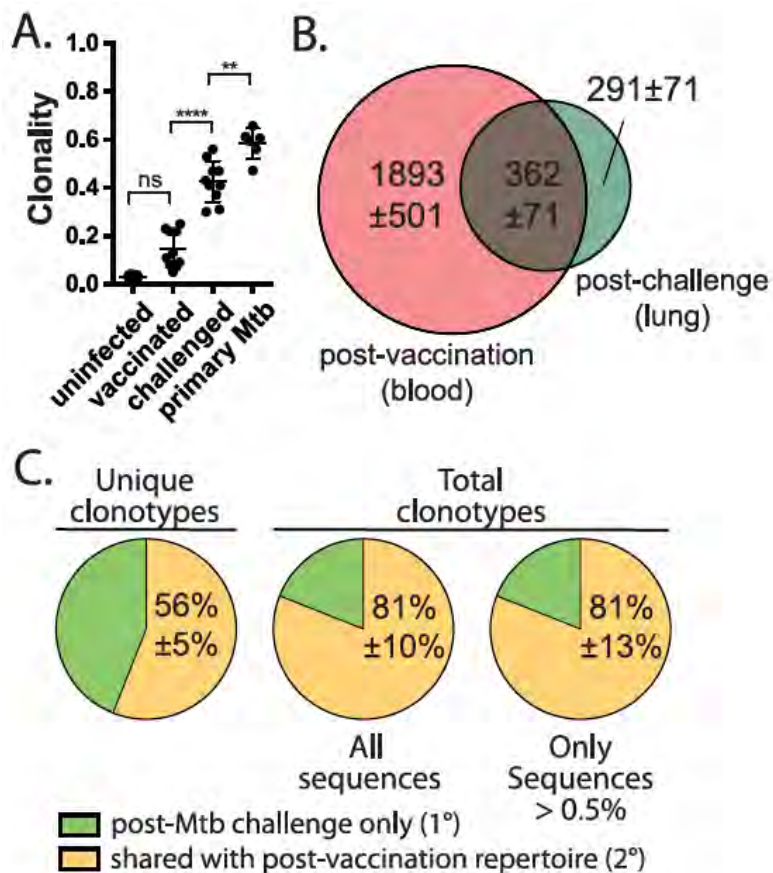


Figure 3.2: TCR β deep sequencing reveals the dual contribution of the primary and secondary effector CD8⁺ T cell response in vaccinated mice challenged with Mtb. (a) Clonality of TB10-specific CD8⁺ T cells from blood 1w after amphi-TB10 vaccination, compared to those isolated from lung in the same individuals 4-5w after Mtb challenge, or compared to those isolated from unvaccinated, Mtb-infected mice [data for primary Mtb-infected mice from (Nunes-Alves et al., 2015)]. Data are from 3-10 individuals/group, independently analyzed from two independent experiments. One-way ANOVA with a Bonferroni post-test was used to compare clonality. * $p < 0.05$, **** $p < 0.0001$. (b) Sharing of unique TCR β DNA sequences between the post-vaccination (blood) and post-Mtb challenge (lung) repertoires of TB10-specific CD8⁺ T cells. Numbers are the average of unique TCR β DNA clonotypes, determined for four subjects, each analyzed individually. (c) The percentage of the lung TB10-specific CD8⁺ TCRs detected either only post-Mtb challenge (e.g., 1° response); or, detected both post-vaccination and post-Mtb challenge (e.g., 2° response). Left, unique clonotypes; center, total TCRs; right, total TCRs that had a frequency of >0.5%.

One week after vaccination, we detected 2254 ± 509 unique clonotypes among peripheral blood TB10-specific CD8⁺ T cells (Fig 3.2b). Four weeks after Mtb challenge, the repertoire of pulmonary TB10-specific CD8⁺ T cells consisted of 652 ± 125 unique clonotypes. On average, 56% (362 ± 71) of the unique clonotypes in the lungs of infected mice were previously detected after vaccination, meeting our definition of 2° effector T cells. Thus, nearly half of the distinct clonotypes specific for TB10.4 in the lungs of vaccinated mice challenged with Mtb were part of a new 1° response. However, not all clonotypes were similarly represented in the lung. While the shared sequences, which we define as 2° effectors, accounted for 56% of the unique clonotypes, they added up to 81% of all TB10-specific CD8⁺ T cells in the lung. A possible bias in our analysis is that clonotypes detected in the lung might not be detected in blood. However, the peripheral blood samples in this cohort of mice were sequenced at the maximum depth available to mitigate this possible confounder. We have found the naïve precursor frequency of TB10-specific CD8⁺ T cells in C57BL/6 mice to be ~ 1 in 20,000 (0.005%) (Nunes-Alves et al., 2015), whereas the frequency of memory TB10-specific CD8⁺ T cells 8-12 weeks after a single vaccination with amphi-TB10 / α CD40 mAb / poly(I:C) is $\sim 1:10$ (Fig 2.2b). Therefore, we focused on only the abundant clonotypes in the lung, using a threshold of 0.005% or 0.5%, and we found that 84% and 81% of the total TCRs were shared, which is similar to when clonotypes of all frequencies are analyzed (Fig 3.2c). Thus, in vaccinated mice subsequently challenged with Mtb, nearly half of the unique clonotypes in the lung

represent a new 1° response and in aggregate they make up ~20% of the total TB10-specific CD8⁺ T cells in the lung. Since the TB10-specific response represents >30% of all CD8⁺ T cells in the lung by 4wpi, the new 1° response expanded from ~1 in 20,000 CD8⁺ T cells (in the naïve repertoire) to ~6% of the CD8⁺ T cells in the lung. Likewise, the 2° response expanded from ~1 in 10 CD8⁺ T cells (in the post-vaccination repertoire) to ~24% of the CD8⁺ T cells in the lung. Thus, the new 1° response underwent a ~1,200-fold expansion compared to a ~2.4-fold expansion for the 2° response.

TCR β deep sequencing identifies TB10-specific CD8⁺ T cell clonotypes that are shared between lung and blood

Our evaluation of the post-vaccination and post-Mtb challenge TCR repertoires in the same individual requires the comparison of T cells in blood (post-vaccine) to lung (post-challenge). To further validate this approach, we asked to what degree the TB10-specific repertoire in the blood and lung are related. TB10-specific CD8⁺ T cells (e.g., tetramer⁺) were simultaneously isolated from the peripheral blood and perfused lung of individual Mtb-challenged mice by flow sorting. Although unique clonotypes exist that are detected only in lung or blood, all of the abundant TCR clonotypes detected in the lung (defined as a frequency >0.5%) were also detected in blood (Fig 3.3a). In each of the mice, two distinct clusters of T cell clonotypes could be identified: each with a similar frequency in blood but significantly different frequencies in lung. Although lung parenchymal

and lung intravascular pools of T cells were not formally distinguished in this experiment, T cells in these newly-defined compartments might also exhibit such clustering by frequency in the lung (Moguche et al., 2015; Sakai et al., 2014b).

There was substantial overlap between the clonotypes detected in blood and in lung (Fig 3.3a,b). We detected 400-600 distinct DNA sequences among pulmonary TB10-specific CD8⁺ T cells (Fig 3.3b). Of these unique sequences, 30% were also detected in the blood, and the remaining 70% were detected only in the lung (Fig 3.3c). Many of the clonotypes unique to the lung compartment were infrequent and in aggregate accounted for only 10% of the total T cells. Thus, 90% of the TB10-specific CD8⁺ T cells found in the lungs of infected mice used TCRs that were detected both in blood and in lung (Fig 3.3c). In fact, if only the highly abundant clonotypes (>0.5%) are considered, more than 99% of pulmonary TB10-specific CD8⁺ T cells use a TCR that is detected in peripheral blood during infection. Thus, clonotypes that are abundant and clonally expanded in the lung are also detected in peripheral blood.

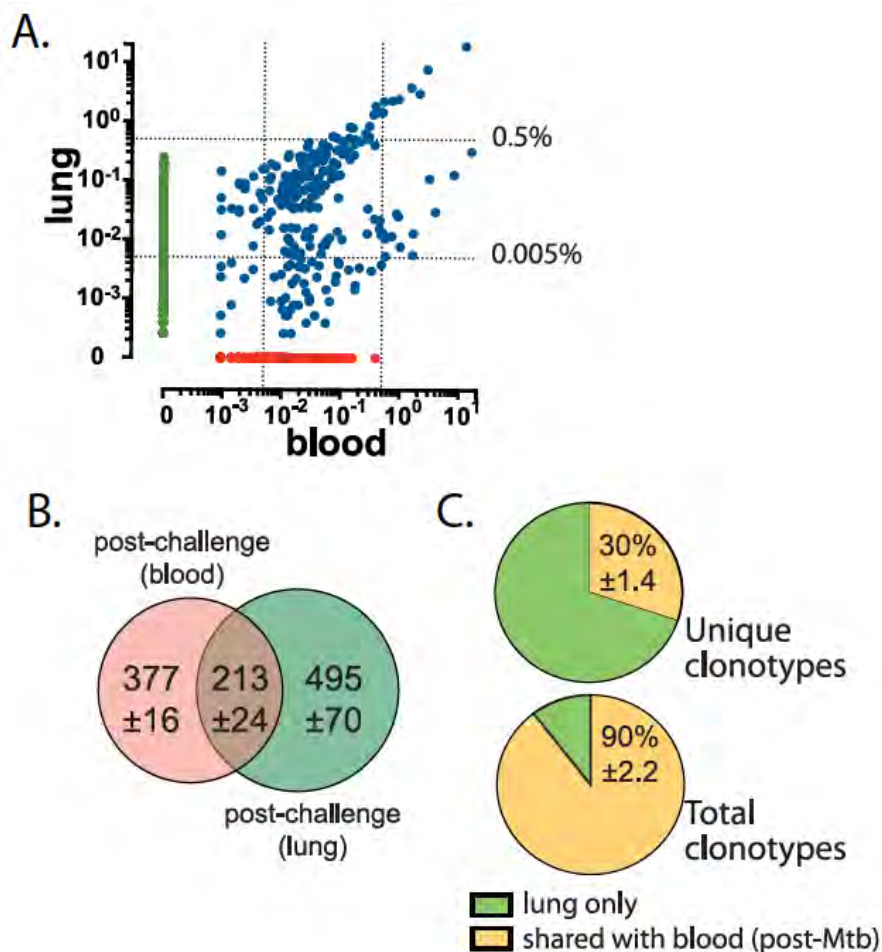


Figure 3.3: The distribution of the TB10-specific CD8⁺ T cell receptor repertoire in lung and blood after aerosol Mtb challenge in vaccinated mice. (a) Representative logarithmic scatter plot showing the frequencies of all clonotypes detected in the blood or in the lung of a single individual 5 weeks after Mtb challenge. Green, lung only; Red, blood only; Blue, Shared. The dotted lines indicate frequencies of 0.005% and 0.5%, respectively. **(b)** Sharing of unique TCRβ DNA sequences between blood and lung repertoires of TB10-specific CD8⁺ T cells in individual mice, 5 weeks after infection. Numbers are the average of unique TCRβ DNA clonotypes, determined for three subjects analyzed individually. **(c)** The percentage of the lung repertoire of TB10-specific CD8⁺ TCRs that were detected only in the lung ("lung only") or detected in the blood and lung ("shared with blood") after Mtb challenge. Left, unique clonotypes; right, total TCRs. Only clonotypes with a frequency of >0.005% were analyzed.

