# MECHANISTIC UNDERSTANDING OF THE REGULATION OF LUNG RESIDENT MEMORY T CELLS INDUCED BY TB VACCINATION STRATEGIES

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# MECHANISTIC UNDERSTANDING OF THE REGULATION OF LUNG RESIDENT MEMORY T CELLS INDUCED BY TB VACCINATION STRATEGIES

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**TITLE:** **Mechanistic understanding of the regulation of lung resident memory T cells induced by TB vaccination strategies**

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## ABSTRACT

In the recent years, it has been well established that primary respiratory viral infection-induced lung resident memory CD8 T cells ($T_{RM}$) characterized by the expression of integrins CD49a and CD103, as well as the early-activation marker CD69, constitute the first line of defense against reinfection. On the other hand, viral vector-based respiratory mucosal (RM) vaccination, as well as parenteral vaccination followed by airway luminal manipulation induce lasting and protective lung T cell immunity towards pulmonary tuberculosis (TB). However, it remains poorly understood whether and how these TB vaccination strategies induce $T_{RM}$ in the lung. As such, within this thesis we will investigate generation of lung CD8 $T_{RM}$ upon different TB vaccination strategies and the underlying mechanisms regulating establishment of such cells. Here using distinct models of replication-deficient adenoviral vector-based TB vaccination, we find that RM vaccination leads to generation of lung CD8 $T_{RM}$ identified by the expression of CD69, CD103, and very late activation Ag 1 (VLA-1). These $T_{RM}$-associated molecules are acquired by CD8 T cells in distinct tissues. In this regard, VLA-1 is acquired during T cell priming in draining mediastinal lymph nodes (dMLNs) and the others acquired after T cells entered the lung. Once in the lung, Ag-specific CD8 $T_{RM}$ continue to express VLA-1 at high levels through the effector/expansion, contraction, and memory phases of T cell responses. We also reveal that VLA-1 is not required for homing of these cells to the lung, but it negatively regulates them in the contraction phase. Furthermore, VLA-1 has a negligible role in the maintenance of such cells in the lung. Separately, we have observed that while parenteral intramuscular vaccination alone does not induce lung CD8 $T_{RM}$, subsequent RM inoculation of an Ag-

dependent, but not a non-specific inflammatory agonist induces lung CD8 $T_{RM}$. Such generation of lung CD8 $T_{RM}$ needs CD4 T cell help. These findings not only fill the current knowledge gap, but also hold important implications in developing effective vaccination strategies towards mucosal intracellular infectious diseases such as acquired immunodeficiency syndrome (AIDS), TB and herpes virus infection.

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## LIST OF ABBREVIATIONS

Ad – adenoviral
AdCh – chimpanzee adenovirus
AdHu – human adenovirus
Ag – antigen
AIDS – acquired immunodeficiency syndrome
APC – antigen presenting cell
BAL – bronchoalveolar lavage
BCG – Bacillus Calmette-Guerin
CC – CC type chemokine
CCR – CC type chemokine receptor
CD – Cluster of Differentiation protein
CpG ODN – CpG oligodeoxynucleotide
CTL – Cytotoxic T lymphocytes
CXC – CXC type chemokine
CXCR – CXC type chemokine receptor
DC – dendritic cell
dLNs – draining lymph nodes
dMLNs – draining mediastinal lymph nodes
DNA – Deoxyribonucleic acid
DTH – delayed type hypersensitivity
Eis – intracellular survival protein
Eomes – eomesodermin
ESAT-6 – early secretory antigenic target 6
FasL – Fas ligand
HIV – human immunodeficiency virus
i.m. – intramuscular
i.n. – intranasal
i.p. – intraperitoneal
i.v. –intravenous
IAV – influenza A virus
IFN – interferon
IL – interleukin
INH – isoniazid
LAM – lipoarabinomannan
LFA-1 – lymphocyte function-associated antigen 1
LPT – lung parenchymal tissue
LRG47 – IFN-inducible protein Irgm1
LV – lung vasculature
LXA4 – lipoxin A4
*M.tb – Mycobacterium tuberculosis*

mAb – monoclonal antibody
ManLAM – mannosylated lipoarabinomannan
MDR – multidrug resistant
MHC – major histocompatibility complex
MVA – Modified Vaccinia Ankara
MΦ – macrophage
NK – natural killer cells
NLTs – nonlymphoid tissues
PAS – para-aminosalicylic acid
PGE2 – prostaglandin E2
PtpA – protein tyrosine phosphatase A
PZA – pyrazinamide
RM – respiratory mucosal
RNA – Ribonucleic acid
s.c. – subcutaneous
S1P1 – sphingosine-1 phosphate receptor 1
SM – streptomycin
TB – tuberculosis
T-bet – T-box expressed in T cells
$T_{CM}$ – central memory T cells
TCR – T cell receptor
TDR – totally drug resistant
$T_{EM}$ – effector memory T cells
TGF-β – transforming growth factor-β
Th- T helper cell
TLR – toll like receptor
TNF – tumor necrosis factor
TNFR – tumor necrosis factor receptor
TRAIL – TNF-related apoptosis-inducing ligand
$T_{RM}$ – tissue-resident memory T cells
VCAM-1 – vascular cell adhesion molecule 1
VLA-1 – very late activation antigen 1
XDR – extensively drug resistant

## DECLARATION OF ACADEMIC ACHIEVEMENT

The two research articles included in the thesis are:

**1. Expression and role of VLA-1 in resident memory CD8 T cell responses to respiratory mucosal viral-vectored immunization against tuberculosis (Chapter 2)** by **Siamak Haddadi**, Niroshan Thanthrige-Don, Sam Afkhami, Amandeep Khera, Mangalakumari Jeyanathan & Zhou Xing. ***Scientific Reports. 2017*** Aug 25;7(1):9525

**SH -** primary author, contributed to the execution and completion of all experiments, data analysis and interpretation, manuscript preparation and revision.
**MJ -** assisted with experimental design, data interpretation, edited and revised the manuscript
**NTD, SA, AK -** assisted with the execution of some key experiments
**ZX –** reviewed experimental design and data interpretation, edited and revised the manuscript

**2. Respiratory mucosal modulation in parenteral TB vaccine immunized hosts induces helper CD4 T cell-dependent resident memory CD8 T cells (Chapter 3)** by **Siamak Haddadi**, Mangalakumari Jeyanathan, Yushi Yao, Maryam Vaseghi-Shanjani, Sam Afkhami & Zhou Xing– Prepared for submission to ***Journal of Immunology.***

**SH -** primary author, contributed to the execution and completion of all experiments, data analysis and interpretation, manuscript preparation and revision.
**MJ -** assisted with experimental design and data interpretation
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**ZX –** reviewed experimental design and data interpretation, edited and revised manuscript

# CHAPTER 1: INTRODUCTION

## 1.1 Tuberculosis history and epidemiology

### 1.1.1 TB epidemiology

Pulmonary TB, which is mainly caused by an intracellular bacterial pathogen *Mycobacterium tuberculosis (M.tb),* along with HIV/AIDS and malaria, is one of the major infectious causes of death. Currently 10.8 million new cases of TB still arise annually, and TB leads to 1.8 million deaths yearly. As a case in point, a quarter of the world's population is latently infected with *M.tb* (1), and about 5-10% of them develop active TB upon immunosuppression (2). The highest rate of active TB per capita has been reported in Sub-Saharan Africa. Moreover, India and China have the greatest burden of TB in the world. In the case of TB, both young children and HIV-positive hosts are considered as high-risk groups (2). In this regard, around one third of the 34 million HIV-infected individuals are co-infected with *Mtb* and one quarter of HIV related deaths are due to TB as a consequence of a loss of CD4 T cell mediated immunity (3). This underlines the importance of cellular arm of immune system in host defense against TB.

The fact that *M.tb* is spread via aerosols generated by coughing and can be infectious in low numbers makes it a major health issue in regions with high population densities and poor socioeconomic conditions as seen in developing world.

### 1.1.2 Historical and current TB chemotherapeutics

The first effective antibiotic against TB, streptomycin (SM) was introduced in 1946, but within 5 years resistance to SM was acquired by the majority of *M.tb* strains worldwide (4). Then, it was determined that the resistance could be avoided if SM was co-administered with para-aminosalicylic acid (PAS) (4). In 1952, isoniazid (INH) was discovered and has

been highly effective at impeding SM resistance and the treatment of TB. Unfortunately, like SM, in regions that TB was treated with INH alone, quickly INH-resistant strains of *M.tb* emerged. Thus, it was suggested that a combination therapy using SM, PAS and INH should be widely adopted for TB treatment (4). While highly effective, this combination therapy was discontinued due to the cost and long-term treatment (4). Further studies showed rifampicin (RMP) to be a new effective TB drug shortening the treatment period (4). The current TB treatment including co-administration of INH, RMP and or pyrazinamide (PZA) is lengthy (6-12 months) (5). In this regard, several reasons, particularly drug availability, cost, and poor compliance, have led to the development of drug-resistant TB cases, including multidrug resistant (MDR), extensively drug resistant (XDR), and totally drug resistant (TDR) TB (5). Thus, there is an urgent need for improved understanding of host defense and developing new TB vaccine strategies to control the current TB epidemics.

### 1.2 Gaps in host defense against pulmonary TB

Upon exposure to *M.tb*-containing aerosol, three clinical outcomes are possible. While only a small frequency (20-25%) of *M.tb*-exposed individuals can quickly clear infection (6), demonstrating the potential role of innate immune responses in some humans, the rest of them progress to latent TB infection (65-75%) or active disease (5-10%). It has been well documented that host defense, in particular innate immunity, is able to rapidly clear primary pulmonary TB infection in a small percentage of humans after *M.tb* exposure (6). Moreover, mounting clinical evidence suggests that some primarily infected hosts can be skin test- or *M.tb*-specific T cell test-negative (7, 8). Immune responses developed in

latent-infected individuals were also shown to markedly reduce the risk of progression to active form of disease upon re-infection (9). Nevertheless, host defense fails to clear infection in the majority of exposed individuals (7). Understanding the gaps in host defense to pulmonary TB is pivotal to design effective vaccination strategies conferring immunity in such human populations. Over thousands of years of coevolution with human beings, *M.tb* has developed complicated strategies to evade host defense and impede bacterial clearance via active immune suppression at different stages of immune responses such as innate immune suppression, delayed antigen (Ag) presentation and Th1 priming, delayed and impaired Th1 immunity in the lung (Figure 1), leading to uncontrolled *M.tb* growth in the lung in early phases of infection and persisting infection in most of the exposed humans (5).

### 1.2.1 Innate immune responses

In unvaccinated individuals, upon the first exposure to *M.tb*, a small frequency (20-25%) of them can quickly clear infection (6), demonstrating the potential role of innate immune responses in conferring protection to some humans. *M.tb* infects lung macrophages (MΦs) and utilizes cell surface receptors, such as complement, mannose, and Fcγ receptors, and DC-SIGN for its entry (10). It can survive and replicate in infected MΦs, particularly before the emergence of *M.tb* specific T cell responses in the lung by multiple mechanisms including inhibition of phagosome maturation, phagosome–lysosome fusion, reactive oxygen and nitrogen species production, apoptosis, and major histocompatibility complex (MHC) class II expression and Ag presentation. Binding of *M.tb* mannosylated lipoarabinomannan (ManLAM) and protein tyrosine phosphatase A (PtpA) to mannose

receptors and V-ATPase pumps consequently hampers phagosome–lysosome fusion and phagosome acidification, respectively (11, 12). *M.tb* virulence factors, including ManLAM, early secretory antigenic target 6 (ESAT-6), and enhanced intracellular survival protein (Eis), inhibit production of proinflammatory cytokines and chemokines by MΦs and enhance the release of anti-inflammatory cytokines, including IL-10 (13-15). In addition, *M.tb* biases MΦ death modalities from apoptosis to necrosis via upregulating lipoxin A4 (LXA4) and downregulating prostaglandin E2 (PGE2) (16). In this regard, Inhibition of apoptosis not only downregulates cross-presentation of *M.tb* Ags to DCs and consequently impairs initiation of Ag-specific T cell responses (16), but also leads to enhanced bacterial viability and dissemination (17, 18).