Selection drives T cell expansion following Mtb infection

To compare the fitness of each TCR clonotype responding to TB10.4 after Mtb challenge, we analyzed matched blood (post-vaccination) and lung (post-Mtb challenge) samples from individual subjects. Each unique TCR β DNA sequence detected in the post-vaccination and post-challenge repertoire of the same individual were classified as “**successful**” if they increased in frequency after Mtb challenge or “**persisters**” if they decreased, but remained present post-challenge. Clonotypes were stratified as “**de novo**” or “**unsuccessful**” if they were present only after Mtb challenge or vaccination, respectively (Fig 3.4a). Interestingly, the two-thirds of the unique clonotypes elicited by vaccination are “unsuccessful” and are not detected after infection (Fig 3.4b). Although this is a large number of unique TCR sequences, they represent only ~18% of the total TB10-specific CD8⁺ T cell response in peripheral blood (Fig 3.4b). Conversely, after Mtb challenge, 25% of the unique clonotypes in the lung were de novo sequences, which accounted for ~16% of the total TB10-specific CD8⁺ T cells in lung (Fig 3.4a). Although each of these TCRs generally had a frequency of <0.5%, occasionally they were at a higher abundance (see Fig 3.4a as an example). Thus, although the naive T cells have the potential to expand more than memory T cells (Fig 2.5e,f, and (M. D. Martin et al., 2012; Mehlhop-Williams and Bevan, 2014; Miyakoda et al., 2012; West et al., 2011)) and make a definable contribution to the recall response to Mtb infection, the majority of the total TCRs responding to Mtb challenge are a clonal population of 2^o effectors derived from a relatively small number of “successful”

vaccine-elicited T cells (Fig 3.4b). Finally, the majority of the vaccine-elicited T cells (67%) fail to expand during Mtb challenge, becoming “persisters” (Fig 3.4b). The fates of these “successful” and “persister” TCRs mirror the observed functions of the higher-affinity TB10Rg4 and lower-affinity TB10Rg3 CD8⁺ T cells, respectively, in our adoptive transfer studies (Fig 3.4b and Fig 2.11a,b).

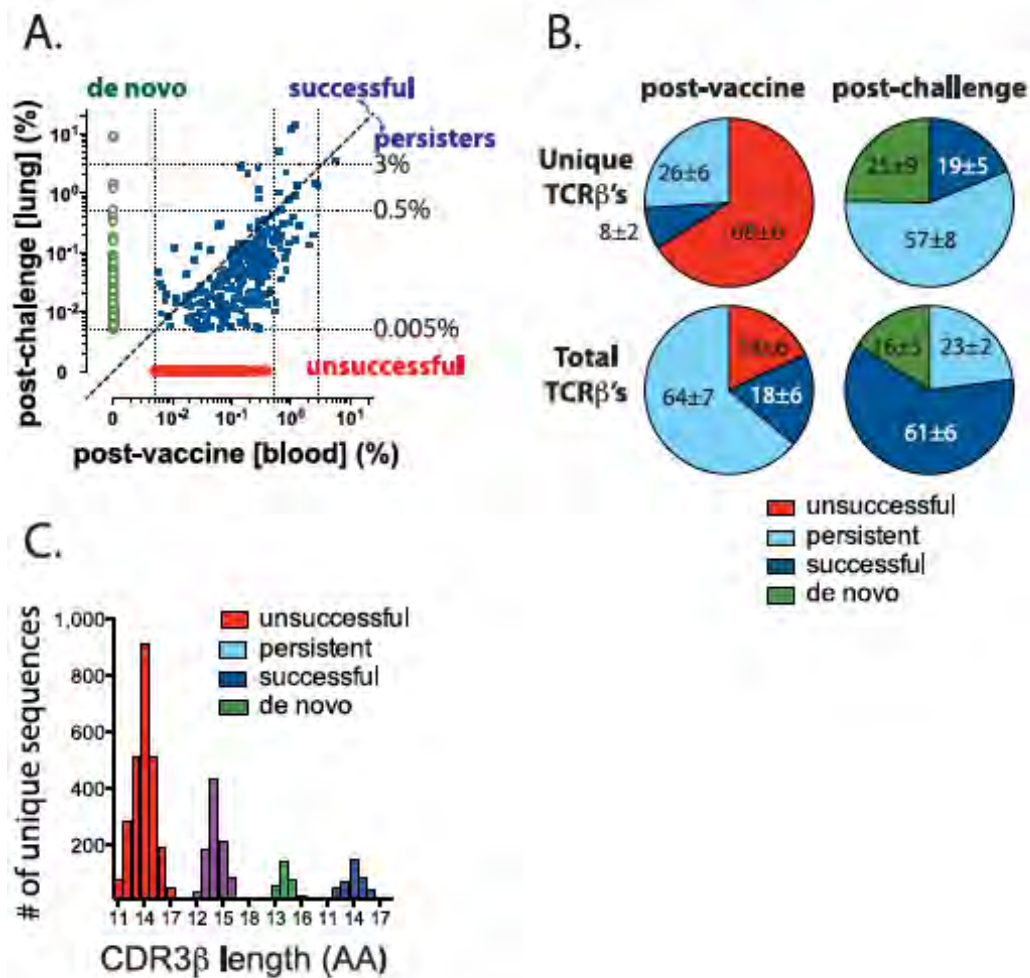


Figure 3.4: Selection drives the expansion of TB10-specific CD8⁺ T cells. (a) Representative logarithmic scatter plot showing the frequencies of all clonotypes detected in the post-vaccination (blood) or in the post-Mtb challenge (lung) repertoire of TB10-specific CD8⁺ T cells. Green, lung only (de novo response); red, blood only (unsuccessful); blue, shared (persistent & successful). The diagonal dashed line separates the successful and the persistent clonotypes. The dotted lines indicate frequencies of 0.005%, 0.5%, and 3%. (b) The proportion of total TCRβ amino acid sequences categorized as “unsuccessful”, “persisters”, “successful”, or “de novo” as defined in the text, from the post-vaccination (blood, left) or post-Mtb challenge (lung, right) repertoires. Top row, unique clonotypes; bottom row, total TCRs. Only clonotypes with a frequency of >0.005% were analyzed. (c) CDR3β length distribution among unique clonotypes categorized as “unsuccessful”, “persisters”, “successful”, or “de novo”.

Our clonality data suggested that infection was driving greater selection than vaccination. To identify structural features that govern TCR success or failure, we analyzed the CDR3 β amino acid sequence of “**persisters**” and “**successful**” TCR β clonotypes detected in the post-vaccination and post-challenge repertoire of the same individual. The CDR3 β length distribution was similar among these four groups, with 14 amino acids being the most common length (Fig 3.4c). To focus on selection, we analyzed clonotypes that were present in both the post-vaccination and post-Mtb challenge repertoire, with a frequency of >0.5% in one of the samples. In addition, we restricted our analysis to clonotypes with a CDR3 β length of 13, 14, or 15, which accounted for most of the sequences (Fig 3.4c). We identified 241 sequences from 7 individual subjects that met these criteria. These highly-represented clonotypes accounted for 60% of the productive sequences detected in the lungs of mice after Mtb challenge. Analysis showed that the “successful” clonotypes had a “DRxN” CDR3 β motif (Fig 3.5). The “DRENSD” motif, which had previously been detected among TB10-specific CD8⁺ T cells after Mtb infection in the absence of vaccination (Nunes-Alves et al., 2015), was expressed by the most successful clones (Fig 3.5). In contrast, “persister” clones lacked the “DRENSD” motif and instead more frequently encoded “RG” (Fig 3.5). A similar motif was identified among those clonotypes with a CDR3 β length of 15 amino acids. The persisters had a motif of “DRggNx”, while the successful clones had a motif of “DRgNQD” (Fig 3.5). Therefore, during Mtb infection, selective pressure constrains structural features of the TCR repertoire that recognize TB10.

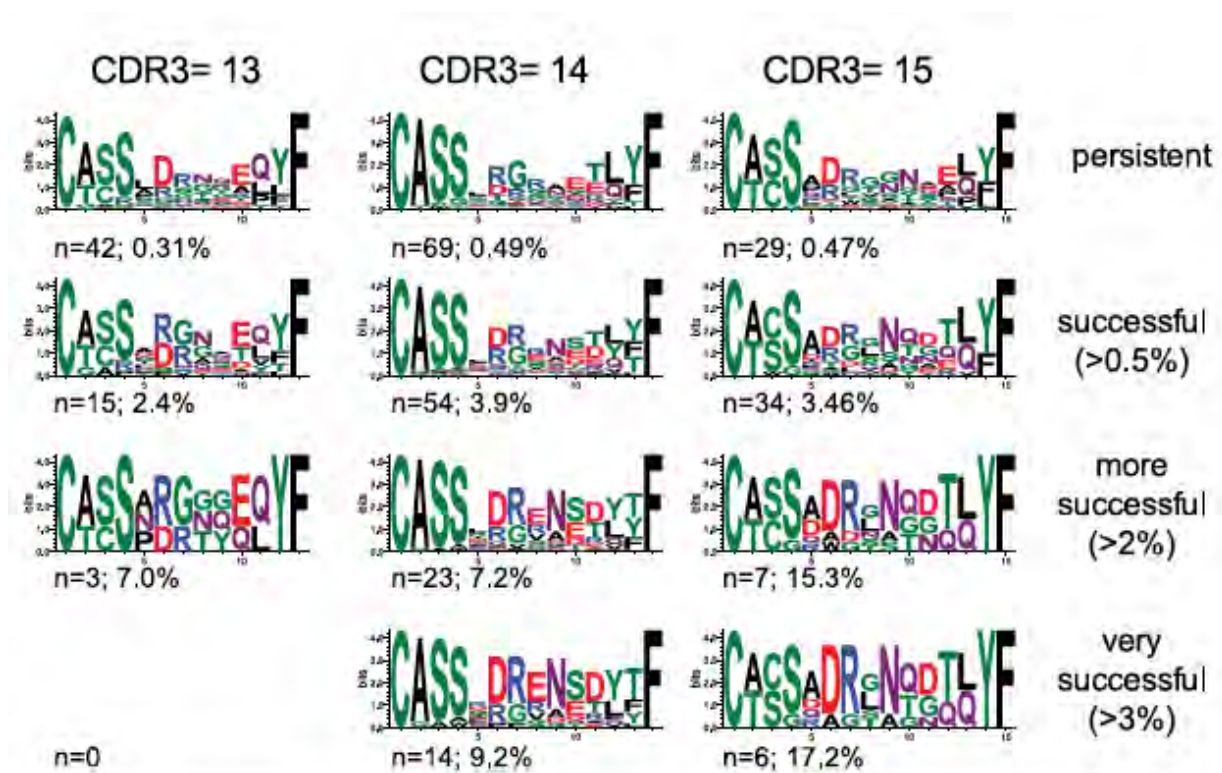


Figure 3.5: High consensus among CDR3 β sequences from “successful” memory TB10-specific CD8⁺ T cells. CDR3 β amino acid motifs were determined for highly prevalent clonotypes (>0.5% in post-vaccination or post-Mtb challenge repertoire), which were identified as “persisters” or “successful” TCR clonotypes with a CDR3 length of 13, 14, or 15 aa. For successful TCRs, different frequency thresholds were chosen (0.5%, 2%, or 3%) to identify structural motifs among highly prevalent clonotypes. The numbers below each sequence refer to the number of unique clonotypes that were used to derive the motif and the average frequency of each clonotype among total productive sequences.

Discussion:

We have shown that TB10-specific CD8⁺ T cells are under extreme selection, and clonal expansions emerge even early during infection. Given our data from the study of naïve and memory TCR retrogenic CD8⁺ T cells specific for TB10.4 (Chapter 2 and (Nunes-Alves et al., 2015)) we infer the observed clonal selection of presumably high-affinity T cells is driven by a paucity of antigen presentation. Although we have not yet directly correlated these memory T cell clonotypes with function, we hypothesize that these same conditions could lead to memory T cell dysfunction during Mtb challenge.

TCR deep sequencing allowed us to track vaccine-elicited memory T cells during active infection. We were able to compare their expansion with that of a new primary response to infection, and determine the most successful T cell clones by their expansion. However, we had two initial concerns with this technique. First, we wondered whether the T cells sampled in the blood after vaccination would accurately represent the entire repertoire of memory T cells that could respond to infection. By sampling a large volume of blood after vaccination, and increasing the depth of sequencing, we were able to detect both highly abundant clonotypes as well as those at such low frequency as to be on par with the naïve precursor frequency for TB10.4-specific CD8⁺ T cells in C57BL/6 mice (~1:20,000). Second, we questioned the congruence between the TCR repertoire found in the lung and the blood. However, by sequencing tetramer⁺ T cells from both the blood and lung during infection, we found >90% overlap of the total TCRs between lung and blood.

We were able to detect ~81% of the memory T cell sequences found post-vaccination in the lung during TB. While it is possible that we missed some of the post-vaccination clonotypes in the remaining 19% found post-infection, it is statistically unlikely that we missed dominant clones unless nearly all of the T cells in a particular clonotypes were inaccessible by the blood compartment (Steinert et al., 2015).

The majority of the unique TCR clonotypes detected in the lungs of infected mice were initially detected post-vaccination. Interestingly, the dominant clonotypes were all derived from a narrow repertoire, suggesting that they were undergoing selection based on their affinity or other structural features during the response to Mtb infection. One such structural feature is the dominant TCR β motif “DRENSD” that we find among TB10-specific CD8⁺ T cells in the lungs post Mtb-challenge (in our vaccinated mice), and is the same motif we previously identified in unvaccinated Mtb-infected mice (Nunes-Alves et al., 2015), representing a highly-clonal response to Mtb. Thus, this motif appears to be highly-selected and independent of vaccination. We have shown that TB10-specific CD8⁺ T cells undergo massive clonal expansion in Mtb-infected mice, characterized by selection of the CDR3 β motif, “DRENSD,” most likely driven by avidity for the TB10.4₄₋₁₁/K^b complex, a hypothesis that we are preparing to test directly (Cukalac et al., 2014; Nunes-Alves et al., 2015). Interestingly, the majority of the clonotypes in the blood after vaccination were “persisters”, in that they did not increase in frequency relative to their abundance 1w post-vaccination in blood. Thus, the

majority of vaccine-elicited TB10₄ 11-specific CD8⁺ T cells persist without expanding significantly during tuberculosis, and we relate this phenomenon to that observed after adoptive transfer of memory TB10Rg3 (lower affinity) CD8⁺ T cells into Mtb-infected mice, which are impaired in their expansion during TB. However, the higher-affinity TCR Rg CD8⁺ T cells (TB10Rg4) could be compared to a “successful” clonotypes from the TCR sequencing data as they displayed improved expansion in response to Mtb infection.

Materials and Methods

Mice. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions at the University of Massachusetts Medical School animal facilities. Mice were 7 to 10 weeks old at the start of all experiments. Infected mice were housed in biosafety level 3 facilities under specific pathogen-free conditions at UMMS.

Vaccination and elicitation antigen-specific CD8⁺ T cells. “Amphi-TB10” (MFVMFVQIMYNYPPAM) peptide was purchased from New England Peptides (Gardner, MA, USA) and reconstituted in DMSO (10mM). High molecular weight polyinosinic:polycytidylic acid [poly(I:C)] was obtained from InvivoGen (San Diego, CA). Anti-CD40 mAb (clone FGK4.5) was purchased from BioXCell (West Lebanon, NH). Vaccines were prepared by mixing 100 micromoles of peptide, 50 µg poly(I:C), and 50 µg αCD40 mAb, in a total volume of 200 µL sterile PBS and administered intravenously. Peripheral blood TB10.4₁₁-specific CD8⁺ T cell responses were sorted by flow cytometry 1 week after vaccination. Mice were rested 12 weeks after vaccination to allow for the development of memory. Mice were then challenged with aerosol Mtb infection and the secondary response was assessed in the lungs by TCRβ deep sequencing 4-5 weeks post Mtb-challenge.

Experimental infection. Mtb (strain Erdman) infections were performed via the

aerosol route using a Glas-Col full body inhalation exposure system (Terre Haute, IN). 1mL Mtb Erdman culture was thawed from a frozen stock of broth culture in Middlebrooke 7H9 medium frozen at -80°C at mid-log phase growth. The same frozen stock was used for all experiments. Mice received an inoculation dose of 100-150 CFU/mouse, as measured within 24 hours of infection. At 4-5 weeks post-infection, mice were euthanized, lungs were aseptically removed, individually homogenized for T cell isolation. The inoculum was quantified in control mice 1 day after aerosol inoculation by plating neat lung homogenate onto 7H11 agar plates.

Flow cytometry and cell sorting. Lungs were removed after perfusion of pulmonary arteries with 10mL of cold RPMI1640. Lung cell suspensions were prepared by coarse dissociation using the GentleMACS tissue dissociator (Miltenyi Biotec, Germany). Tissue was digested for 30-60 min at 37°C with 250 U/mL collagenase (Sigma) in complete RPMI1640 [10% heat inactivated FCS (Sigma), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 10mM β -mercaptoethanol, 50 mg/ml streptomycin and 50 U/ml penicillin (all from Invitrogen)] followed by homogenization in the GentleMACS dissociator and sequential straining through 70 μ m and 40 μ m nylon cell strainers (Falcon). CD8⁺ T cells were enriched prior to surface antibody staining using positive selection (Mouse CD8 T cell isolation kit, Miltenyi Biotec) followed by magnetic separation. Surface staining was performed with antibodies specific for mouse CD3 ϵ (clone

145-2C11), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD19 (clone 6D5), CD44 (clone IM7) (all from Biolegend, CA, USA). TB10.4₁₁-loaded H-2K^b tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA, USA). Dual-tetramer staining was performed as described (Moon et al., 2009) using PE- and APC-conjugated TB10.4₁₁-loaded tetramers together to accurately enumerate low-frequency events and minimize false-positive signal. We sorted CD8⁺CD4 tetramer⁺⁺CD44^H T cells from blood, one week after vaccination with amphi-TB10/CD40/poly(I:C). Twelve weeks after vaccination, the same mice were infected with Mtb, and 4-5 weeks later, CD8⁺CD4 tetramer⁺⁺CD44^H T cells were again sorted from the lungs. An amine-reactive viability dye, Zombie Aqua (Biolegend), was used to exclude necrotic cells. All samples from Mtb-infected mice were sorted using a FACS Aria II under BSL-3 conditions, followed by genomic DNA extraction. DNA samples were heated to 85°C for 1 hour to kill any remaining bacteria prior to removing samples from the BSL-3.

Next generation sequencing. Genomic DNA was purified from sorted TB10 tetramer⁺ CD44⁺CD8⁺ T cell populations using the QIAamp DNA Mini kit (Qiagen). High-throughput TCR β sequencing was performed by Adaptive Biotechnologies Corp. (Seattle, WA) (<http://www.immunoseq.com>) and analyzed using the ImmunoSEQ analyser toolset (Lepore et al., 2014). Clonality was calculated as entropy of the frequency distribution $1 - (\text{entropy} / \log_2[\# \text{ unique TCRs}])$. Entropy,

a measure of diversity within a complex data set, is also known as the Shannon-Wiener index, Shannon's diversity index or Shannon's entropy (Sherwood et al., 2013; Stewart et al., 1997). Transforming entropy in this manner results in a clonality score on a scale between 0-1. A score of "0" indicates that every TCR is unique; a score of "1" means that every TCR is the same. WebLogo 3 was used to identify CDR3 β motifs (<http://weblogo.threeplusone.com>). S6 Supporting Information identifies the different samples, their characteristics, and their inclusion in the different analyses and figures.

Preface to Chapter IV

This chapter comprises the discussion of the work in the dissertation. The discussion includes both new, original text, as well as text from three recently published manuscripts, written by Stephen Carpenter, including:

Carpenter, S. M., Nunes-Alves, C., Booty, M. G., Way, S. S., & Behar, S. M. (2016). A Higher Activation Threshold of Memory CD8+ T Cells Has a Fitness Cost That Is Modified by TCR Affinity during Tuberculosis. *PLoS Pathogens*, 12(1), e1005380. <http://doi.org/10.1371/journal.ppat.1005380>

Behar, S. M., Carpenter, S. M., Booty, M. G., Barber, D. L., & Jayaraman, P. (2014). Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interruptus. *Seminars in Immunology*, 26(6), 559–577. <http://doi.org/10.1016/j.smim.2014.09.003>

Nunes-Alves, C., Booty, M. G., Carpenter, S. M., Jayaraman, P., Rothchild, A. C., & Behar, S. M. (2014). In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology*, 12(4), 289–299. <http://doi.org/10.1038/nrmicro3230>

CHAPTER IV

Discussion

It is unclear why vaccinated individuals, or those with prior Mtb infection, do not reliably exhibit protection from Mtb reinfection (Fine, 1995; Millet et al., 2013; van Rie et al., 1999). An often-cited benefit of recall immunity is its speed compared to naïve T cell responses, as shown during acute viral infection (Kedzierska et al., 2006), homeostatic proliferation (Sprent and Tough, 1994) and sterile antigen stimulation (Veiga-Fernandes et al., 2000). Both memory and naïve CD8⁺ T cells have been shown to expand equally well early during acute inflammation and in the presence of abundant antigen (M. D. Martin et al., 2012; Mehlhop-Williams and Bevan, 2014). However, the contribution of memory T cells may decline under certain circumstances (M. D. Martin et al., 2012; Mehlhop-Williams and Bevan, 2014; Miyakoda et al., 2012). West et al. find that memory CD8⁺ T cells proliferate poorly during a model of chronic infection (LCMV clone 13), possibly because persistently high antigen loads induce T cell exhaustion (West et al., 2011). Mehlhop-Williams and Bevan find that following vaccination with immune-complexed ovalbumin (OVA), a higher antigen threshold is required to trigger proliferation of memory T cells than naïve T cells (Mehlhop-Williams and Bevan, 2014). Finally, while memory OT-1 cells outperform naïve OT-1 cells after infection with *Listeria monocytogenes* expressing OVA; naïve OT-1 cells

dominated after infection with *Plasmodium berghei* ANKA expressing OVA (Miyakoda et al., 2012). These studies raise the possibility that T cell vaccination could fail if the environment during infection hinders the expansion of memory T cells, which could minimize their contribution. A common thread in these studies is that memory T cells are disadvantaged when antigen is limiting, particularly during states of persistent inflammation. One limitation in these studies, however, is the use of OT-I cells and microbes engineered to over-express OVA rather than T cells specific for native microbial antigens. This study is the first to evaluate the behavior of memory CD8⁺ T cells specific for a native immunodominant Mtb antigen using TCR retrogenic CD8⁺ T cells during infection. Furthermore, using peptide-challenge in two systems we show that the relative expansion of naïve and memory CD8⁺ T cells is determined by the amount of antigen present in their environment. Given the difficulty of developing a vaccine for tuberculosis, the fate of Mtb-specific memory T cells during challenge is an important question.