*M.tb* acquisition by dendritic cells (DCs) occurs through direct phagocytosis (19) or interaction with apoptotic MΦs and neutrophils containing *M.tb* bacilli (16, 20). *M.tb*-imposed innate immune suppression in the lung reduces the rate of DC recruitment to the site of infection, as well as lung draining lymph nodes (dLNs) (21, 22). Like infected MΦs, *M.tb* reduces the expression of MHC class II and costimulatory molecules on DCs and enhances the production of the Th1-inhibitory cytokines such as IL-10 and type 1 interferons (IFN-α/β) (23-25). Also, *M.tb* impedes autophagy, a pathway of delivering cytoplasmic and nuclear Ags to MHC molecules, leading to diminished Ag presentation to CD4 and CD8 T cells. Moreover, *M.tb*-imposed inhibition of apoptosis impedes Ag cross-presentation by DC, leading to impaired activation of CD8 T cells (10). Regarding the differential impact of *M.tb* on migratory DC subsets, while CD11b+ DCs, but not CD103+ DCs, are required for the activation of Th1 cells in dLNs upon *M.tb* infection, CD103+

DCs directly impede CD11b+ DC–mediated Th1 cell activation by producing the immunosuppressive cytokine IL-10 (26).

### 1.2.2 T cell-mediated immune responses

T cell-mediated immunity is pivotal in host defense against pulmonary TB. However, *M.tb*-imposed innate suppression leads to delayed Th1 cell priming in dLNs by 10–14 days post infection in mice (22). *M.tb* further delays recruitment of primed circulating Th1 cells to the lung, which takes 14–21 days to happen (21, 27), through immunosuppressive mechanisms including *M.tb* ManLAM-mediated inhibition of chemokine production by MΦs and DCs, as well as desensitization of primed T cells for S1P-mediated chemotaxis during T cell egression from dLNs (28, 29). Delayed Th1 responses are also documented in nonhuman primates, that is around several weeks for Ag-specific T cells to emerge in the lung (30). Clinical evidence suggests that it takes weeks for the development of T cell responses in the lung following *M.tb* exposure in humans, probably in a dose-dependent manner (31). Even after T cell arrival to the site of infection, the full functionality of Th1 immunity remains limited due to the *M.tb*-imposed immunosuppressive microenvironment of the lung (32, 33). However, such Ag-specific T cells are still vital for host defense, as they control bacterial burden and inhibit systemic dissemination of *M.tb* by empowering the granuloma (33). CD4 T cells are considered as the major T cell subset generated upon *M.tb* infection, and they are pivotal to immune protection (34). CD8 T cells also play a role in protection against TB (35), but their generation occurs after CD4 T cell activation and they constitute a minor T cell subset in both circulation and lung granuloma (5). Compared

to CD4 T cells, the relative CD8 T cell contribution to overall immunity against TB in humans remains to be determined.

Experimental studies confirm that CD8 T cells are required for optimal protection against TB (36). For example, murine models of virus-vectored TB vaccination leading to CD8 T cell generation have revealed a critical role for these cells in host defense against TB (5). CD8 T cells exert their bactericidal effect via production of type 1 cytokines (IFN-γ and tumor necrosis factor-α (TNF-α)) and cell-mediated cytotoxicity (CTL activity).

Both IFN-γ and TNF-α increase MΦs bactericidal activity through production of reactive nitrogen and oxygen species and LRG47 (37-39). Moreover, IFN-γ and TNF-α are required for granuloma formation (40) and the maintenance of granuloma (41). While IFN-γ production by CD8 T cells can inhibit bacterial growth, it is not enough to replace IFN-γ production by CD4 T cells. This shows that CD4 and CD8 T cells may have distinct functions during *M.tb* infection. Some of the functions mediated by CD8 T cells require CD4 T cell help (42, 43). However, *M.tb* infection inhibits MHC class II expression in MΦs in response to IFN-γ (44, 45), which may prevent the recognition of infected cells by CD4 T cells. This highlights a unique role for CD8 T cells in controlling *M.tb* burden, as CD8, but not CD4, T cell depletion gives rise to increased bacterial replication during latent *M.tb* infection in mice (46).

CTL activity of CD8 T cells is mediated by perforin, Fas ligand (FasL), TNFR, and granulysin (47, 48). However, the significance of CTL activity in TB immunity in humans remains unknown. Experimental studies demonstrate that in the absence of perforin, mice usually succumb to *M.tb* late during infection, suggesting that CTL activity is more critical

during chronic (49) or even latent (46) infection. The majority of killing strategies used by CD8 T cells towards *M.tb* infected cells induce apoptosis which dampens intracellular bacterial viability (50).

### *1.2.3* Cytokines in adaptive immunity to *M.tb*

IFN-γ, IL-12, and Th1 responses are required for protective anti-*M.tb* responses. IFN-γ plays a critical role in immunity to TB in both humans and in animal models (51-55). The major source for this cytokine is CD4 T cells, followed by CD8 T cells, natural killer (NK) cells, γδ T cells, and CD1-restricted T cells (56-58). IFN-γ plays critical roles during *M.tb* infection including direct activation of MΦs, as well as immunoregulation and anti-inflammatory functions that impede immunopathology. However, T cells remain capable of antimycobacterial activities even in the absence of IFN-γ (59).

The establishment of IFN-γ-producing T cells against primary *M.tb* infection is dependent on IL-12 (p40/p35) (56, 57, 60-62), which is mainly produced by *M.tb*–activated DCs (56, 61), in part through downstream pathways of TLR signaling (63). Not only is IL-12 required for the initiation of IFN-γ-producing T cell responses to *M.tb*, but continued IL-12p70 production is also necessary for the expansion and maintenance of Th1 responses in the lungs that are needed to keep control of chronic *M.tb* infection (63). In the absence of IL-12p70, IL-23 could partially elicit the generation of Th1 responses during *M.tb* infection (64), while this compensatory mechanism is not enough to control the infection (64, 65). As the uncontrolled activity of IFN-γ and TNF-α can be detrimental to the host during *M.tb* infection (57), various immunoregulatory mechanisms are in place to limit immunopathology, such as those mediated by Foxp3+ regulatory T cells (66-69) and IL-10

(56, 61, 70, 71). IL-10 is secreted by various cellular sources during *M.tb* infection. Thus, a fine-tuned balance between these regulators and IFN-γ/ TNF-α is critical to control *M.tb* infection with limited immunopathology.

Besides Th1 responses, IL-17-producing T cells are also induced during *M.tb* infection. However, IFN-γ acts to restrain the IL-17-producing T cell population, demonstrating that this counterregulatory pathway may be pivotal in limiting *M.tb*-associated immunopathology (72). Thus, during *M.tb* infection, IFN-γ inhibits CD4 T cell production of IL-17, and subsequently inhibits neutrophil survival and the accumulation of pathogenic neutrophils in the lung, leading to reduced lung pathology and improved disease outcome (73). During *M.tb* infection, IL-17 is mainly secreted by γδ T cells and double negative T cells, but not CD4 T cells (74). IL-17 may contribute to the formation of granuloma and Th1 responses upon BCG immunization (75), as well as granuloma formation during *M.tb* infection (76). However, IL-23, required for the initiation of Th17 response during *M.tb* infection, is inessential for protection and Th1 response if IL-12p70 is accessible (64).

Taken together, understanding the defects in host defense against *M.tb* infection is important to developing effective TB vaccination strategies aimed to fill these gaps for enhanced protection against pulmonary TB.

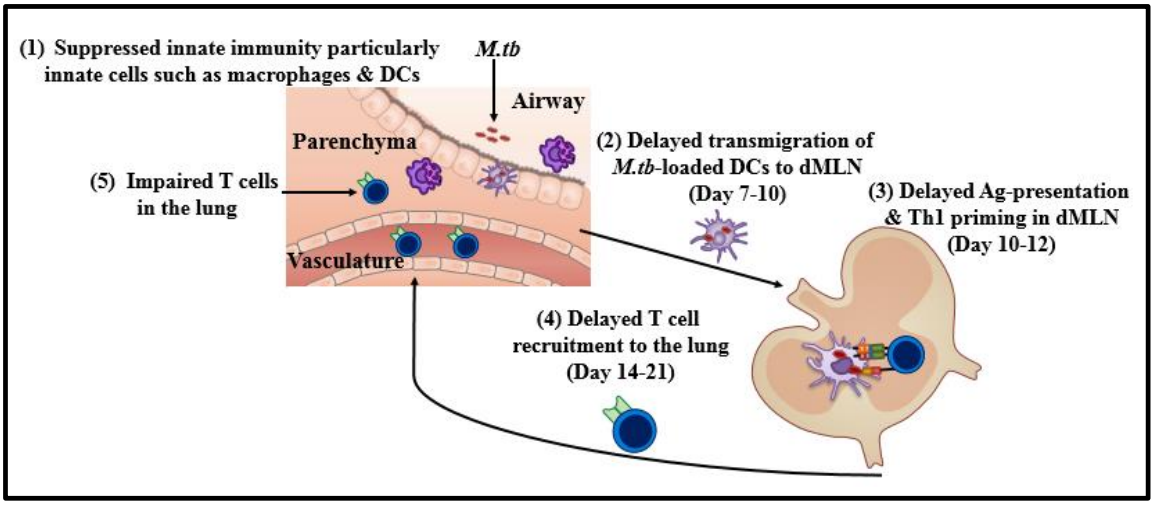


**Figure 1. Gaps in host defense towards pulmonary TB.** Features of host defense in unvaccinated individuals in response to pulmonary *Mycobacterium tuberculosis* (*M.tb*) infection. Upon arrival to the respiratory tract of an unvaccinated host, *M.tb* infects macrophages (MΦs) and dendritic cells (DCs), and consequently through suppression of innate immune responses it delays *M.tb*-infected DC migration to the draining mediastinal lymph nodes (dMLNs) by 7–10 days. Then, Th1 priming occurs in dMLNs by 10–12 days post infection. Consequently, Ag-specific T cells enter the lung en masse only by 14–21 days after infection. Such natural immunity generated in unvaccinated hosts is ineffective in controlling *M.tb* growth in lung compartments (airway and parenchyma), accounting for active disease or latent TB in most of the exposed hosts.

## 1.3 BCG vaccination

Bacillus Calmette-Guerin (BCG), attenuated *Mycobacterium bovis* bacilli derived from a virulent strain of *M. bovis* after over 13 years of continuous *in vitro* passage, is the only approved TB vaccine that has been widely utilized for more than 60 years in most countries. The first clinical trials conducted from 1921 to 1927 in France and Belgium, revealed high efficacy of BCG in protection against childhood TB (77). In France, BCG was administered via oral route, but it was stopped as a consequence of Lubeck disease, in which 67 of 249 babies received the vaccine died due to unintended contamination of the vaccine with virulent *M.tb* (78). As vaccine distribution and local production grew before establishment of standardized seed lots, many local BCG strains were developed with differences in genetic and antigenic composition (79, 80). BCG is administered as a single intradermal dose soon after birth in all countries with high risk of TB infection. While BCG is highly efficacious to end TB epidemic in Europe, its efficacy has been disappointing in developing countries (81), particularly in areas with high incidences of TB (82). While parenteral BCG vaccination has been proven efficacious towards childhood disseminated TB, it has an exceedingly variable efficacy (0-80%) against adult pulmonary TB (82). Moreover, BCG-induced immunity is not life-long as it declines within 10-15 years of vaccination and cannot be boosted by repeated BCG administration (5). There are other factors contributing to the ineffectiveness of parenteral BCG vaccination in humans, in particular the prior sensitization to environmental mycobacteria, the route of BCG vaccination (83), as well as using genetically distinct strains of BCG (79, 80).