Early initiation and sustained proliferation of a memory recall response are two characteristics that could affect the success of memory T cells during Mtb infection. We previously hypothesized that delayed initiation of T cell immunity is associated with susceptibility to Mtb (Chackerian et al., 2002). Indeed, even a transient delay in T cell priming impairs control of Mtb in the lung (Tian et al., 2005). Conversely, vaccinating C3H mice, whose adaptive immune response is delayed by ~1 week compared to C57BL/6 mice, with a DNA vaccine promoted an early recall response and CD8⁺ T cell-mediated reduction in bacterial CFU (Y. Wu et al.,

2008). CD8⁺ T cell vaccination in CD4^{-/-} mice also led to protection after Mtb challenge (Derrick et al., 2004). In contrast, CD8⁺ T cell vaccination in C57BL/6 mice did not reduce bacterial CFU after Mtb challenge despite a robust memory response to vaccination ((Lindenstrøm et al., 2014) and Figs 2.2a,c, 2.3c). We reason this is due to the rapid 1^o adaptive immune response outnumbering the 2^o response, preventing memory T cells from contributing meaningfully to combat infection. These studies highlight the potential benefits of an early T cell response and higher affinity T cells in controlling Mtb growth; however, the specific characteristics critical to the successful expansion of memory T cells during TB have not yet been evaluated.

Our results comparing the relative expansion of TB10Rg3 and TB10Rg4 to naïve TB10.4-specific CD8⁺ T cells during TB show the crucial contribution of TCR affinity to the successful expansion of the 2^o effector CD8⁺ T cells. In our adoptive co-transfer experiments, the response of memory TB10Rg3 and naïve TB10Rg3 CD8⁺ T cells results in domination by the 1^o effectors by d21. As naïve and memory TB10Rg3 CD8⁺ T cells use an identical TCR and were co-transferred at a 1:1 ratio, factors other than TCR affinity or precursor frequency must affect T cell fitness after activation. However, there is enormous TCR diversity in individuals with intact immune systems and the success of individual clonotypes can be influenced by TCR affinity, particularly in environments characterized by little antigen presentation. Thus, in co-transfer experiments using the higher-affinity memory TB10Rg4 and naïve TB10Rg4 CD8⁺ T cells, we also observed skewing in favor of

the 1° effector response, but the effect was much smaller. The role of TCR affinity is also demonstrated by co-transfer experiments using the higher-affinity memory TB10Rg4 CD8⁺ T cells and the lower-affinity naïve TB10Rg3 CD8⁺ T cells. Now we observe the opposite result: the memory TB10Rg4 cells (which have a higher TCR affinity) dominate the response by d21. Importantly, both TB10Rg3 and TB10Rg4 were dominant clones isolated from different individuals after aerosol Mtb infection (Nunes-Alves et al., 2015). Thus, eliciting memory T cells with high affinity for pMHC may make them more likely to successfully compete and expand during Mtb challenge. During natural infection in vaccinated individuals, competition between memory and naïve T cells of differing affinities is expected to occur; therefore, increasing the affinity of memory T cells should improve their fitness.

An important property of memory T cell responses is the increase in the number of T cells specific for the eliciting antigen (Schmidt et al., 2008). Thus, the speed of the recall response is based in part on the greater precursor frequency of T cells that recognize the antigen challenge, independent of any increase in proliferative rate. By adjusting the ratio of naïve to memory T cells to a 1:1 ratio in our adoptive transfer studies, we control for any effect of T cell frequency. In contrast, our TCR sequencing of TB10 tetramer⁺ T cells from intact mice, post-vaccination and post-challenge, addresses differences between the 1° and 2° T cell responses in individuals in which both the avidity and abundance of individual clonotypes is allowed to vary. The majority (>80%) of unique TCR clonotypes

obtained from the lungs of infected mice were previously detected post-vaccination. Interestingly, the dominant clonotypes were derived from a narrow repertoire, suggesting that they were undergoing selection based on structural features during the response to Mtb infection. We speculate that high affinity is the most likely factor that would lead to the selection of these clonotypes. One dominant structural TCR β motif observed was “DRENSD”, found among TB10-specific CD8⁺ T cells in the lungs post Mtb-challenge (in our vaccinated mice). This is the same motif we previously identified as the dominant motif for TB10-specific CD8⁺ T cells that made up the primary response in unvaccinated Mtb-infected mice (Nunes-Alves et al., 2015). Overall, we find that both the primary and secondary TB10-specific CD8⁺ T cell responses to Mtb are highly-clonal and share a common “public” TCR motif. This motif appears to be highly-selected and independent of vaccination.

For the majority of the experiments in these manuscripts, we used a peptide / α CD40 / poly(I:C) vaccination strategy that induces protection against viral infection and promotes tumor eradication (Cho et al., 2011; S. Lee et al., 2012; Phoolcharoen et al., 2011). The memory CD8⁺ T cells produced by using this vaccine strategy for TB10.4 were potent cytolytic effectors and cytokine-producers, and were able to attenuate infection when transferred to immunocompromised mice. Two other CD8⁺ T cell vaccines aimed at individual Mtb epitopes have also lowered bacterial burdens in intact mice, although the vaccinated hosts had a delayed adaptive immune response (Y. Wu et al., 2008) or were deplete of CD4⁺

T cells (Derrick et al., 2004). A similarly potent vaccination strategy that elicits TB10-specific CD8⁺ T cells failed to protect intact mice against Mtb challenge (Lindenstrøm et al., 2014). In no TB vaccines, CD4 or CD8-focused, however, do we observe continued decline of bacterial CFU in the lungs, indicative of continuously functional T cells. Lindenstrøm et al. attribute the lack of protection to limited presentation of the TB10.4₄₋₁₁ epitope by infected cells during priming due to inefficient proteolytic cleavage of the TB10.4₄₋₁₁ epitope (Lindenstrøm et al., 2014). Limited antigen presentation by infected cells could explain why TB10-specific CD8⁺ T cells undergo selection and clonal expansion and could be detrimental for memory T cell responses in tuberculosis. First, it is unknown whether current vaccine strategies elicit T cells of sufficient avidity to recognize the sparse antigen presented by infected cells. Second, memory T cells generated by vaccination may drop out of the response to infection if they require a higher antigen threshold for activation, independent of TCR affinity (Mehlhop-Williams and Bevan, 2014). Why are class I MHC antigens poorly presented? In the case of TB10.4, the abundance of the protein in infected cells may be limiting. Alternatively, Mtb infection may inhibit class I MHC antigen presentation or infected macrophages in the lung may not be able to cross-present Mtb antigens. Remarkably, there exists little published data showing the direct recognition of Mtb-infected macrophages by CD8⁺ T cells (Lewinsohn et al., 2006; Serbina et al., 2000). Transfer of activated TB10Rg3 and TB10Rg4 CD8⁺ T cells to immunocompromised mice confers protection against Mtb in a TAP1-dependent

manner (Nunes-Alves et al., 2015). We conclude from these data that infected macrophages present the TB10.4₁₁ epitope in vivo. Although our current data demonstrate TCR selection, whether TCR avidity affects recognition of Mtb-infected macrophages and bacterial killing remain to be determined.

An important question is whether this idea of a low antigen state can be generalized to other antigens. We previously studied memory CD8⁺ T cells elicited by vaccination with a recombinant vaccinia virus (rVV, strain WR) expressing the Mtb antigen, EspA (Rv3616) or TB10.4 in BALB/c mice (Woodworth et al., 2011). Two weeks after Mtb challenge, the numbers of EspA- and TB10.4-specific CD8⁺ T cells were significantly greater in the lungs of vaccinated mice compared to control mice, indicating a successful recall response. However, there were no differences in the numbers of TB10.4- or EspA-specific CD8⁺ T cells in vaccinated vs. control mice four weeks after Mtb challenge. Thus, despite using a different vaccination strategy (rVV) and different antigens (EspA, TB10.4), we observe similar results, namely that memory CD8⁺ T cell responses are less fit than primary CD8⁺ T cell responses. Furthermore, the decreased fitness of TB10.4-specific memory CD8⁺ T cells is not solely a consequence of the peptide/CD40/poly(I:C) vaccination strategy. The limited antigen recognition by Ag85b-specific CD4⁺ T cells (Bold et al., 2011; Egen et al., 2011; Grace and Ernst, 2015) and the transient benefit of antibiotic-induced memory (Jung et al., 2005; Kamath and Behar, 2005) suggest relevance beyond CD8⁺ T cells.

Rational vaccine design should aim to elicit protective T cells by optimizing

their action on infected cells in several ways: Vaccine-elicited memory T cells must rapidly expand and generate secondary effector T cells that undergo sustained proliferation following activation. Whereas the functions of primary effector T cells are heterogeneously expressed, vaccination can lead to more homogenous expression of effector functions during the recall response which may have a greater protective potential. Primed effector and memory T cells should efficiently traffic to sites of infection, but the kinetics of the response must be balanced with respect to T cell subsets and limit the potential for T cell exhaustion, excessive inflammatory pathology or an ineffective or an ineffective response that hinders T cell-target contact.

As memory T cells are a potent arm of adaptive immunity, impairing memory T cell function becomes a plausible step in the bacterium's evolution as a pathogen. This complicates vaccine development against TB, as a successful candidate may need to generate high affinity T cells in order to compete with naïve, or vaccine-elicited, lower affinity T cells during TB. If a vaccine were to preferentially stimulate high affinity T cells, we predict that such T cells would be more fit during Mtb challenge. Although some argue that generating central memory T cells should be the goal of vaccination against Mtb (Lindenstrøm et al., 2013; Vogelzang et al., 2014), we and others argue that generating resident effector memory cells may be more important (Hansen et al., 2011; 2013; Jameson and Masopust, 2009; Stary et al., 2015), as CD8⁺ T cells residing at the site of infection may be poised to initiate an earlier response. Screening known Mtb

antigens for their ability to induce early memory T cell expansion during infection, and focusing on vaccines that generate high affinity T cells specific for those antigens could be an important next step in rational TB vaccine design.

References

- Ahmed, R., Gray, D., 1996. Immunological memory and protective immunity: understanding their relation. 272, 54–60.
- Ahonen, C.L., Doxsee, C.L., McGurran, S.M., Riter, T.R., Wade, W.F., Barth, R.J., Vasilakos, J.P., Noelle, R.J., Kedl, R.M., 2004. Combined TLR and CD40 triggering induces potent CD8+ T cell expansion with variable dependence on type I IFN. *J Exp Med* 199, 775–784. doi:10.1084/jem.20031591
- Aleksic, M., Dushek, O., Zhang, H., Shenderov, E., Chen, J.-L., Cerundolo, V., Coombs, D., van der Merwe, P.A., 2010. Dependence of T cell antigen recognition on T cell receptor-peptide MHC confinement time. *Immunity* 32, 163–174. doi:10.1016/j.immuni.2009.11.013
- Arstila, T.P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., Kourilsky, P., 1999. A direct estimate of the human alphabeta T cell receptor diversity. *Science* 286, 958–961.
- Axelsson-Robertson, R., Loxton, A.G., Walzl, G., Ehlers, M.M., Kock, M.M., Zumla, A., Maeurer, M., 2013. A broad profile of co-dominant epitopes shapes the peripheral Mycobacterium tuberculosis specific CD8+ T-cell immune response in South African patients with active tuberculosis. *PLoS ONE* 8, e58309. doi:10.1371/journal.pone.0058309
- Axelsson-Robertson, R., Weichold, F., Sizemore, D., Wulf, M., Skeiky, Y.A.W., Sadoff, J., Maeurer, M.J., 2010. Extensive major histocompatibility complex class I binding promiscuity for Mycobacterium tuberculosis TB10.4 peptides and immune dominance of human leucocyte antigen (HLA)-B*0702 and HLA-B*0801 alleles in TB10.4 CD8 T-cell responses. *Immunology* 129, 496–505. doi:10.1111/j.1365-2567.2009.03201.x
- Baur, K., Brinkmann, K., Schwenecker, M., Pätzold, J., Meisinger-Henschel, C., Hermann, J., Steigerwald, R., Chaplin, P., Suter, M., Hausmann, J., 2010. Immediate-early expression of a recombinant antigen by modified vaccinia virus ankara breaks the immunodominance of strong vector-specific B8R antigen in acute and memory CD8 T-cell responses. *Journal of virology* 84, 8743–8752. doi:10.1128/JVI.00604-10
- Behar, S.M., Dascher, C.C., Grusby, M.J., Wang, C.R., Brenner, M.B., 1999. Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. *J Exp Med* 189, 1973–1980.
- Behar, S.M., Divangahi, M., Remold, H.G., 2010. Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? *Nat Rev Microbiol* 8, 668–674. doi:10.1038/nrmicro2387
- Billeskov, R., Vingsbo-Lundberg, C., Andersen, P., Dietrich, J., 2007. Induction of CD8 T cells against a novel epitope in TB10.4: correlation with mycobacterial virulence and the presence of a functional region of difference-1. *J Immunol* 179, 3973–3981.
- Black, G.F., Dockrell, H.M., Crampin, A.C., Floyd, S., Weir, R.E., Bliss, L.,

- Sichali, L., Mwaungulu, L., Kanyongoloka, H., Ngwira, B., Warndorff, D.K., Fine, P.E., 2001. Patterns and implications of naturally acquired immune responses to environmental and tuberculous mycobacterial antigens in northern Malawi. *Journal of Infectious Diseases* 184, 322–329. doi:10.1086/322042
- Blythe, M.J., Zhang, Q., Vaughan, K., de Castro, R., Salimi, N., Bui, H.-H., Lewinsohn, D.M., Ernst, J.D., Peters, B., Sette, A., 2007. An analysis of the epitope knowledge related to Mycobacteria. *Immunome Res* 3, 10. doi:10.1186/1745-7580-3-10
- Bold, T.D., Banaei, N., Wolf, A.J., Ernst, J.D., 2011. Suboptimal activation of antigen-specific CD4⁺ effector cells enables persistence of *M. tuberculosis* in vivo. *PLoS Pathog* 7, e1002063. doi:10.1371/journal.ppat.1002063
- Brandt, L., Feino Cunha, J., Weinreich Olsen, A., Chilima, B., Hirsch, P., Appelberg, R., Andersen, P., 2002. Failure of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infect Immun* 70, 672–678.
- Brewer, T.F., 2000. Preventing tuberculosis with bacillus Calmette-Guérin vaccine: a meta-analysis of the literature. *CLIN INFECT DIS* 31 Suppl 3, S64–7. doi:10.1086/314072
- Brewer, T.F., Colditz, G.A., 1995. Relationship between bacille Calmette-Guérin (BCG) strains and the efficacy of BCG vaccine in the prevention of tuberculosis. *CLIN INFECT DIS* 20, 126–135.
- Bruns, H., Meinken, C., Schauenberg, P., Härter, G., Kern, P., Modlin, R.L., Antoni, C., Stenger, S., 2009. Anti-TNF immunotherapy reduces CD8⁺ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans. *J. Clin. Invest.* 119, 1167–1177. doi:10.1172/JCI38482
- Cambier, C.J., Takaki, K.K., Larson, R.P., Hernandez, R.E., Tobin, D.M., Urdahl, K.B., Cosma, C.L., Ramakrishnan, L., 2014. Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature* 505, 218–222. doi:10.1038/nature12799
- Carpenter, S.M., Nunes-Alves, C., Booty, M.G., Way, S.S., Behar, S.M., 2016. A Higher Activation Threshold of Memory CD8⁺ T Cells Has a Fitness Cost That Is Modified by TCR Affinity during Tuberculosis. *PLoS Pathog* 12, e1005380. doi:10.1371/journal.ppat.1005380
- Casrouge, A., Beaudoin, E., Dalle, S., Pannetier, C., Kanellopoulos, J., Kourilsky, P., 2000. Size estimate of the alpha beta TCR repertoire of naive mouse splenocytes. *J Immunol* 164, 5782–5787.
- Chackerian, A.A., Alt, J.M., Perera, T.V., Dascher, C.C., Behar, S.M., 2002. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. *Infect Immun* 70, 4501–4509.
- Chen, C.Y., Huang, D., Wang, R.C., Shen, L., Zeng, G., Yao, S., Shen, Y., Halliday, L., Fortman, J., McAllister, M., Estep, J., Hunt, R., Vasconcelos, D., Du, G., Porcelli, S.A., Larsen, M.H., Jacobs, W.R., Haynes, B.F., Letvin, N.L.,

- Chen, Z.W., 2009. A Critical Role for CD8 T Cells in a Nonhuman Primate Model of Tuberculosis. *PLoS Pathog* 5, e1000392. doi:10.1371/journal.ppat.1000392.g004
- Chen, W., Norbury, C.C., Cho, Y., Yewdell, J.W., Bennink, J.R., 2001. Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. *J Exp Med* 193, 1319–1326.
- Cho, H.I., Celis, E., 2009. Optimized Peptide Vaccines Eliciting Extensive CD8 T-Cell Responses with Therapeutic Antitumor Effects. *Cancer Research* 69, 9012–9019. doi:10.1158/0008-5472.CAN-09-2019
- Cho, H.I., Lee, Y.R., Celis, E., 2011. Interferon γ limits the effectiveness of melanoma peptide vaccines. *Blood* 117, 135–144. doi:10.1182/blood-2010-08-298117
- Collins, F.M., 1971. Immunogenicity of various mycobacteria and the corresponding levels of cross-protection developed between species. *Infect Immun* 4, 688–696.
- Crofts, J.P., Andrews, N.J., Barker, R.D., Delpech, V., Abubakar, I., 2010. Risk factors for recurrent tuberculosis in England and Wales, 1998-2005. *Thorax* 65, 310–314. doi:10.1136/thx.2009.124677
- Cukalac, T., Chadderton, J., Handel, A., Doherty, P.C., Turner, S.J., Thomas, P.G., La Gruta, N.L., 2014. Reproducible selection of high avidity CD8+ T-cell clones following secondary acute virus infection. *Proc Natl Acad Sci USA* 111, 1485–1490. doi:10.1073/pnas.1323736111
- Curtis, M.M., Rowell, E., Shafiani, S., Negash, A., Urdahl, K.B., Wilson, C.B., Way, S.S., 2010. Fidelity of pathogen-specific CD4+ T cells to the Th1 lineage is controlled by exogenous cytokines, interferon-gamma expression, and pathogen lifestyle. *Cell Host and Microbe* 8, 163–173. doi:10.1016/j.chom.2010.07.006
- Davis, J.M., Ramakrishnan, L., 2009. The Role of the Granuloma in Expansion and Dissemination of Early Tuberculous Infection. *Cell* 136, 37–49. doi:10.1016/j.cell.2008.11.014
- Davis, M.M., Bjorkman, P.J., 1988. T-cell antigen receptor genes and T-cell recognition. *Nature* 334, 395–402. doi:10.1038/334395a0
- Davis, M.M., Boniface, J.J., Reich, Z., Lyons, D., Hampl, J., Arden, B., Chien, Y., 1998. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* 16, 523–544. doi:10.1146/annurev.immunol.16.1.523
- Derrick, S.C., Repique, C., Snoy, P., Yang, A.L., Morris, S., 2004. Immunization with a DNA vaccine cocktail protects mice lacking CD4 cells against an aerogenic infection with *Mycobacterium tuberculosis*. *Infect Immun* 72, 1685–1692. doi:10.1128/IAI.72.3.1685-1692.2004
- Diel, R., Loddenkemper, R., Niemann, S., Meywald-Walter, K., Nienhaus, A., 2011. Negative and positive predictive value of a whole-blood interferon- γ release assay for developing active tuberculosis: an update. *Am. J. Respir. Crit. Care Med.* 183, 88–95. doi:10.1164/rccm.201006-0974OC

- Divangahi, M., Behar, S.M., Remold, H., 2013. Dying to live: how the death modality of the infected macrophage affects immunity to tuberculosis. *Adv. Exp. Med. Biol.* 783, 103–120. doi:10.1007/978-1-4614-6111-1_6
- Egen, J.G., Rothfuchs, A.G., Feng, C.G., Horwitz, M.A., Sher, A., Germain, R.N., 2011. Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity* 34, 807–819. doi:10.1016/j.immuni.2011.03.022
- Elias, D., Britton, S., Aseffa, A., Engers, H., Akuffo, H., 2008. Poor immunogenicity of BCG in helminth infected population is associated with increased in vitro TGF-beta production. *Vaccine* 26, 3897–3902. doi:10.1016/j.vaccine.2008.04.083
- Fine, P.E., 1995. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 346, 1339–1345.
- Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A., Bloom, B.R., 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* 178, 2249–2254.
- Flynn, J.L., Goldstein, M.M., Triebold, K.J., Koller, B., Bloom, B.R., 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc Natl Acad Sci USA* 89, 12013–12017.
- Fraser, K.A., Schenkel, J.M., Jameson, S.C., Vezys, V., Masopust, D., 2013. Preexisting high frequencies of memory CD8+ T cells favor rapid memory differentiation and preservation of proliferative potential upon boosting. *Immunity* 39, 171–183. doi:10.1016/j.immuni.2013.07.003
- Gallegos, A.M., Xiong, H., Leiner, I.M., Sušac, B., Glickman, M.S., Pamer, E.G., van Heijst, J.W.J., 2016. Control of T cell antigen reactivity via programmed TCR downregulation. *Nat Immunol* 17, 379–386. doi:10.1038/ni.3386
- Geldenhuys, H., Mearns, H., Miles, D.J.C., Tameris, M., Hokey, D., Shi, Z., Bennett, S., Andersen, P., Kromann, I., Hoff, S.T., Hanekom, W.A., Mahomed, H., Hatherill, M., Scriba, T.J., H4:IC31 Trial Study Group, van Rooyen, M., Bruce McClain, J., Ryall, R., de Bruyn, G., H4:IC31 Trial Study Group, 2015. The tuberculosis vaccine H4:IC31 is safe and induces a persistent polyfunctional CD4 T cell response in South African adults: A randomized controlled trial. *Vaccine* 33, 3592–3599. doi:10.1016/j.vaccine.2015.05.036
- Govern, C.C., Paczosa, M.K., Chakraborty, A.K., Huseby, E.S., 2010. Fast on-rates allow short dwell time ligands to activate T cells. *Proc Natl Acad Sci USA* 107, 8724–8729. doi:10.1073/pnas.1000966107
- Grace, P.S., Ernst, J.D., 2015. Suboptimal Antigen Presentation Contributes to Virulence of *Mycobacterium tuberculosis* In Vivo. *The Journal of Immunology* 196, 1501494–364. doi:10.4049/jimmunol.1501494
- Haluszczak, C., Akue, A.D., Hamilton, S.E., Johnson, L.D.S., Pujanauski, L., Teodorovic, L., Jameson, S.C., Kedl, R.M., 2009. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells

- bearing markers of homeostatic expansion. *Journal of Experimental Medicine* 206, 435–448. doi:10.1084/jem.20081829
- Hansen, S.G., Ford, J.C., Lewis, M.S., Ventura, A.B., Hughes, C.M., Coyne-Johnson, L., Whizin, N., Oswald, K., Shoemaker, R., Swanson, T., Legasse, A.W., Chiuchiolo, M.J., Parks, C.L., Axthelm, M.K., Nelson, J.A., Jarvis, M.A., Piatak, M., Lifson, J.D., Picker, L.J., 2011. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature* 473, 523–527. doi:10.1038/nature10003
- Hansen, S.G., Piatak, M., Ventura, A.B., Hughes, C.M., Gilbride, R.M., Ford, J.C., Oswald, K., Shoemaker, R., Li, Y., Lewis, M.S., Gilliam, A.N., Xu, G., Whizin, N., Burwitz, B.J., Planer, S.L., Turner, J.M., Legasse, A.W., Axthelm, M.K., Nelson, J.A., Früh, K., Sacha, J.B., Estes, J.D., Keele, B.F., Edlefsen, P.T., Lifson, J.D., Picker, L.J., 2013. Immune clearance of highly pathogenic SIV infection. *Nature* 502, 100–104. doi:10.1038/nature12519
- Harriff, M.J., Cansler, M.E., Toren, K.G., Canfield, E.T., Kwak, S., Gold, M.C., Lewinsohn, D.M., 2014. Human lung epithelial cells contain *Mycobacterium tuberculosis* in a late endosomal vacuole and are efficiently recognized by CD8⁺ T cells. *PLoS ONE* 9, e97515. doi:10.1371/journal.pone.0097515
- Hartman, M.L., Kornfeld, H., 2011. Interactions between naïve and infected macrophages reduce *Mycobacterium tuberculosis* viability. *PLoS ONE* 6, e27972. doi:10.1371/journal.pone.0027972
- Henao-Tamayo, M., Ordway, D.J., Orme, I.M., 2014. Memory T cell subsets in tuberculosis: what should we be targeting? *Tuberculosis (Edinb)* 94, 455–461. doi:10.1016/j.tube.2014.05.001
- Hoang, T.T.K.T., Nansen, A., Roy, S., Billeskov, R., Aagaard, C., Elvang, T., Dietrich, J., Andersen, P., 2009. Distinct differences in the expansion and phenotype of TB10.4 specific CD8 and CD4 T cells after infection with *Mycobacterium tuberculosis*. *PLoS ONE* 4, e5928. doi:10.1371/journal.pone.0005928
- Holst, J., Szymczak-Workman, A.L., Vignali, K.M., Burton, A.R., Workman, C.J., Vignali, D.A.A., 2006. Generation of T-cell receptor retrogenic mice. *Nat Protoc* 1, 406–417. doi:10.1038/nprot.2006.61
- Horsburgh, C.R., 2004. Priorities for the treatment of latent tuberculosis infection in the United States. *N Engl J Med* 350, 2060–2067. doi:10.1056/NEJMsa031667
- Horsburgh, C.R., Rubin, E.J., 2011. Clinical practice. Latent tuberculosis infection in the United States. *N Engl J Med* 364, 1441–1448. doi:10.1056/NEJMcp1005750
- Irwin, S.M., Izzo, A.A., Dow, S.W., Skeiky, Y.A.W., Reed, S.G., Alderson, M.R., Orme, I.M., 2005. Tracking antigen-specific CD8 T lymphocytes in the lungs of mice vaccinated with the Mtb72F polyprotein. *Infect Immun* 73, 5809–5816. doi:10.1128/IAI.73.9.5809-5816.2005
- Jabbari, A., Harty, J.T., 2006. Secondary memory CD8⁺ T cells are more protective but slower to acquire a central-memory phenotype. *J Exp Med*

- 203, 919–932. doi:10.1084/jem.20052237
- Jameson, S.C., Masopust, D., 2009. Diversity in T Cell Memory: An Embarrassment of Riches. *Immunity* 31, 859–871. doi:10.1016/j.immuni.2009.11.007
- Jenkins, M.K., Chu, H.H., McLachlan, J.B., Moon, J.J., 2010. On the composition of the preimmune repertoire of T cells specific for Peptide-major histocompatibility complex ligands. *Annu Rev Immunol* 28, 275–294. doi:10.1146/annurev-immunol-030409-101253
- Jenkins, M.K., Moon, J.J., 2012. The role of naive T cell precursor frequency and recruitment in dictating immune response magnitude. *The Journal of Immunology* 188, 4135–4140. doi:10.4049/jimmunol.1102661
- Joshi, N.S., Cui, W., Chandele, A., Lee, H.K., Urso, D.R., Hagman, J., Gapin, L., Kaech, S.M., 2007. Inflammation Directs Memory Precursor and Short-Lived Effector CD8+ T Cell Fates via the Graded Expression of T-bet Transcription Factor. *Immunity* 27, 281–295. doi:10.1016/j.immuni.2007.07.010
- Jung, Y.-J., Ryan, L., LaCourse, R., North, R.J., 2005. Properties and protective value of the secondary versus primary T helper type 1 response to airborne *Mycobacterium tuberculosis* infection in mice. *J Exp Med* 201, 1915–1924. doi:10.1084/jem.20050265
- Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., Ahmed, R., 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4, 1191–1198. doi:10.1038/ni1009
- Kagina, B.M.N., Tameris, M.D., Geldenhuys, H., Hatherill, M., Abel, B., Hussey, G.D., Scriba, T.J., Mahomed, H., Sadoff, J.C., Hanekom, W.A., 018-402 Clinical Lab study team, Mansoor, N., Hughes, J., de Kock, M., Whatney, W., Africa, H., Krohn, C., Veldsman, A., Kany, A.L.K., Douoguih, M., Pau, M.G., Hendriks, J., McClainc, B., Benko, J., Snowden, M.A., Hokey, D.A., 2014. The novel tuberculosis vaccine, AERAS-402, is safe in healthy infants previously vaccinated with BCG, and induces dose-dependent CD4 and CD8T cell responses. *Vaccine* 32, 5908–5917. doi:10.1016/j.vaccine.2014.09.001
- Kamath, A., Woodworth, J.S.M., Behar, S.M., 2006. Antigen-specific CD8+ T cells and the development of central memory during *Mycobacterium tuberculosis* infection. *J Immunol* 177, 6361–6369.
- Kamath, A.B., Behar, S.M., 2005. Anamnestic responses of mice following *Mycobacterium tuberculosis* infection. *Infect Immun* 73, 6110–6118. doi:10.1128/IAI.73.9.6110-6118.2005
- Kamath, A.B., Woodworth, J.S., Xiong, X., Taylor, C., Weng, Y., Behar, S.M., 2004. Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after *Mycobacterium tuberculosis* infection. *J Exp Med* 200, 1479–1489. doi:10.1084/jem.20041690
- Kato-Maeda, M., Hopewell, P.C., Ernst, J.D., 2016. Chapter 35: Tuberculosis, in: Broaddus, V.C., Ernst, J.D., King, T.E., Jr., Lazarus, S.C., Murray, J.F.,

- Nadel, J.A., Slutsky, A.S., Gotway, M.B. (Eds.), Murray and Nadel's Textbook of Respiratory Medicine. Philadelphia, PA, pp. 593–628.
- Kedzierska, K., La Gruta, N.L., Turner, S.J., Doherty, P.C., 2006. Establishment and recall of CD8⁺ T-cell memory in a model of localized transient infection. *Immunol. Rev.* 211, 133–145. doi:10.1111/j.0105-2896.2006.00386.x
- Kotturi, M.F., Scott, I., Wolfe, T., Peters, B., Sidney, J., Cheroutre, H., Herrath, von, M.G., Buchmeier, M.J., Grey, H., Sette, A., 2008. Naive precursor frequencies and MHC binding rather than the degree of epitope diversity shape CD8⁺ T cell immunodominance. *The Journal of Immunology* 181, 2124–2133.
- Lee, J., Hartman, M., Kornfeld, H., 2009. Macrophage Apoptosis in Tuberculosis. *Yonsei Med J* 50, 1–11. doi:10.3349/ymj.2009.50.1.1
- Lee, S., Stokes, K.L., Currier, M.G., Sakamoto, K., Lukacs, N.W., Celis, E., Moore, M.L., 2012. Vaccine-elicited CD8⁺ T cells protect against respiratory syncytial virus strain A2-line19F-induced pathogenesis in BALB/c mice. *Journal of virology* 86, 13016–13024. doi:10.1128/JVI.01770-12
- Leemans, J.C., Juffermans, N.P., Florquin, S., van Rooijen, N., Vervoordeldonk, M.J., Verbon, A., van Deventer, S.J., van der Poll, T., 2001. Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. *J Immunol* 166, 4604–4611.
- Lepore, M., Kalinichenko, A., Kalinichenko, A., Colone, A., Paleja, B., Singhal, A., Tschumi, A., Lee, B., Poidinger, M., Zolezzi, F., Quagliata, L., Sander, P., Newell, E., Bertolotti, A., Terracciano, L., De Libero, G., Mori, L., 2014. Parallel T-cell cloning and deep sequencing of human MAIT cells reveal stable oligoclonal TCR β repertoire. *Nat Commun* 5, 3866. doi:10.1038/ncomms4866
- Lewinsohn, D.M., Grotzke, J.E., Heinzl, A.S., Zhu, L., Owendale, P.J., Johnson, M., Alderson, M.R., 2006. Secreted proteins from Mycobacterium tuberculosis gain access to the cytosolic MHC class-I antigen-processing pathway. *J Immunol* 177, 437–442.
- Lin, P.L., Flynn, J.L., 2015. CD8 T cells and Mycobacterium tuberculosis infection. *Semin Immunopathol* 37, 239–249. doi:10.1007/s00281-015-0490-8
- Lindenstrøm, T., Aagaard, C., Christensen, D., Agger, E.M., Andersen, P., 2014. High-frequency vaccine-induced CD8⁺ T cells specific for an epitope naturally processed during infection with Mycobacterium tuberculosis do not confer protection. *Eur J Immunol* 44, 1699–1709. doi:10.1002/eji.201344358
- Lindenstrøm, T., Knudsen, N.P.H., Agger, E.M., Andersen, P., 2013. Control of chronic mycobacterium tuberculosis infection by CD4 KLRG1- IL-2-secreting central memory cells. *The Journal of Immunology* 190, 6311–6319. doi:10.4049/jimmunol.1300248
- Liu, H., Moynihan, K.D., Zheng, Y., Szeto, G.L., Li, A.V., Huang, B., Van Egeren, D.S., Park, C., Irvine, D.J., 2014. Structure-based programming of lymph-node targeting in molecular vaccines. *Nature* 507, 519–522.