Since many environmental mycobacterial species are immunologically cross-reactive (84-86), with some of the shared major immunodominant Ags such as the Ag85 complex proteins, repeated exposure to these mycobacteria can sensitize humans and trigger cell-mediated immune responses (87-89). This has been supported by high levels of responsiveness to such Ags in individuals pre-exposed to a high level of such mycobacteria (87-89) which may subsequently dampen the efficacy of human BCG vaccination. For example, BCG is effective in human trials from which tuberculin-skin-test-positive (and therefore sensitized) donors have been excluded (90). Furthermore, BCG vaccination of neonates before being exposed to environmental mycobacteria demonstrates effective defense against childhood forms of TB (90-92). The prevalent latent TB is considered as a major source of mycobacterial sensitization and might be an even larger single source of sensitization compared to environmental mycobacteria. In fact, there are two major interpretations about deleterious impact of the prior sensitization to mycobacteria on the efficacy of BCG vaccination. First, the masking hypothesis suggests that exposure to environmental mycobacteria gives rise to some level of protective immunity to TB that limits further protective effect of a subsequent BCG vaccination (93). Second, the blocking hypothesis postulates that the prior sensitization inhibits the replication and dissemination of BCG (94, 95). which has been supported in many human trials of BCG vaccination as observed by a lower level of skin-test conversion and smaller delayed type hypersensitivity (DTH) reaction upon BCG vaccination in areas with high levels of sensitization compared with areas where there is less exposure of environmental mycobacteria (96-98). On the other hand, the ineffectiveness of parenteral intradermal BCG vaccination can also be

attributable to its route of administration, as it fails to quickly induce a Th1 response in the lung mucosa upon *M.tb* infection. Indeed, while BCG vaccination accelerates T cell recruitment to the lung by 4-5 days (83), there is still a 10-12-day window of unchecked *M.tb* growth within the lung. As BCG provides reliable protection against disseminated form of TB in childhood, it might be considered unethical to examine and deploy a vaccine strategy that does not contain BCG. Thus, there is an urgent need for development of effective TB vaccines that can be used as booster upon BCG immunization (99).

### 1.4 Novel TB vaccines and vaccination strategies

Currently, a dozen TB vaccine candidates including mycobacterial-organism based, protein-based, and viral vector boost vaccines are in various stages of clinical trials (Figure 2), with most of them being administered via the parenteral route for prophylactic or therapeutic applications. Until now, the initial efficacy evaluations of novel TB vaccines have failed to reveal enhanced anti-TB protection over that induced by primary BCG immunization in infants or adults. Ideally, a successful TB vaccine strategy should induce a state of protective immunity comprising trained innate immunity (defined by imprinting *M.tb*-target innate cells in the lung prophylactically before *M.tb* exposure to avoid *M.tb* imposed immunosuppression on innate cells), anti-*M.tb* surface antibodies, and long lasting tissue-resident memory T cells ($T_{RM}$) (composed of balanced CD4 and CD8 T cell responses to multistage *M.tb* Ags in the lung (5). Among distinct TB vaccine platforms are viral vector platforms, particularly adenoviral (Ad) vector TB vaccines that have been considered as one of the most promising vaccine candidates (5).

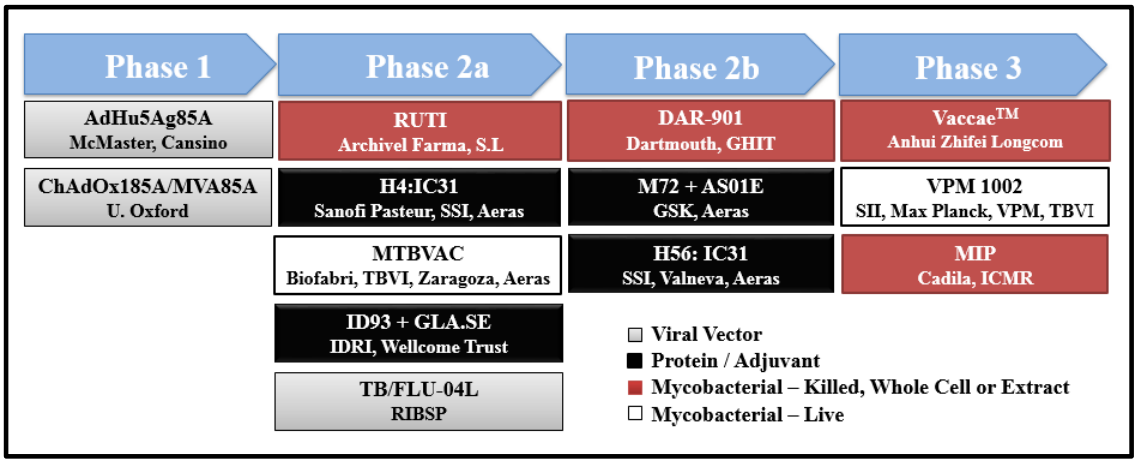


**Figure 2. Global TB vaccine pipeline.** The vaccine candidates (Viral vector, protein based, and mycobacterial organism based including killed, whole cell or extract, as well as live candidate vaccines) currently undergoing various phases of clinical assessment are shown.

### 1.4.1 Adenoviral vector-based TB vaccines

Among different viral vector platforms, recombinant Ad vectors have the features which make them suitable platforms for vaccine design. First, they have promising safety records as demonstrated by their initial use as vectors for gene therapy in humans (100). Second, recombinant Ad vectors have such broad tissue tropism for mucosal surfaces that they have been widely used for RM delivery of vaccines against mucosal infectious agents such as *M.tb* and influenza virus (100). Third, certain Ad vectors are highly immunogenic, as they can elicit robust, long-lived T cell responses to vector-encoded Ags (100).

The serotype 5 human adenovirus (AdHu5) is the most immunogenic and explored serotype for TB vaccines (101). In this regard, our lab has developed one of the best-characterized AdHu5-based TB vaccine candidates that utilizes the first-generation (E1/E3-deficient) recombinant AdHu5 vector to express an immunodominant *M.tb* Ag 85A under control of the murine cytomegalovirus promoter (AdHu5Ag85A) (102-104). This Ag (Ag85A) is a mycolyl transferase required for the incorporation of mycolic acids into the *M.tb* cell wall, as well as the synthesis of cord factor (105). Both preclinical and clinical studies demonstrate promising results including its safety and immunogenicity (99). AdHu5Ag85A may also be safely used in immunocompromised populations (106). However, the prevalence of preexisting anti-AdHu5 immunity in humans may compromise the vaccine efficacy in humans. One alternative strategy to avoid this problem would be utilizing the alternative routes of vaccination (107). In this regard, clinical studies support RM route of vaccination for AdHu5-based vaccines as much lower levels of preexisting anti-AdHu5 antibodies were found in the lung mucosa compared to the peripheral blood

(107). Other potential strategies to avoid preexisting anti-AdHu5 immunity would be utilization of rare serotypes of human adenoviruses such as AdHu35, or nonhuman adenoviruses including chimpanzee adenoviruses (107).

AdHu35 has been used for developing a trivalent TB vaccine, and compared to AdHu5, is much less immunogenic and often needs repeated inoculations in animals and humans. The inferior immunogenicity elicited by AdHu35 is due to its potent inducibility of type 1 IFNs (IFNα/β), which dampens the expression of transgene and induces MΦ inactivation (5).

Chimpanzee adenovirus-based (AdCh) vaccines have the capacity to overcome the presence of preexisting anti-AdHu5 neutralizing antibodies. Furthermore, these vectors have a potent immunogenicity compared to group C human adenovirus serotypes 5 and 6 in mice and primates (108). Technically, AdCh vectors could propagate in commercially available cell lines used for AdHu5-based vaccines without development of replication competent viruses, and Chimpanzee Ad serotype 68 (AdCh68) compared with AdHu5 demonstrates similarities in terms of infectivity, reproducibility and expression of large size transgenes (109). However, AdCh68 elicits stronger CD8 T cell responses (110) and it does not cross-react with antibodies in patients and murine models infected with various serotypes of human adenoviruses such as AdHu5 (111). The chimpanzee-based Ad vector TB vaccines including ChAdOx1.85A and AdCh68Ag85A, express Ag85A (112, 113). ChAdOx1.85A is being used in a booster regimen with Modified Vaccinia Ankara expressing Ag85A (MVA85A) to confer anti-TB protection in BCG-immunized hosts. Currently ChAdOx1.85A is under assessment in clinical trials (113). AdCh68Ag85A,

which was designed in our lab and based on chimpanzee adenovirus serotype 68, expresses the same transgene cassette as AdHu5Ag85A. It is safe, and its immunogenicity is comparable and even superior to that induced by AdHu5Ag85A. AdCh68Ag85A induces minimal levels of type-1 IFN (112).

### 1.4.2 Parenteral and respiratory mucosal routes of TB vaccination

Experimental evidence shows that if a TB vaccine strategy quickly recruits anti-*M.tb* T cells into the restricted entry sites of the lung including the lung parenchymal tissue (LPT) and airways (referred to bronchoalveolar lavage or BAL), it confers robust protection against pulmonary TB (114). In this regard, parenteral TB vaccine strategies are unlikely to confer significantly improved lung protection in most humans, as similar to *M.tb*-exposed naïve hosts, the lung environment of parenterally TB vaccine immunized hosts is still prone to *M.tb*-imposed innate immune suppression, and anti-*M.tb* T cells are sequestered in lung vasculature (LV). In fact, T cell localization in the restricted sites of the lung (LPT and BAL) is tightly regulated via selective expression of tissue-specific homing molecules including chemokine receptors (CCRs), selectins, and integrins on antigen (Ag)-experienced T cells. Likely, the site or route of vaccination, the type of vaccinated tissue, and the type of vaccine all determine the migration pattern of Ag-experienced T-cells (115-117). More interestingly, the geographical localization of Ag-experienced T cells is not a terminal event which may change or can be modified during a secondary vaccination or immunomodulation. Non-haematopoietic cells in peripheral tissues, as well as resident stromal cells and DCs in the secondary lymphoid organs all play a role in T cell tissue distribution (118). While constitutive homing of T cells into the restricted entry sites is

tightly regulated, T cell trafficking may become less restricted in inflammatory conditions. For example, during inflammation, upregulation of inflammatory chemokines and ligands for integrins might compensate for the requirements for tissue-specific homing of T cells into inflamed peripheral tissues (119). However, compared to other mucosal tissue sites such as the gut, the nature of the molecules involved T cell homing to the lung remains poorly defined.

Mounting evidence demonstrates that compared with parenteral vaccination, a well-designed RM vaccination strategy, provides robust protection against pulmonary TB (99, 120-122). An Effective RM vaccination should fill the gap in host defense against pulmonary TB through induction of a state of protective immunity at the *M.tb* entry site of the respiratory tract comprising trained-innate immunity (defined by imprinting *M.tb*-target innate cells in the lung prophylactically before *M.tb* exposure to avoid *M.tb* imposed immunosuppression on innate cells), anti-*M.tb* surface antibodies, and permanently noncirculating *M.tb*-specific tissue resident memory CD4 and CD8 T cells residing in the lung restricted entry sites. Such strategy is likely capable of conferring effective levels of protection or even sterilizing immunity (5).

There has been an interest in delivering mycobacterial organism-based vaccines such as BCG via RM route to the lung of experimental animals, particularly nonhuman primates (123-125). While parenterally repeated prime-boost BCG vaccination is inefficient in humans (126), the safety and effectiveness of BCG administration via the RM route in parenterally BCG-primed humans remains to be established. RM route of BCG administration in humans might cause undesired inflammation (127), and BCG

vaccination is not recommended by WHO in HIV-infected children. Moreover, compared to viral vector-based platforms, BCG is not effective as a CD8 T cell activator (5).

Several *M.tb* protein Ag-based TB vaccines have been examined through the RM route in murine or guinea pig models (128-131). These vaccines are adjuvanted with Ag-independent inflammatory agonists such as TLR4 agonists, or encapsulated within polymers, with encouraging results. The main concerns for RM application of protein-based TB vaccines in humans include the fact that often three repeated administrations are required, and the safe, effective immune adjuvants suitable for RM delivery remain to be developed. Moreover, these vaccines are poor inducers of CD8 T cells. Thus, further studies are required to fully characterise protein-based vaccines with suitable mucosal adjuvants for human RM application (5).

Replication-deficient viral vector-based TB vaccines expressing immunodominant *M.tb* protein Ags are amenable for human RM application owing to their general safety, built-in immune adjuvanticity, and capability to elicit trained innate immunity (100). The major candidates include MVA viral-, type 5 human Ad (AdHu5)-, type 35 human Ad (AdHu35)-, chimpanzee Ad (AdCh)-, and influenza A viral (TB/FLU-04L) vector TB vaccines.

MVAg85A has undergone the most advanced clinical evaluation. Single RM, but not parenterally intradermal, delivery of MVA85A to BCG-vaccinated humans gives rise to higher magnitude of activated T cells in the lung (132). While whether RM delivery of MVA85A enhances protection in humans remains to be determined, intradermal-delivered

MVA85A has failed to enhance protection against pulmonary TB in BCG-vaccinated individuals (133, 134). Moreover, while MVA85A induces highly durable CD4 T cell responses, this vaccine is a poor inducer of CD8 T cells (135).

RM delivery of AdHu5Ag85A has successfully gone through various experimental studies from murine to nonhuman primate models. Furthermore, such vaccination strategy does not lead to production of anti-Th1 cytokines including type 1 IFNs (IFNα/β), which contributes to its high immunogenicity (136). In this regard, murine studies have shown that RM delivery of AdHu5Ag85A mounts strong, durable cytotoxic IFNγ+TNFα+ CD8 T cell, and to a less extent CD4 T-cell, responses at the lung mucosa which is correlated with protection towards pulmonary TB (104, 137). Experimental studies in other animal models including guinea pig, bovine, and nonhuman primates have also demonstrated strong protection of RM AdHu5Ag85A immunized hosts (138-142). Of importance, single RM, but not intramuscular (i.m.), inoculation of AdHu5Ag85A in BCG-primed humanized mice leads to higher levels of activated CD4 T cells in the lung and confers robust protection against pulmonary TB (143). Intramuscular AdHu5Ag85A vaccination in BCG-primed humans was safe and immunogenic and induced polyfunctional CD4 and CD8 T cell responses (144, 145). Since the preexisting anti-AdHu5 immunity may compromise the vaccine efficacy in humans, one alternative strategy to avoid this problem is to deliver the vaccine via alternative routes (107). In this regard, clinical studies support RM route of vaccination for AdHu5-based vaccines as little preexisting anti-AdHu5 antibodies can be found in the lung mucosa compared to the peripheral blood (107). Currently a second phase

1 clinical trial evaluating the safety and immunogenicity of AdHu5Ag85A delivered by inhaled aerosol in BCG-vaccinated human volunteers is on-going.

As a rare human serotype of human adenoviruses, AdHu35 can bypass the issue associated with preexisting anti-AdHu5 immunity, and it has been used as a trivalent vaccine expressing *M.tb* immunodominant Ags including Ag85A, Ag85B (acute stage *M.tb* Ags), and TB10.4 (chronic stage *M.tb* Ag). However, compared to AdHu5, AdHu35 is much less immunogenic and often needs to be repeatedly inoculated in animals and humans. While RM administration of an AdHu35 TB vaccine confers moderate protection in murine models, it fails to protect nonhuman primates against pulmonary TB. The inferior immunogenicity elicited by AdHu35 is due to its potent inducibility of type 1 IFNs (IFNα/β), which dampens the expression of transgene and induces MΦ inactivation (5).

Chimpanzee adenovirus-based (AdCh) vaccines have the capacity to overcome the presence of preexisting anti-AdHu5 immunity as anti-AdHu5 antibodies do not cross-react with AdCh. Chimpanzee-based Ad vector TB vaccines including ChAdOx1.85A and AdCh68Ag85A express Ag85A (112, 113). Currently ChAdOx1.85A is under assessment in clinical trials. A murine study using ChAdOx1.85A has demonstrated that while RM vaccination with ChAdOx1.85A fails to markedly improve anti-TB protection in BCG-immunized hosts, it enhances protection when combined in a booster regimen with MVA85A (113). AdCh68Ag85A, which was designed in our lab based on chimpanzee adenovirus serotype 68, expresses the same transgene cassette as AdHu5Ag85A. A murine model of RM vaccination with AdCh68Ag85A has demonstrated that this vaccine is safe, and induces minimal levels of type-1 IFN (112). Compared to AdHu5Ag85A, this vaccine

elicits higher levels of long-lasting Ag-specific CD8 T cells within the lung. Interestingly, while there are comparable levels of protection upon RM vaccination with both AdHu5Ag85A and AdCh68Ag85A, the lungs from hosts vaccinated with the latter reveals less pathology as shown by reduced formation of granulomatous lesions (a hallmark of pulmonary TB infection).

Together the above-mentioned studies encourage RM delivery of viral vector-based TB vaccines to enhance lung immunity against pulmonary TB in humans.

### 1.5 Memory T cells

Memory T cells elicited following natural infection or vaccination have a critical role in host defense against infectious diseases. In the past, it was believed that cellular T cell immune responses induced by natural infection or vaccination persist only in the form of effector ($T_{EM}$) or central ($T_{CM}$) memory T cells. $T_{CM}$ recirculate between blood and secondary lymphoid organs (SLOs). Moreover, while some sub-populations of $T_{EM}$ recirculate among blood, SLOs and non-lymphoid organs, others are restricted in blood (117, 146, 147). In the recent years, the third subset of memory T cells has been recognized that persist in the site of infection without recirculation and are called as tissue-resident memory T cells ($T_{RM}$) (Figure 3) (148).

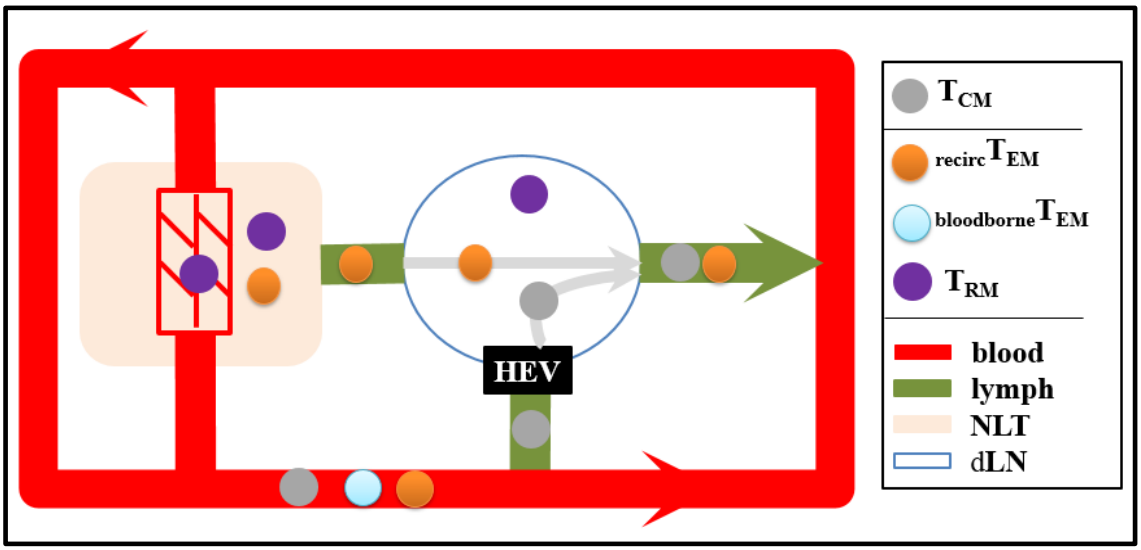


**Figure 3. Trafficking characteristics of memory T cell subsets.** The schematic illustrates the trafficking patterns of several subsets of memory T cells. $T_{CM}$ cells recirculate in blood and secondary lymphoid organs (SLOs) and enter draining lymph nodes (dLNs) through high endothelial venules (HEVs). Some subsets of $T_{EM}$ recirculate from blood to nonlymphoid tissues (NLTs) and move through lymphatics and SLOs on the way to renter the bloodstream (recirc $T_{EM}$), although other subsets are restricted in blood (bloodborne $T_{EM}$). $T_{RM}$ stay within NLTs, SLOs, and some local vasculature sites without recirculation.

### 1.5.1 Tissue-resident memory T cells ($T_{RM}$)

$T_{RM}$ have been described within nonlymphoid tissues, SLOs, and some vascular compartments such as liver sinusoids (Figure 3) (147). Lung $T_{RM}$ induced following a primary respiratory viral infection constitute the first line of defense to reinfection (149-151). $T_{RM}$ exert their effector functions through direct cytotoxic effects on infected cells and trigger a cascade of inflammatory events, resulting in innate cell activation and recruitment of circulating memory cells and leading to a state of tissue-wide pathogen alert (Figure 4) (152). Lung CD8 $T_{RM}$ are typically defined by the expression of $T_{RM}$ features including integrins CD49a (153, 154) and CD103 (149, 151), as well as the early-activation marker CD69 (149, 151). Intriguingly, while CD103 and CD69 are expressed by CD8 $T_{RM}$ in all mucosal sites, CD49a is exclusively expressed by lung and skin CD8 $T_{RM}$ (153-155). Lung CD4 $T_{RM}$ express high levels of CD49a, CD69 and integrin $\alpha_L$ (CD11a), but only a small fraction of them express CD103 (156).

CD49a (integrin $\alpha_1$) exclusively pairs with CD29 (integrin $\beta_1$) to form VLA-1 ($\alpha_1\beta_1$). VLA-1 is expressed by mesenchymal cells including endothelial cells and fibroblasts, as well as activated T cells after T cell priming (157, 158). VLA-1 binds to collagen and laminin (159), with preference for collagen IV in the basement membrane of lung epithelium, and retains T cells in the vicinity of the lung airways (160). VLA-1 contributes to T cell migration (161), retention (153), and survival (162), as well as TNF-α secretion (159). Accordingly, CD49a deficiency or blockade on $T_{RM}$ cells following primary influenza infection inhibits protection against secondary heterosubtypic infections which is associated with reduction in the number of $T_{RM}$ (153). Of note, VLA-1 protects

airway luminal effector CD8 T cells from passive apoptosis which is induced following reduction in Ag dose- and IL-2-dependent proliferation (163). However, the expression and function of VLA-1 in $T_{RM}$ responses following RM replication-deficient viral-based vaccination has remained unclear.