- doi:10.1038/nature12978
- Majlessi, L., Rojas, M.-J., Brodin, P., Leclerc, C., 2003. CD8⁺-T-cell responses of Mycobacterium-infected mice to a newly identified major histocompatibility complex class I-restricted epitope shared by proteins of the ESAT-6 family. *Infect Immun* 71, 7173–7177.
- Martin, C.J., Booty, M.G., Rosebrock, T.R., Nunes-Alves, C., Desjardins, D.M., Keren, I., Fortune, S.M., Remold, H.G., Behar, S.M., 2012. Efferocytosis is an innate antibacterial mechanism. *Cell Host and Microbe* 12, 289–300. doi:10.1016/j.chom.2012.06.010
- Martin, C.J., Peters, K.N., Behar, S.M., 2014. Macrophages clean up: efferocytosis and microbial control. *Current Opinion in Microbiology* 17, 17–23. doi:10.1016/j.mib.2013.10.007
- Martin, M.D., Condotta, S.A., Harty, J.T., Badovinac, V.P., 2012. Population dynamics of naive and memory CD8 T cell responses after antigen stimulations in vivo. *The Journal of Immunology* 188, 1255–1265. doi:10.4049/jimmunol.1101579
- McShane, H., Pathan, A.A., Sander, C.R., Keating, S.M., Gilbert, S.C., Huygen, K., Fletcher, H.A., Hill, A.V.S., 2004. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nature Medicine* 10, 1240–1244. doi:10.1038/nm1128
- Mehlhop-Williams, E.R., Bevan, M.J., 2014. Memory CD8⁺ T cells exhibit increased antigen threshold requirements for recall proliferation. *Journal of Experimental Medicine* 211, 345–356. doi:10.1084/jem.20131271
- Millet, J.-P., Shaw, E., Orcau, À., Casals, M., Miro, J.M., Caylà, J.A., Barcelona Tuberculosis Recurrence Working Group, 2013. Tuberculosis recurrence after completion treatment in a European city: reinfection or relapse? *PLoS ONE* 8, e64898. doi:10.1371/journal.pone.0064898
- Mittrücker, H.-W., Steinhoff, U., Köhler, A., Krause, M., Lazar, D., Mex, P., Miekley, D., Kaufmann, S.H.E., 2007. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc Natl Acad Sci USA* 104, 12434–12439. doi:10.1073/pnas.0703510104
- Miyakoda, M., Kimura, D., Honma, K., Kimura, K., Yuda, M., Yui, K., 2012. Development of memory CD8⁺ T cells and their recall responses during blood-stage infection with *Plasmodium berghei* ANKA. *The Journal of Immunology* 189, 4396–4404. doi:10.4049/jimmunol.1200781
- Moguche, A.O., Shafiani, S., Clemons, C., Larson, R.P., Dinh, C., Higdon, L.E., Cambier, C.J., Sissons, J.R., Gallegos, A.M., Fink, P.J., Urdahl, K.B., 2015. ICOS and Bcl6-dependent pathways maintain a CD4 T cell population with memory-like properties during tuberculosis. *Journal of Experimental Medicine* 212, 715–728. doi:10.1084/jem.20141518
- Mogues, T., Goodrich, M.E., Ryan, L., LaCourse, R., North, R.J., 2001. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *J Exp Med* 193, 271–

280.

- Molloy, A., Laochumroonvorapong, P., Kaplan, G., 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guérin. *J Exp Med* 180, 1499–1509.
- Moon, J.J., Chu, H.H., Hataye, J., Pagán, A.J., Pepper, M., McLachlan, J.B., Zell, T., Jenkins, M.K., 2009. Tracking epitope-specific T cells. *Nat Protoc* 4, 565–581. doi:10.1038/nprot.2009.9
- Moon, J.J., Chu, H.H., Pepper, M., McSorley, S.J., Jameson, S.C., Kedl, R.M., Jenkins, M.K., 2007. Naive CD4+ T Cell Frequency Varies for Different Epitopes and Predicts Repertoire Diversity and Response Magnitude. *Immunity* 27, 203–213. doi:10.1016/j.immuni.2007.07.007
- Moraco, A.H., Kornfeld, H., 2014. Cell death and autophagy in tuberculosis. *Semin. Immunol.* 26, 497–511. doi:10.1016/j.smim.2014.10.001
- Nunes-Alves, C., Booty, M.G., Carpenter, S.M., Jayaraman, P., Rothchild, A.C., Behar, S.M., 2014. In search of a new paradigm for protective immunity to TB. *Nat Rev Microbiol* 12, 289–299. doi:10.1038/nrmicro3230
- Nunes-Alves, C., Booty, M.G., Carpenter, S.M., Rothchild, A.C., Martin, C.J., Desjardins, D., Steblenko, K., Kløverpris, H.N., Madansein, R., Ramsuran, D., Leslie, A., Correia-Neves, M., Behar, S.M., 2015. Human and Murine Clonal CD8+ T Cell Expansions Arise during Tuberculosis Because of TCR Selection. *PLoS Pathog* 11, e1004849. doi:10.1371/journal.ppat.1004849
- Obar, J.J., Jellison, E.R., Sheridan, B.S., Blair, D.A., Pham, Q.-M., Zickovich, J.M., Lefrançois, L., 2011. Pathogen-induced inflammatory environment controls effector and memory CD8+ T cell differentiation. *The Journal of Immunology* 187, 4967–4978. doi:10.4049/jimmunol.1102335
- Obar, J.J., Khanna, K.M., Lefrançois, L., 2008. Endogenous Naive CD8+ T Cell Precursor Frequency Regulates Primary and Memory Responses to Infection. *Immunity* 28, 859–869. doi:10.1016/j.immuni.2008.04.010
- Oddo, M., Renno, T., Attinger, A., Bakker, T., MacDonald, H.R., Meylan, P.R., 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular Mycobacterium tuberculosis. *J Immunol* 160, 5448–5454.
- Orme, I.M., 2006. Preclinical testing of new vaccines for tuberculosis: a comprehensive review. *Vaccine* 24, 2–19. doi:10.1016/j.vaccine.2005.07.078
- Ottenhoff, T.H.M., Kaufmann, S.H.E., 2012. Vaccines against Tuberculosis: Where Are We and Where Do We Need to Go? *PLoS Pathog* 8, e1002607. doi:10.1371/journal.ppat.1002607.t001
- Palmer, C.E., Long, M.W., 1966. Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *Am. Rev. Respir. Dis.* 94, 553–568. doi:10.1164/arrd.1966.94.4.553
- Penn-Nicholson, A., Geldenhuys, H., Burny, W., van der Most, R., Day, C.L., Jongert, E., Moris, P., Hatherill, M., Ofori-Anyinam, O., Hanekom, W., Vaccine Study Team, Bollaerts, A., Demoitie, M.-A., Kany Luabeya, A.K., De Ruymaeker, E., Tameris, M., Lapierre, D., Scriba, T.J., 2015. Safety and

- immunogenicity of candidate vaccine M72/AS01E in adolescents in a TB endemic setting. *Vaccine* 33, 4025–4034. doi:10.1016/j.vaccine.2015.05.088
- Peperzak, V., Veraar, E.A.M., Xiao, Y., Babala, N., Thiadens, K., Brugmans, M., Borst, J., 2013. CD8⁺ T cells produce the chemokine CXCL10 in response to CD27/CD70 costimulation to promote generation of the CD8⁺ effector T cell pool. *The Journal of Immunology* 191, 3025–3036. doi:10.4049/jimmunol.1202222
- Phoolcharoen, W., Dye, J.M., Kilbourne, J., Piensook, K., Pratt, W.D., Arntzen, C.J., Chen, Q., Mason, H.S., Herbst-Kralovetz, M.M., 2011. A nonreplicating subunit vaccine protects mice against lethal Ebola virus challenge. *Proc Natl Acad Sci USA* 108, 20695–20700. doi:10.1073/pnas.1117715108
- Plotkin, S.A., 2010. Correlates of protection induced by vaccination. *Clin. Vaccine Immunol.* 17, 1055–1065. doi:10.1128/CVI.00131-10
- Plumlee, C.R., Sheridan, B.S., Cicek, B.B., Lefrançois, L., 2013. Environmental Cues Dictate the Fate of Individual CD8. *Immunity* 39, 347–356. doi:10.1016/j.immuni.2013.07.014
- Ramakrishnan, L., 2012. Revisiting the role of the granuloma in tuberculosis. *Nature Reviews Immunology* 12, 352–366. doi:10.1038/nri3211
- Reiley, W.W., Calayag, M.D., Wittmer, S.T., Huntington, J.L., Pearl, J.E., Fountain, J.J., Martino, C.A., Roberts, A.D., Cooper, A.M., Winslow, G.M., Woodland, D.L., 2008. ESAT-6-specific CD4 T cell responses to aerosol *Mycobacterium tuberculosis* infection are initiated in the mediastinal lymph nodes. *Proc Natl Acad Sci USA* 105, 10961–10966. doi:10.1073/pnas.0801496105
- Sakai, S., Kauffman, K.D., Schenkel, J.M., McBerry, C.C., Mayer-Barber, K.D., Masopust, D., Barber, D.L., 2014a. Cutting edge: control of *Mycobacterium tuberculosis* infection by a subset of lung parenchyma-homing CD4 T cells. *The Journal of Immunology* 192, 2965–2969. doi:10.4049/jimmunol.1400019
- Sakai, S., Kauffman, K.D., Schenkel, J.M., McBerry, C.C., Mayer-Barber, K.D., Masopust, D., Barber, D.L., 2014b. Cutting Edge: Control of *Mycobacterium tuberculosis* Infection by a Subset of Lung Parenchyma-Homing CD4 T Cells. *The Journal of Immunology* 192, 1400019–2969. doi:10.4049/jimmunol.1400019
- Sallusto, F., Lenig, D., Förster, R., Lipp, M., Lanzavecchia, A., 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401, 708–712. doi:10.1038/44385
- Sassetti, C.M., Boyd, D.H., Rubin, E.J., 2003. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol* 48, 77–84.
- Schmidt, N.W., Podyminogin, R.L., Butler, N.S., Badovinac, V.P., Tucker, B.J., Bahjat, K.S., Lauer, P., Reyes-Sandoval, A., Hutchings, C.L., Moore, A.C., Gilbert, S.C., Hill, A.V., Bartholomay, L.C., Harty, J.T., 2008. Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. *Proc Natl Acad Sci USA* 105, 14017–14022. doi:10.1073/pnas.0805452105

- Scriba, T.J., Tameris, M., Mansoor, N., Smit, E., van der Merwe, L., Isaacs, F., Keyser, A., Moyo, S., Brittain, N., Lawrie, A., Gelderbloem, S., Veldsman, A., Hatherill, M., Hawkridge, A., Hill, A.V.S., Hussey, G.D., Mahomed, H., McShane, H., Hanekom, W.A., 2010. Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells. *Eur J Immunol* 40, 279–290. doi:10.1002/eji.200939754
- Serbina, N.V., Liu, C.C., Scanga, C.A., Flynn, J.L., 2000. CD8+ CTL from lungs of *Mycobacterium tuberculosis*-infected mice express perforin in vivo and lyse infected macrophages. *J Immunol* 165, 353–363.
- Sewell, A.K., 2012. Why must T cells be cross-reactive? *Nature Reviews Immunology* 12, 669–677. doi:10.1038/nri3279
- Shafiani, S., Dinh, C., Ertelt, J.M., Moguche, A.O., Siddiqui, I., Smigielski, K.S., Sharma, P., Campbell, D.J., Way, S.S., Urdahl, K.B., 2013. Pathogen-specific Treg cells expand early during mycobacterium tuberculosis infection but are later eliminated in response to Interleukin-12. *Immunity* 38, 1261–1270. doi:10.1016/j.immuni.2013.06.003
- Sherwood, A.M., Emerson, R.O., Scherer, D., Habermann, N., Buck, K., Staffa, J., Desmarais, C., Halama, N., Jaeger, D., Schirmacher, P., Herpel, E., Kloor, M., Ulrich, A., Schneider, M., Ulrich, C.M., Robins, H., 2013. Tumor-infiltrating lymphocytes in colorectal tumors display a diversity of T cell receptor sequences that differ from the T cells in adjacent mucosal tissue. *Cancer Immunol Immunother* 62, 1453–1461. doi:10.1007/s00262-013-1446-2
- Skjõt, R.L., Oettinger, T., Rosenkrands, I., Ravn, P., Brock, I., Jacobsen, S., Andersen, P., 2000. Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infect Immun* 68, 214–220.
- Skjõt, R.L.V., Brock, I., Arend, S.M., Munk, M.E., Theisen, M., Ottenhoff, T.H.M., Andersen, P., 2002. Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. *Infect Immun* 70, 5446–5453. doi:10.1128/IAI.70.10.5446-5453.2002
- Slütter, B., Pewe, L.L., Kaech, S.M., Harty, J.T., 2013. Lung airway-surveilling CXCR3(hi) memory CD8(+) T cells are critical for protection against influenza A virus. *Immunity* 39, 939–948. doi:10.1016/j.immuni.2013.09.013
- Sprent, J., Tough, D.F., 1994. Lymphocyte life-span and memory. *265*, 1395–1400.
- Strydom, G., Olive, A., Radovic-Moreno, A.F., Gondek, D., Alvarez, D., Basto, P.A., Perro, M., Vrbanac, V.D., Tager, A.M., Shi, J., Yethon, J.A., Farokhzad, O.C., Langer, R., Starnbach, M.N., Andrian, von, U.H., 2015. A mucosal vaccine against *Chlamydia trachomatis* generates two waves of protective memory T cells 348, aaa8205–aaa8205. doi:10.1126/science.aaa8205
- Steinert, E.M., Schenkel, J.M., Fraser, K.A., Beura, L.K., Manlove, L.S., Igyártó, B.Z., Southern, P.J., Masopust, D., 2015. Quantifying Memory CD8 T Cells