CD103 (integrin $\alpha_E$) pairs with Ly69 (integrin $\beta_7$) and is highly expressed by airway luminal influenza-specific CD8 T cells, as well as migratory $CD103^+$ DCs in the lung (164, 165). CD103 expression by influenza-specific CD8 T cells occurs following homing to the lung airways in a CD4 T cell-dependent way (149). In this regard, inducible CD103 expression is mediated through the effect of the active form of transforming growth factor-β (TGF-β), which is generated by both epithelial cells and DC subsets (166). CD103 binds to E-cadherin, an adherence junctional protein on adjacent cells in epithelium. Of note, CD103 interaction with E-cadherin affects both cellular shape and motility within the epithelial tissue, which restricts lymphocyte movement through epithelial cell layers (166). CD103 increases the killing capacity of cytolytic lymphocytes through formation of stable synapses with target cells (167), and signal transduction through binding to its ligand which increases T cell functions, such as lytic activity and lytic granule polarization (166). CD103 also plays a role in the retention of influenza-specific CD8 T cells in lung airways after clearance of infection, and this process requires persisting Ag presentation (164, 168, 169). However, how the persisting Ag presentation regulates CD103 expression and what would be the role CD103 during the early stages of $T_{RM}$ development still remain to be clarified.

The lectin CD69 expression is transiently acquired by activated T cells upon T cell receptor (TCR ) stimulation, but constitutively expressed on resident cells (170). CD69 as

an antagonist of sphingosine-1 phosphate receptor $_1$ ($S1P_1$), a receptor responsive to S1P gradients, downregulates $S1P_1$ expression through its internalization and degradation, thereby inhibiting T cell egress from both SLOs (secondary lymphoid organs) and non-lymphoid tissues (171). Indeed, CD69 expression persists in many non-lymphoid tissues without the requirement for constitutive specific Ag recognition (172, 173), suggesting CD69 as a potential marker of tissue residence. Nevertheless, CD69 deficiency on CD8 T cells, only leads to reduction, but not ablation, of CD8 T cell populations (174).

CD11a (integrin $\alpha_L$) pairs with CD18 (integrin $\beta_2$) to form LFA-1 ($\alpha_L\beta_2$). LFA-1 is expressed by activated T cells upon T cell priming, and its binding to ICAM-1 on endothelial cells leads to leukocyte migration across endothelial barriers into the surrounding tissues (175, 176). Like CD103, LFA-1 also increases the killing capacity of cytolytic lymphocytes through formation of stable synapses with target cells (167). While LFA-1 expression by airway luminal T cells decreases over time, CD103 expression on these cells may compensate for the low LFA-1 expression and maintain effective cytolytic responses to reinfections. However, the expression and function of LFA-1 in mucosally manipulated parenteral vaccination-induced $T_{RM}$, has remained unclear.

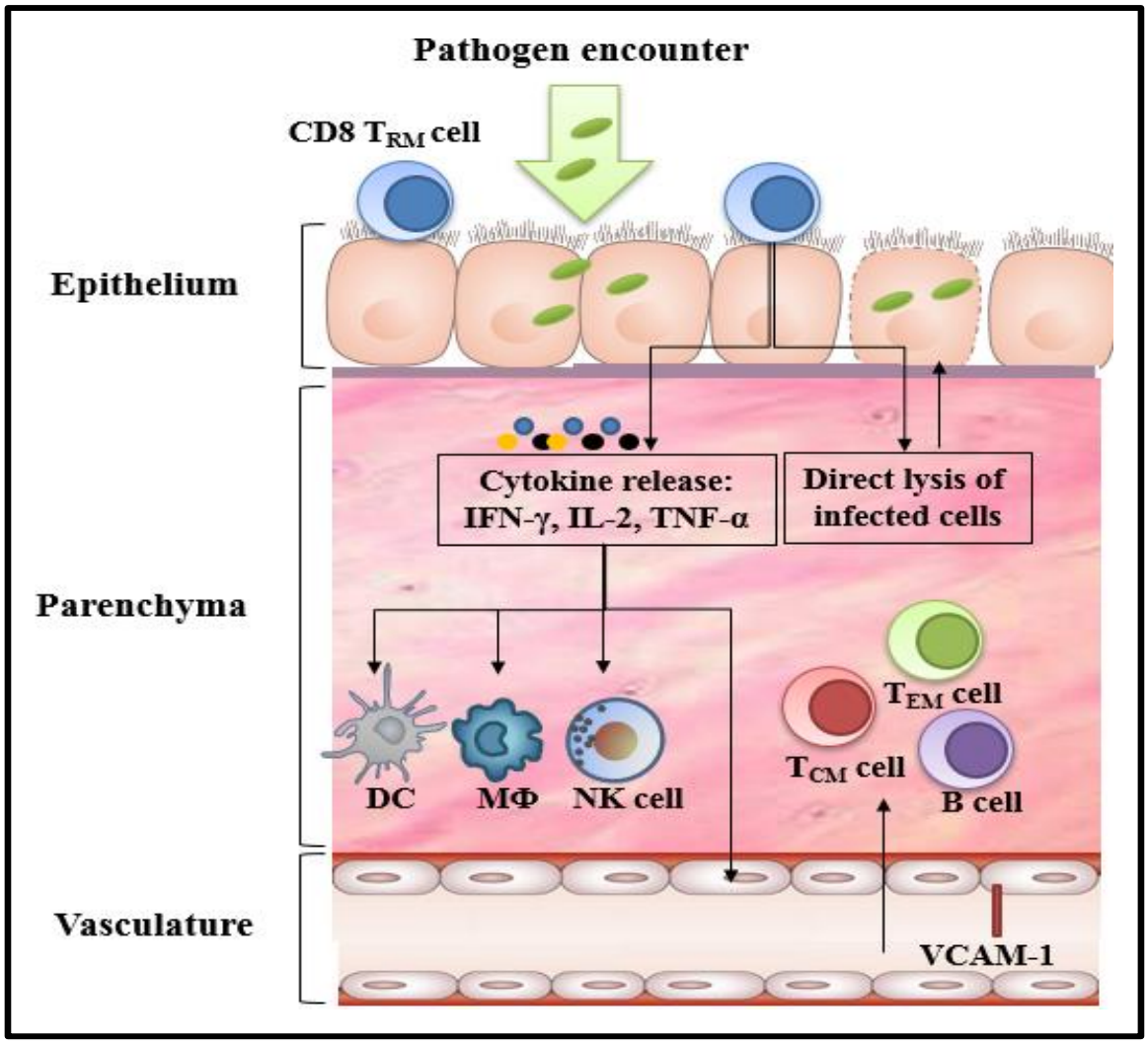


**Figure 4. Protective functions of respiratory mucosal surface CD8 $T_{RM}$ upon infection.** Tissue-resident memory T cells ($T_{RM}$) respond quickly to local pathogen re-encounter. The early phase of local immune responses in mucosal sites is mediated by CD8 $T_{RM}$ through the release of inflammatory cytokines such as interferon-γ (IFNγ), interleukin-2 (IL-2) and tumour necrosis factor-α (TNFα), as well as direct cytotoxic effects on infected cells. Local inflammatory cytokine release by $T_{RM}$ triggers a cascade of immune cell- and tissue-specific events, including the recruitment and activation of dendritic cells (DCs), macrophages (MΦ) and natural killer (NK) cells, as well as upregulation of vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells in local blood vessels, leading to increased localization of memory CD8 T cells and B cells from bloodstream. All these events result in an anti-microbial state in the tissue.

## 1.6 Induction of lung $T_{RM}$ following TB vaccination

Based on the information learned from models of respiratory viral infections and other mucosal infections, it is now well accepted that induction of $T_{RM}$ at the site of pathogen entry is critical for protection towards infection (99, 114, 177-179). Furthermore, while the mechanisms underlying lung $T_{RM}$ development and maintenance remain incompletely understood, the presence of local specific-Ags (114, 180), specific niches for $T_{RM}$ (180, 181), local inflammatory cues (174, 182-184), as well as the site of infection and the natural tissue tropism of infectious agents (185) all have been demonstrated to play a role (114, 180). In murine models of respiratory influenza A virus (IAV) infection, lung $T_{RM}$ are expanded and maintained for a long term in specific niches (186) established at the site of regeneration upon tissue damage, whereas circulating $T_{EM}$ are widely distributed in intact areas of the lung (180). In this regard, cytokines including TGF-β (149) and TNF-α (184) have been observed to play a role in generation of lung CD8 $T_{RM}$, coincided with downregulation of transcription factors including T-box expressed in T cells (T-bet) and eomesodermin (Eomes) (149, 184). In comparison, interleukin- 33 (IL-33) is only required for optimal generation of these cells without affecting $T_{RM}$ phenotype (184). IL-15 has been implicated for long-term survival of lung CD8 $T_{RM}$, which is dependent on residual T-bet expression (182). Moreover, natural tissue tropism of infectious agents plays a role in $T_{RM}$ establishment, as compared to systemic viruses, respiratory viruses have a greater capability to trigger lung $T_{RM}$ formation upon RM infection (185). All these studies suggest the superiority of RM to parenteral infection or vaccination in generation of $T_{RM}$ in the lung mucosa.

To this end, we have shown that RM vaccination with a viral vector-based TB vaccine is superior to parenteral vaccination in protection against pulmonary TB (99, 114). In fact, CD8 T cells induced by RM viral vector-based TB vaccination persist in the lung for a long term in an Ag-dependent manner, and retain their protective potential similar to those lung $T_{RM}$ cell induced following respiratory viral infection (99, 114). However, it remains unknown whether and how RM replication-deficient viral vector TB vaccination also induces lung $T_{RM}$.

While parenteral influenza infection fails to elicit lung $T_{RM}$, if it is followed by the airway delivery of specific-Ag together with a non-specific inflammatory agents such as CpG (prime-pull strategy), influenza-specific CD8 $T_{EM}$ differentiate into CD8 $T_{RM}$ by creating de novo $T_{RM}$ niches (180). To this end, we have shown that parenteral vaccination with distinct TB vaccines followed by mucosal delivery of inflammatory specific-Ags elicits lasting and protective T cell immunity in the lung restricted entry sites (114). However, it remains unknown whether RM delivery of immune-modulators after parenteral replication-deficient viral vector-based vaccination (prime-pull strategy) also induces lung $T_{RM}$, and if so, what are the underlying mechanisms.

## 1.7 Rationale, central hypothesis and objectives of study

**Rationale:** It has been demonstrated that RM vaccination with a replication-deficient viral vector-based TB vaccine is superior to parenteral vaccination against pulmonary TB (99, 114). In this regard, respiratory vaccination induced CD8 T cells in the lung persist for a long time in an Ag-dependent way and retain their protective potential similar to those lung $T_{RM}$ cells induced following respiratory viral infection (99, 114). However, it remains

to be investigated whether RM replication-deficient viral vector-based vaccination also induces lung $T_{RM}$ expressing $T_{RM}$-associated molecules, and if so, what would be their functions. Furthermore, parenteral route of vaccination is being tested with most of TB vaccines in clinical studies while safe and convenient, this strategy unlikely induces long-lasting and protective T cell immunity at the restricted entry sites of the lung. Recently, the concept of $T_{RM}$ generation at the lung mucosa through RM immune manipulation of parenterally viral pathogen-infected hosts (prime-pull strategy) has emerged, which requires local lung inflammatory signals as well as specific-Ag (180). To this end, we have shown that parenteral TB vaccine vaccination followed by RM delivery of inflammatory specific-Ag, but not non-specific inflammatory agonists, induces lasting and protective T cell-mediated immunity in the lung towards pulmonary TB (114). In this regard, it remains to be understood whether such prime-pull TB vaccination strategy also generates lung $T_{RM}$, and if so, what inductive cellular & molecular mechanisms govern generation of $T_{RM}$ in the lung. Finding the answers to these questions is not only important in filling the current knowledge gap, but also helps develop strategies to improve the protective efficacy of parenteral vaccination towards mucosal intracellular infectious diseases such as AIDS and TB, as well as lung cancers.

**Hypothesis**: Respiratory mucosal, but not parenteral, viral-vectored TB vaccination leads to the generation of CD8 $T_{RM}$ in the lung mucosa, which is closely associated with enhanced immune protection. While various respiratory mucosal immune modulatory strategies can recruit circulating CD8 T cells into the lung of parenterally vaccinated hosts, only the strategy containing specific-Ag can lead to the development of CD8 $T_{RM}$. Such

induction of lung $T_{RM}$ is dependent on the stage of parenteral vaccine-induced T cell responses and local lung immune signals.

**Objectives:**

1) To address whether RM adenovirus-vectored TB vaccination induces CD8 $T_{RM}$ in the lung, and if so, whether $T_{RM}$-associated molecules play any critical role(s) in different phases of T cell responses.

2) To address whether RM delivery of a specific-Ag or a non-specific inflammatory agonist in hosts parenterally vaccinated with an adenovirus-vectored TB vaccine induce lung CD8 $T_{RM,}$ and if so, what are underlying mechanism(s) that drive CD8 $T_{RM}$ generation.

# CHAPTER 2: Expression and role of VLA-1 in resident memory CD8 T cell responses to respiratory mucosal viral-vectored immunization against tuberculosis

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**Expression and role of VLA-1 in resident memory CD8 T cell responses to respiratory mucosal viral-vectored immunization against tuberculosis**

It has been shown that RM vaccination with a replication-deficient viral vector-based TB vaccine is superior to parenteral vaccination against pulmonary TB. In this regard, respiratory vaccination induced CD8 T cells in the lung persist for a long-term in an Ag-dependent manner and retain their protective potential like those lung CD8 $T_{RM}$ induced upon respiratory viral infection. However, it remains to be understood whether RM replication-deficient viral vector-based TB vaccination also induces lung $T_{RM}$ expressing $T_{RM}$-associated molecules, and if so, what would be their functions. In this study we evaluated generation of lung CD8 $T_{RM}$ following RM replication-deficient Ad vector-based TB vaccination, as well as the function of selected $T_{RM}$-associated molecules.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

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# Expression and role of VLA-1 in resident memory CD8 T cell responses to respiratory mucosal viral-vectored immunization against tuberculosis

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**Lung resident memory T cells ($T_{RM}$) characterized by selective expression of mucosal integrins VLA-1 ($\alpha1\beta1$) and CD103 ($\alpha_E\beta7$) are generated following primary respiratory viral infections. Despite recent progress, the generation of lung $T_{RM}$ and the role of mucosal integrins following viral vector respiratory mucosal immunization still remains poorly understood. Here by using a replication-defective viral vector tuberculosis vaccine, we show that lung Ag-specific CD8 T cells express both VLA-1 and CD103 following respiratory mucosal immunization. However, VLA-1 and CD103 are acquired in differential tissue sites with the former acquired during T cell priming in the draining lymph nodes and the latter acquired after T cells entered the lung. Once in the lung, Ag-specific CD8 T cells continue to express VLA-1 at high levels through the effector/expansion, contraction, and memory phases of T cell responses. Using a functional VLA-1 blocking mAb, we show that VLA-1 is not required for trafficking of these cells to the lung, but it negatively regulates them in the contraction phase. Furthermore, VLA-1 plays a negligible role in the maintenance of these cells in the lung. Our study provides new information on vaccine-inducible lung $T_{RM}$ and shall help develop effective viral vector respiratory mucosal tuberculosis vaccination strategies.**

Immunological memory acquired following natural infection or immunization has a critical role in host defence against infectious diseases. T cell immune responses induced by natural infection or immunization persists in the form of effector ($T_{EM}$) or central ($T_{CM}$) memory T cells[1]. In the recent years it has become clear that some of the effector memory T cells reside in non-lymphoid tissues, the site of infection, following pathogen clearance and are considered as non-circulating memory cells named resident memory T cells ($T_{RM}$) which play a critical role in immune protection[2–6].

$T_{RM}$ are typically defined by the expression of surface markers including integrin molecules. Interaction of integrins on T cells with extracellular matrix proteins is believed to play a critical role in T cell trafficking and retention in non-lymphoid mucosal tissues[7,8]. Furthermore, integrin molecules have also been implicated in regulation of T cell differentiation[9,10] and survival-related signalling pathways[11]. In this regard $T_{RM}$ persisting in the lung after acute respiratory viral infection selectively express integrins $\alpha_1\beta_1$ (also known as VLA-1/CD49a) and $\alpha_E\beta_7$ (CD103), as well as early-activation marker CD69, and provide robust protection against subsequent infections[5,6]. In particular, abundant VLA-1-expressing $T_{RM}$ were induced in murine lungs by influenza infection, and VLA-1 was shown to play a role in retention and survival, but not in trafficking, of influenza-specific CD8 T cells in the lung[12,13]. The VLA-1-expressing $T_{RM}$ have also been seen in human lungs and such lung $T_{RM}$ appear unique in that they differ from their skin and gut counterparts in their frequency[6,14,15]. Nevertheless, much still

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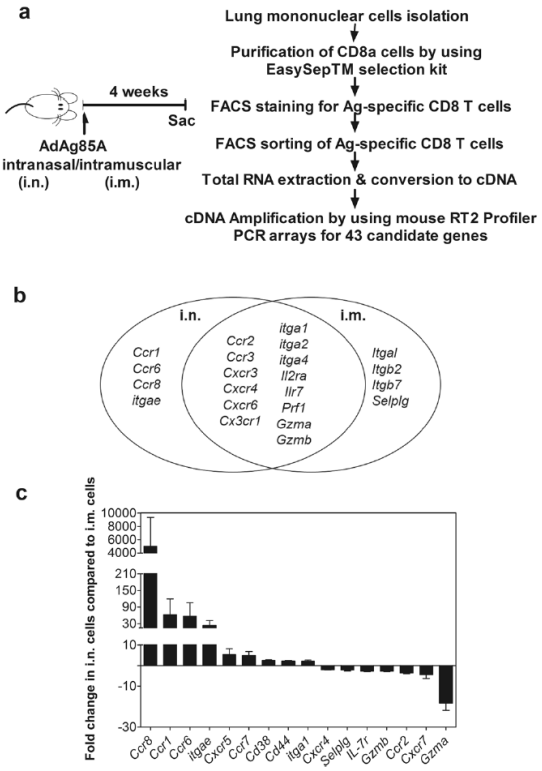


**Figure 1.** Expression of candidate genes by Ag-specific CD8 T cells induced by replication-defective viral-vectored respiratory mucosal immunization. (**a**) Experimental schema and flow chart showing the workflow. (**b**) Venn diagram depicts genes that are commonly expressed on both respiratory mucosal (i.n.) and parenteral intramuscular (i.m.) immunization-induced Ag-specific CD8 T cells, and the genes that are uniquely expressed on i.n.- and i.m.-immunization induced Ag-specific CD8 T cells. (**c**) Bar graph shows mean ± S.E.M. fold changes of genes expressed by i.n. immunization-induced Ag-specific CD8 T cells compared to i.m. immunization-induced Ag-specific CD8 T cells. Data represent mean fold changes calculated from 3 independent experiments.

remains to be understood about the development of $T_{RM}$ and the functional role of $T_{RM}$-associated integrins such as VLA-1 in the lung following respiratory mucosal viral infection.

Viral vector respiratory mucosal route of immunization has emerged as a new strategy for generating effective protective immunity against mucosal pathogens such as *Mycobacterium tuberculosis*[16–18] and enhanced knowledge in vaccine-induced lung $T_{RM}$ will help improve such strategies. Among the most promising respiratory viral vector vaccines are the recombinant human or chimpanzee adenovirus, MVA and sendai virus expressing selected immunodominant microbial antigens shown to be protective against mucosal infections including tuberculosis (TB), RSV, HIV or herpes virus[19–25]. These viral vectors are designed to be replication-defective for improved safety and are yet capable of infection to induce long-lasting T cell responses[26]. However, many differences exist in the immune responses elicited to various viral species, and replication effective (wild type) vs replication-deficient viral infections, and differential innate immune activation, and antigenic expression can all influence $T_{RM}$ generation[27]. Up to date, it still remains to be determined whether replication-defective viral vector respiratory mucosal immunization induces $T_{RM}$ in the lung and what are the functional roles of integrin molecules in the regulation of $T_{RM}$.

In the current study we have used a replication-defective adenovirus-vectored TB vaccine to investigate the $T_{RM}$ properties of respiratory mucosal immunization-induced Ag-specific T cells and the role of $T_{RM}$-associated integrin VLA-1 in such T cell responses. Our study shows that replication-defective viral vector respiratory mucosal TB immunization promotes lung $T_{RM}$ generation. However, $T_{RM}$ integrins, VLA-1 and CD103, were acquired in different phases of T cell responses and differential tissue sites. We further show that VLA-1 was not involved in T cell trafficking to the lung but rather, it played a regulatory role in the contraction phase of T cell responses in the lung. Furthermore, we found that VLA-1 is dispensable for $T_{RM}$ maintenance during the memory phase.

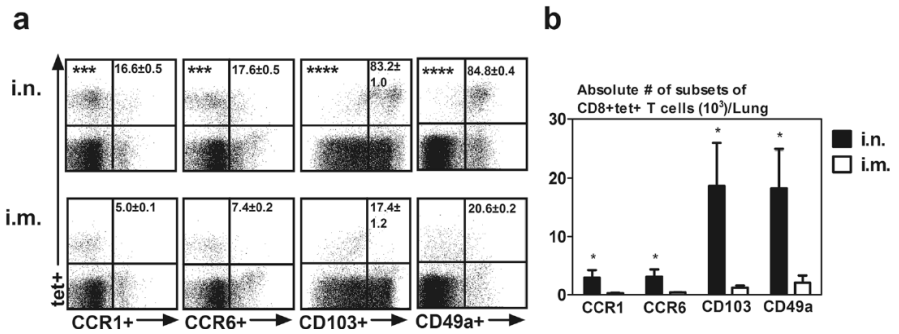


**Figure 2.** Protein expression of $T_{RM}$ surface markers by replication-defective viral-vectored respiratory mucosal immunization-induced Ag-specific CD8 T cells in the lung. Lung mononuclear cells from mice immunized with viral vector vaccine via either respiratory mucosal (i.n.) or parenteral (i.m.) route for four weeks were immunostained for surface markers CCR1, CCR6, CD103 and CD49a and analyzed using flow cytometry. (**a**) Representative dot plots showing frequencies of tet+CCR1, tet+CCR6+, tet+CD103+, tet+CD49a+CD8 T cells out of total CD8+tet+T cells in the lung of i.n. and i.m. immunized mice. (**b**) Bar graph showing absolute numbers of tet+CCR1, tet+CCR6+, tet+CD103+, tet+CD49a+CD8 T cells in the lung of i.n. and i.m. immunized mice. Data are presented as mean ± S.E.M. of three mice per group, representative of three independent experiments. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ compared with i.m. immunization.