- Reveals Regionalization of Immunosurveillance. *Cell* 161, 737–749.
doi:10.1016/j.cell.2015.03.031
- Stenger, S., Hanson, D.A., Teitelbaum, R., Dewan, P., Niazi, K.R., Froelich, C.J., Ganz, T., Thoma-Uszynski, S., Melián, A., Bogdan, C., Porcelli, S.A., Bloom, B.R., Krensky, A.M., Modlin, R.L., 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *282*, 121–125.
- STEWART, C.J., 1956. Eradication of tuberculosis; impressions derived from a Scandinavian tour. *Br Med J* 1, 451–454.
- Stewart, J.J., Lee, C.Y., Ibrahim, S., Watts, P., Shlomchik, M., Weigert, M., Litwin, S., 1997. A Shannon entropy analysis of immunoglobulin and T cell receptor. *Mol. Immunol.* 34, 1067–1082.
- Sutherland, J.S., Young, J.M., Peterson, K.L., Sanneh, B., Whittle, H.C., Rowland-Jones, S.L., Adegbola, R.A., Jaye, A., Ota, M.O.C., 2010. Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. *The Journal of Immunology* 184, 6537–6544. doi:10.4049/jimmunol.1000399
- Swain, S.L., Bradley, L.M., Croft, M., Tonkonogy, S., Atkins, G., Weinberg, A.D., Duncan, D.D., Hedrick, S.M., Dutton, R.W., Huston, G., 1991. Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunol. Rev.* 123, 115–144.
- Swain, S.L., Weinberg, A.D., English, M., 1990. CD4+ T cell subsets. Lymphokine secretion of memory cells and of effector cells that develop from precursors in vitro. *J Immunol* 144, 1788–1799.
- Tameris, M.D., Hatherill, M., Landry, B.S., Scriba, T.J., Snowden, M.A., Lockhart, S., Shea, J.E., McClain, J.B., Hussey, G.D., Hanekom, W.A., Mahomed, H., McShane, H., MVA85A 020 Trial Study Team, 2013. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* 381, 1021–1028. doi:10.1016/S0140-6736(13)60177-4
- Tchilian, E.Z., Desel, C., Forbes, E.K., Bandermann, S., Sander, C.R., Hill, A.V.S., McShane, H., Kaufmann, S.H.E., 2009. Immunogenicity and protective efficacy of prime-boost regimens with recombinant (delta)ureC hly+ *Mycobacterium bovis* BCG and modified vaccinia virus ankara expressing *M. tuberculosis* antigen 85A against murine tuberculosis. *Infect Immun* 77, 622–631. doi:10.1128/IAI.00685-08
- Tian, T., Woodworth, J., Sköld, M., Behar, S.M., 2005. In vivo depletion of CD11c+ cells delays the CD4+ T cell response to *Mycobacterium tuberculosis* and exacerbates the outcome of infection. *J Immunol* 175, 3268–3272.
- Tube, N.J., Pagán, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S., Jenkins, M.K., 2013. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* 153, 785–796. doi:10.1016/j.cell.2013.04.007
- Turner, J., D'Souza, C.D., Pearl, J.E., Marietta, P., Noel, M., Frank, A.A.,

- Appelberg, R., Orme, I.M., Cooper, A.M., 2001. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. *Am. J. Respir. Cell Mol. Biol.* 24, 203–209. doi:10.1165/ajrcmb.24.2.4370
- van Pinxteren, L.A., Cassidy, J.P., Smedegaard, B.H., Agger, E.M., Andersen, P., 2000. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. *Eur J Immunol* 30, 3689–3698. doi:10.1002/1521-4141(200012)30:12<3689::AID-IMMU3689>3.0.CO;2-4
- van Rie, A., Warren, R., Richardson, M., Victor, T.C., Gie, R.P., Enarson, D.A., Beyers, N., van Helden, P.D., 1999. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. *N Engl J Med* 341, 1174–1179. doi:10.1056/NEJM199910143411602
- Vanguri, V., Govern, C.C., Smith, R., Huseby, E.S., 2013. Viral antigen density and confinement time regulate the reactivity pattern of CD4 T-cell responses to vaccinia virus infection. *Proc Natl Acad Sci USA* 110, 288–293. doi:10.1073/pnas.1208328110
- Veiga-Fernandes, H., Walter, U., Bourgeois, C., McLean, A., Rocha, B., 2000. Response of naïve and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol* 1, 47–53. doi:10.1038/76907
- Velayati, A.A., Masjedi, M.R., Farnia, P., Tabarsi, P., Ghanavi, J., Ziazarifi, A.H., Hoffner, S.E., 2009. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran. *Chest* 136, 420–425. doi:10.1378/chest.08-2427
- Venturi, V., Price, D.A., Douek, D.C., Davenport, M.P., 2008. The molecular basis for public T-cell responses? *Nature Reviews Immunology* 8, 231–238. doi:10.1038/nri2260
- Verver, S., Warren, R.M., Beyers, N., Richardson, M., van der Spuy, G.D., Borgdorff, M.W., Enarson, D.A., Behr, M.A., van Helden, P.D., 2005. Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. *Am. J. Respir. Crit. Care Med.* 171, 1430–1435. doi:10.1164/rccm.200409-1200OC
- Vezys, V., Masopust, D., Kemball, C.C., Barber, D.L., O'Mara, L.A., Larsen, C.P., Pearson, T.C., Ahmed, R., Lukacher, A.E., 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J Exp Med* 203, 2263–2269. doi:10.1084/jem.20060995
- Vitetta, E.S., Berton, M.T., Burger, C., Kepron, M., Lee, W.T., Yin, X.M., 1991. Memory B and T cells. *Annu Rev Immunol* 9, 193–217. doi:10.1146/annurev.iy.09.040191.001205
- Vogelzang, A., Perdomo, C., Zedler, U., Kuhlmann, S., Hurwitz, R., Gengenbacher, M., Kaufmann, S.H.E., 2014. Central Memory CD4+ T Cells Are Responsible for the Recombinant *Bacillus Calmette-Guérin* Δ*ureC*:hly Vaccine's Superior Protection Against Tuberculosis. *J INFECT DIS* 210, 1928–1937. doi:10.1093/infdis/jiu347
- Wallgren, A., 1948. The time-table of tuberculosis. *Tubercle*.

- West, E.E., Youngblood, B., Tan, W.G., Jin, H.-T., Araki, K., Alexe, G., Konieczny, B.T., Calpe, S., Freeman, G.J., Terhorst, C., Haining, W.N., Ahmed, R., 2011. Tight regulation of memory CD8(+) T cells limits their effectiveness during sustained high viral load. *Immunity* 35, 285–298. doi:10.1016/j.immuni.2011.05.017
- Wolf, A.J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K., Ernst, J.D., 2008. Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. *Journal of Experimental Medicine* 205, 105–115. doi:10.1084/jem.20071367
- Woodworth, J.S., Shin, D.S., Volman, M., Nunes-Alves, C., Fortune, S.M., Behar, S.M., 2011. *Mycobacterium tuberculosis* directs immunofocusing of CD8+ T cell responses despite vaccination. *J Immunol* 186, 1627–1637. doi:10.4049/jimmunol.1002911
- Woodworth, J.S., Wu, Y., Behar, S.M., 2008. *Mycobacterium tuberculosis*-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo. *The Journal of Immunology* 181, 8595–8603.
- World Health Organization, 2015. Global tuberculosis report 2015 1–204.
- Wu, S., Zhang, Y., Sun, F., Chen, M., Zhou, L., Wang, N., Zhan, S., 2013. Adverse Events Associated With the Treatment of Multidrug-Resistant Tuberculosis: A Systematic Review and Meta-analysis. *Am J Ther* 1. doi:10.1097/01.mjt.0000433951.09030.5a
- Wu, Y., Woodworth, J.S., Shin, D.S., Morris, S.L., Behar, S.M., 2008. Vaccine-elicited 10-kilodalton culture filtrate protein-specific CD8+ T cells are sufficient to mediate protection against *Mycobacterium tuberculosis* infection. *Infect Immun* 76, 2249–2255. doi:10.1128/IAI.00024-08
- Yewdell, J.W., 2006. Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. *Immunity* 25, 533–543. doi:10.1016/j.immuni.2006.09.005
- Yewdell, J.W., Del Val, M., 2004. Immunodominance in TCD8+ responses to viruses: cell biology, cellular immunology, and mathematical models., in: Presented at the *Immunity*, pp. 149–153. doi:10.1016/j.immuni.2004.06.015
- YOUMANS, G.P., PARLETT, R.C., YOUMANS, A.S., 1961. The significance of the response of mice to immunization with viable unclassified mycobacteria. *Am. Rev. Respir. Dis.* 83, 903–905. doi:10.1164/arrd.1961.83.6.903
- Zak, D.E., Penn-Nicholson, A., Scriba, T.J., Thompson, E., Suliman, S., Amon, L.M., Mahomed, H., Erasmus, M., Whatney, W., Hussey, G.D., Abrahams, D., Kafaar, F., Hawkrige, T., Verver, S., Hughes, E.J., Ota, M., Sutherland, J., Howe, R., Dockrell, H.M., Boom, W.H., Thiel, B., Ottenhoff, T.H.M., Mayanja-Kizza, H., Crampin, A.C., Downing, K., Hatherill, M., Valvo, J., Shankar, S., Parida, S.K., Kaufmann, S.H.E., Walzl, G., Aderem, A., Hanekom, W.A., ACS and GC6-74 cohort study groups, 2016. A blood RNA signature for tuberculosis disease risk: a prospective cohort study. *Lancet*. doi:10.1016/S0140-6736(15)01316-1

Zumla, A., Chakaya, J., Centis, R., D'Ambrosio, L., Mwaba, P., Bates, M., Kapata, N., Nyirenda, T., Chanda, D., Mfinanga, S., Hoelscher, M., Maeurer, M., Migliori, G.B., 2015. Tuberculosis treatment and management--an update on treatment regimens, trials, new drugs, and adjunct therapies. *Lancet Respir Med* 3, 220–234. doi:10.1016/S2213-2600(15)00063-6