## Results

**Viral-vectored respiratory mucosal immunization induces Ag-specific CD8 T cells in the lung with distinct gene expression profile.** To begin investigating whether respiratory mucosal immunization promotes lung $T_{RM}$ generation, we first set out to characterize the properties of vaccine-induced Ag-specific CD8 T cells in the lung. An adenovirus-vectored tuberculosis vaccine (AdAg85A) was used as a model replication-defective viral vector vaccine and this vaccine, when delivered via respiratory mucosal and parenteral intramuscular routes, induced Ag-specific CD8 T cell responses in the lung[21,28]. Interestingly, using intravascular immunostaining it has recently been shown that >95% of T cells in a naive lung are trapped in the pulmonary vasculature and bona fide lung tissue T cells were detected only after respiratory mucosal infection or immunization[29,30]. Thus using such intravascular immunostaining we first verified Ag-specific T cell distribution in the lung at 4 weeks following respiratory mucosal and parenteral route of immunization. We found that the vast majority of Ag-specific CD8 T cells induced by respiratory mucosal immunization were bona fide lung tissue T cells. In sharp contrast, most of the Ag-specific CD8 T cells induced by parenteral intramuscular immunization were located in the lung vasculature. To determine the unique properties of respiratory mucosal immunization-induced (i.n.) lung tissue Ag-specific memory CD8 T cells, we compared gene expression of these cells with gene profile in parenteral AdAg85A immunization-induced (i.m.) intravascular Ag-specific CD8 T cells at 4 weeks post-immunization and in naïve CD8 T cells. Such comparisons help identify the genes commonly induced by both routes of immunization and those uniquely expressed in respiratory mucosal immunization-induced lung Ag-specific CD8 T cells. Genes encoding for chemokine receptors, integrin heterodimers, and some activation makers (Supplementary Table 1) implicated in T cell trafficking, maintenance and differentiation[8] were profiled in FACS sorting-purified Ag-specific (Ag85A-tetramer-positive) CD8 T cells (CD8+tet+T cells) by using a custom-made PCR array (Fig. 1a). The relative gene expression was determined using data from the real-time cycler and the ΔΔCT method as previously described[31]. For our first goal, that is, identifying gene profile related to immunization, we focused on comparison of i.n. with control and i.m. with control (Fig. 1b). We found a set of genes commonly induced in Ag-specific CD8 T cells by both i.n. and i.m. immunization compared to naïve CD8 T cells (Fig. 1b). However, the genes encoding proteins CCR1, CCR6, CCR8, and CD103 (*itage*) were uniquely induced in i.n. immunization-induced CD8 T cells (Fig. 1b). Levels of *Ccr1, Ccr6, Ccr8* and *itage* gene expression by i.n. immunization-induced T cells were at least 30-fold higher than those by i.m. immunization (Fig. 1c). In addition, expression of *Cxcr5, Ccr7, CD34*, *CD44* and *itga1* (α1 integrin of VLA-1 or CD49a) genes also increased by more than 2 fold in i.n. immunization-induced memory CD8 T cells (Fig. 1c). Taken together, these data indicate that viral vector mediated respiratory mucosal TB immunization induces lung tissue Ag-specific memory CD8 T cells with a unique set of genes that are implicated in T cell mucosal tissue trafficking and maintenance.

**Viral-vectored respiratory mucosal immunization induces Ag-specific CD8 T cells expressing $T_{RM}$ surface markers.** Based on their unique gene expression profile and differential localities in the lung, we next selected to determine protein expression levels of CCR1, CCR6, CD103 (*itage*) and CD49a (*itga1*or VLA-1) on respiratory mucosal immunization-induced Ag-specific memory CD8 T cells at 4 weeks post-immunization. Although some genes such as *Cxcr5* and *Ccr7* were also increased in these cells, they were not included in our protein expression analysis as they pertains more to the homing of T cells to secondary lymphoid organs[32]. Nor was CCR8 protein examined due to limited murine immunoreagents. By flow cytometry only a smaller frequency of CD8+tet+T cells (~20%) expressed CCR1 and CCR6 protein in the lung of i.n. immunized animals (Fig. 2a).

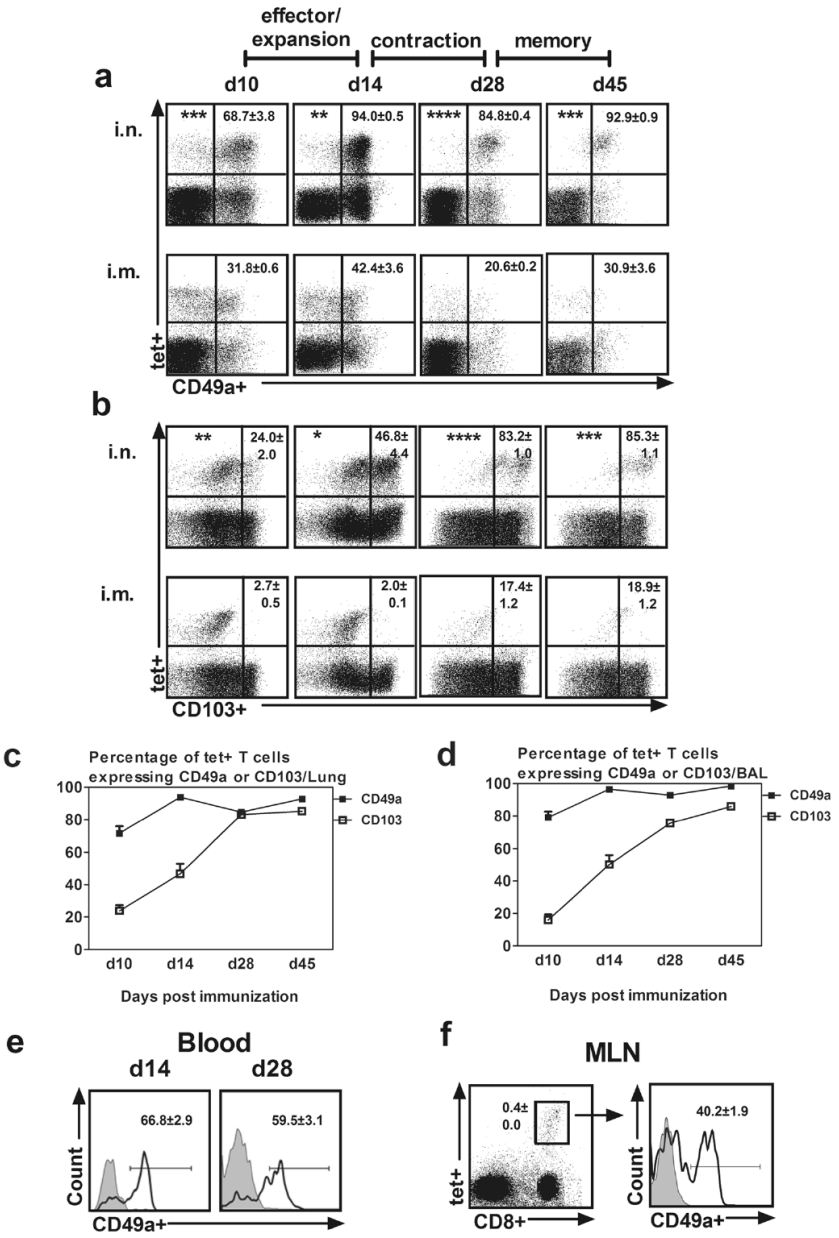


**Figure 3.** Expression of $T_{RM}$ surface markers on replication-defective viral-vectored respiratory mucosal immunization-induced Ag-specific CD8 T cells in different phases of T cell responses. Mononuclear cells from lung, BAL, peripheral blood and mediastinal lymph nodes (MLN) obtained at designated time points post-immunization were immunostained for CD49a and CD103 and analyzed using flow cytometry. (**a**/**b**) Representative dot plots showing frequencies of tet+ CD49a+ and tet+ CD103+ CD8 T cells out of total CD8+tet+T cells in the lung of respiratory mucosal (i.n.) and parenteral (i.m.) immunized mice in the effector/expansion phase (d10/d14), contraction phase (d14/d28) and memory phase (d28/d45) of T cell responses. (**c**) Line graph comparing kinetic changes in the expression of CD49a and CD103 on Ag-specific CD8 T cells in the lung induced by viral vector respiratory mucosal immunization. (**d**) Line graph comparing kinetic changes in the expression of CD49a and CD103 on Ag-specific CD8 T cells in the bronchoalveolar lavage fluid (BAL) induced by respiratory mucosal immunization. (**e**) Representative histograms showing frequencies of CD8+tet+T cells expressing CD49a in the blood at d14 and d28 post- viral vector respiratory mucosal

immunization. (**f**) Representative dot plot showing the frequency of CD8+tet+T cells out of total T cells in MLN at d14 post- viral vector respiratory mucosal immunization, and the representative histogram showing the frequency of CD8+tet+T cells expressing CD49a. Data are presented as mean ± S.E.M. of three mice per group per time point, representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with i.m. immunization.

In sharp contrast, >80% of Ag-specific CD8 T cells expressed $T_{RM}$ surface markers CD103 and CD49a (VLA-1) (Fig. 2a). In consistent with increased frequencies, we also observed significantly higher numbers of Ag-specific CD8 T cells expressing CD103 or CD49a than those expressing CCR1 or CCR6 in the lung (Fig. 2a). In comparison, very few Ag-specific memory CD8 T cells induced by i.m. immunization expressed $T_{RM}$ surface markers CD103 and CD49a (VLA-1). Together, these data demonstrate that respiratory mucosal TB immunization generates Ag-specific T cells with typical properties of $T_{RM}$ cells in the lung.

**Viral-vectored respiratory mucosal immunization-induced Ag-specific CD8 T cells acquires VLA-1 expression in the draining lymph node.** Having established that the majority of respiratory mucosal TB immunization-induced lung tissue Ag-specific CD8 T cells express classical resident memory surface markers, CD103 and CD49a[5], we sought to systematically examine the kinetic expression of these $T_{RM}$ surface markers during various phases (effector/expansion, contraction and memory) of T cell responses following respiratory mucosal immunization. To this end we first characterized the phases of T cell responses following viral vector immunization. The CD8+tet+T cells in the lung significantly increased at 10 days and peaked at 14 days post-respiratory mucosal immunization, consistent with the effector/expansion phase of T cell responses (Supplementary Fig. 1). Between 14 and 28 days, the number of CD8+tet+T cells markedly decreased by more than 80% from the peak time indicative of the contraction phase of T cell responses. From 28 days until 45 days, the number of CD8+tet+T cells in the lung remained stable, hence being in the memory phase of T cell responses. In comparison, parenteral intramuscular (i.m.) immunization led to much smaller levels of Ag-specific T cell responses in the lung in various phases (Supplementary Fig. 1).

We next examined CD103 and CD49a expression on Ag-specific CD8 T cells in the lung in different phases of T cell responses. The majority of CD8+tet+T cells in the lung of i.n. immunized animals expressed CD49a upon arrival at the lung and in the expansion/effector phase (d10–d14) and became further enriched for CD49a expression in the contraction (d14–d28) and memory phases (d28–d45) (Fig. 3a/c). In contrast to CD49a, only a small frequency of CD8+tet+T cells (24% at d10) expressed CD103 in the expansion/effector phase and it progressively increased over the contraction and memory phases (46% at d14 up to 85% at d45) (Fig. 3b/c). The expression profile of CD49a and CD103 on airway luminal (BAL) CD8+tet+T cells was identical to that of the lung cells. Upon closer examination, from d28 onward the majority of CD8+tet+T cells in the lung and airway lumen co-expressed both CD49a and CD103 indicating the acquisition of a bona fide $T_{RM}$ property (Supplementary Fig. 2a/b). In comparison, parenteral intramuscular (i.m.) immunization-induced Ag-specific CD8 T cells only temporarily expressed CD49a and mostly lacked CD103 expression in different phases of T cell responses (Fig. 3a/b). These data suggest that following respiratory mucosal TB immunization the Ag-specific CD8 T cells acquired CD49a (VLA-1) expression before their arrival at the lung whereas they acquired CD103 expression after they entered the lung.

To determine the geographical origin of CD49a acquisition, we examined the CD49a expression on CD8+tet+T cells in the circulation and mediastinal lymph node (MLN), the draining lymph node of the lung. Indeed, a significant number of CD8+tet+T cells in the blood expressed CD49a in the effector phase of T cell responses (d14) (Fig. 3e), consistent with its marked expression on such T cells primed in MLN (d14) (Fig. 3f). Circulating CD8+tet+T cells continued to show high levels of CD49a expression in the memory phase (d28) (Fig. 3e). These data suggest that although respiratory mucosal TB immunization-induced Ag-specific T cells in the lung co-express both CD49a and CD103, these $T_{RM}$ markers are acquired in distinct tissue sites with CD49a (VLA-1) expressed on respiratory mucosal vaccine-induced T cells even before they home to the lung.

**VLA-1 is not required for trafficking of viral-vectored respiratory mucosal immunization-induced Ag-specific CD8 T cells to the lung.** Having demonstrated that the prominent CD49a expression on Ag-specific CD8 T cells outside and within the lung, we postulated that VLA-1 played a role in the trafficking of Ag-specific CD8 T cells to the lung. To address this question, we blocked CD49a during the initial stage of T cell activation (d6–d12) before Ag-specific CD8 T cells arrived *en masse* at the lung (Fig. 4a) by using a well-established CD49a functional blocking antibody (CD49a mAb) delivered via intraperitoneal route[33]. Analysis of CD49a expression on Ag-specific CD8 T cells in the MLN, blood, lung and BAL confirmed complete blockade of CD49a receptor following delivery of CD49a mAb but the isotype control antibody had no effect (Supplementary Fig. 3). Of interest, CD49a blockade did not change the recruitment of CD8+tet+T cells to the lung and airway lumen during the effector/expansion phase (Fig. 4b). These data suggest that VLA-1 does not play a significant role in T cell trafficking to the lung mucosal sites during the initial phase of T cell activation following viral vector mediated respiratory mucosal TB immunization.

**VLA-1 negatively regulates viral-vectored vaccine-induced Ag-specific CD8 T cells during the contraction phase in the lung.** Integrins such as VLA-1 have previously been implicated in intracellular signalling pathways to regulate cell survival and cell death[9,10] which may be involved in the contraction phase of T cell responses. To determine whether VLA-1 was involved in regulating the contraction of antigen-specific T cells following their effector/expansion responses in the lung, immunized mice were treated with CD49a mAb

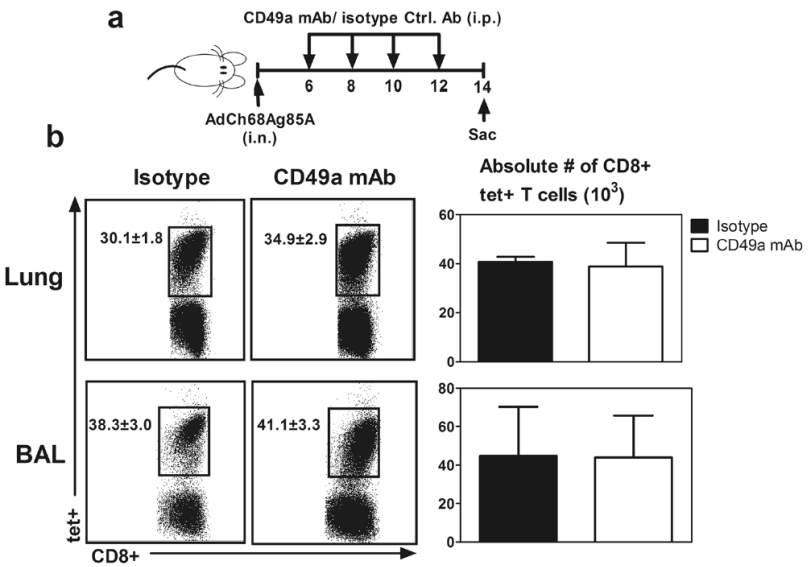


**Figure 4.** Role of VLA-1 in trafficking of replication-defective viral vector respiratory mucosal immunization-induced Ag-specific CD8 T cells to the lung during the effector phase of T cell responses. (**a**) Experimental schema depicting the timing of administration of CD49a blocking mAb and isotype control antibody. Blocking/isotype antibodies were administered 6–12 days post- viral vector respiratory mucosal immunization when Ag-specific tet+ CD8 T cells clonally expanded in the draining lymph nodes and in the process of homing to the lung. (**b**) Representative dot plots showing frequencies of CD8+tet+T cells out of total CD8 T cells in the lung and bronchoalveolar lavage fluid (BAL) of the control and CD49a-blocked mice. Bar graphs comparing absolute numbers of Ag-specific tet+ CD8 T cells in the lung and BAL. Data are presented as mean ± S.E.M. of three mice per group from one experiment.

starting from day 14 post-immunization to block VLA-1 pathway and CD8 + tet+ T cells were examined without Ag re-stimulation at day 18 (Fig. 5a). CD49a blockade led to increased frequencies of CD8+tet+T cells both in the lung and airway (Fig. 5b). It also led to 2–3 times more CD8+tet+T cells in the lung, compared to the isotype control (Fig. 5b). Using an intravascular staining protocol we found that the rise in CD8+tet+T cells in the lung of anti-VLA-1 treated animals occurred only in the lung parenchymal tissue (LPT) while the CD8+tet+T cells in the lung vasculature (LV) remained comparable in numbers to isotype Ab treated animals (Fig. 5c). This finding suggests that VLA-1negatively regulates only the CD8+tet+T cells in the LPT but not those in the LV during the contraction phase.

That VLA-1 blockade increases the number of vaccine-activated CD8+tet+T cell during the contraction phase raised the question whether VLA-1 affects T cell contraction via regulating T cell proliferation. To test this possibility, in separate experiments we examined the proliferation rate of Ag-specific CD8 T cells with and without CD49a blockade by using an *in vivo* BrdU incorporation assay. T cell BrdU labeling was accomplished following repeated intranasal deliveries of BrdU (Fig. 5a). We found the rates of CD8+tet+T cell proliferation in the lung and BAL of animals with CD49a blockade (CD49a mAb) were comparable to isotype controls (Fig. 5d), suggesting that VLA-1 impacts T cell contraction independent of regulation of T cell proliferation.

Apart from tetramer specificity and proliferation of CD8+tet+T cell following CD49a blockade, we further examined other functional properties of CD8 T cells including IFN-γ production and degranulation as indicated by CD107a expression upon *ex vivo* Ag re-stimulation. Indeed, compared to their control counterparts, *in vivo* CD49a blockade led to significantly increased frequencies and numbers of Ag-specific CD8 T cells capable of IFN-γ production (Fig. 6a) and degranulation (Fig. 6b) upon *ex vivo* Ag re-stimulation. Furthermore, we found that *in vivo* CD49a blockade also led to significantly increased production of IFN-γ and degranulation marker CD107a per cell basis measured by mean fluorescent intensity (MFI) of signals (Fig. 6a/b).

That VLA-1 blockade increased the number of vaccine-activated CD8+tet+T cell during the contraction phase also raised the question whether VLA-1 affects T cell contraction via altering the viral clearance as such affecting the level of Ag85A protein in the lung. Thus, in a separate experiment we quantified the antigenic (Ag85A) load in the lung following immunization in isotype and CD49a blocking antibody treated animals (Fig. 5a). Mouse either left without immunization (naïve) or immunized and sacrificed 3 days post-immunization was used as negative and positive controls, respectively. Western blot analysis of the total lung protein showed comparable levels of Ag85A protein in isotype and CD49a blocking antibody treated animals, suggesting that the differential contraction level of CD8+tet+T cell in CD49a blocking antibody treated animals is independent of antigenic load in the lung (Fig. 6c).

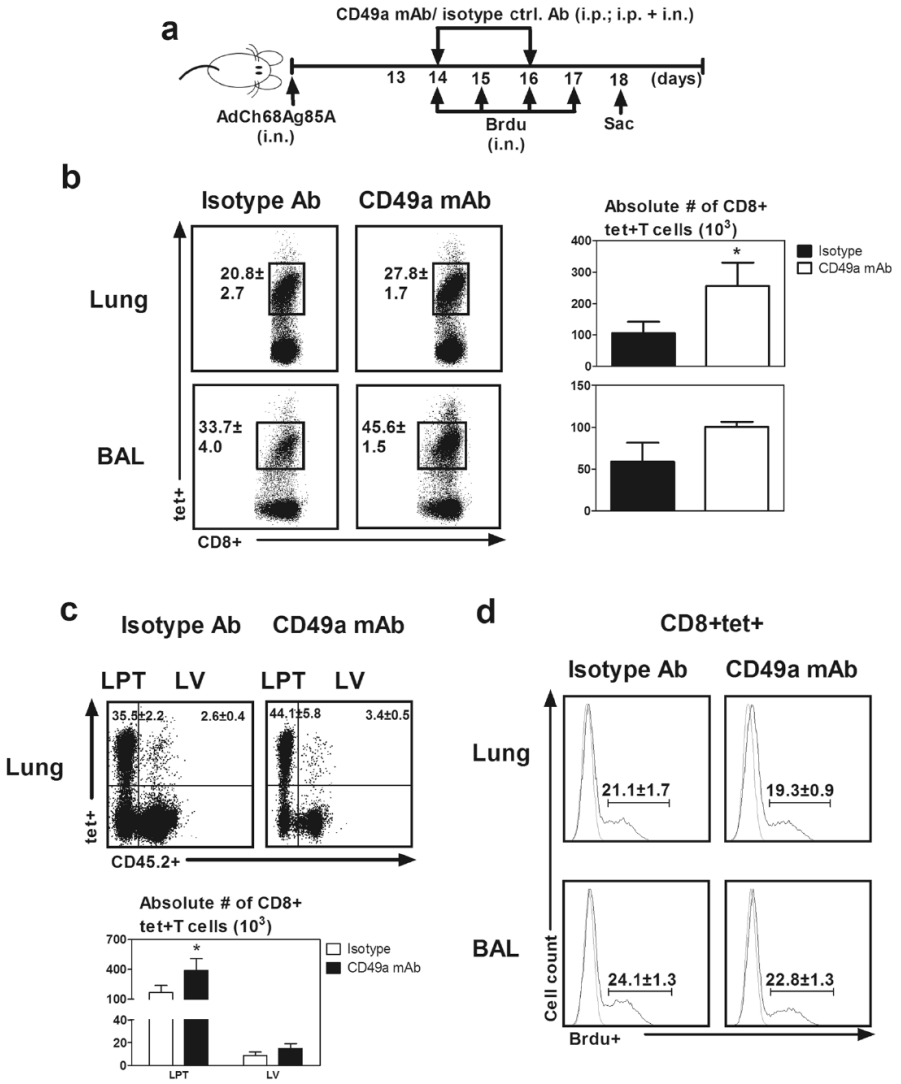


**Figure 5.** Role of VLA-1 in regulation of replication-defective viral-vectored respiratory mucosal immunization-induced Ag-specific CD8 T cells in the lung during the contraction phase of T cell responses. (**a**) Experimental schema depicting the timing of administration of CD49a blocking mAb and isotype control antibody. Blocking and isotype control antibodies were administered at d14 and d16 post- viral vector respiratory mucosal immunization when Ag-specific CD8 T cells in the lung sharply declined. Mice were sacrificed 3 min after i.v. injection of fluorochrome conjugated CD45.2 mAb to differentiate T cells in the lung vasculature from those located in the lung parenchyma. In separate experiments, the mice were treated as above and bromodeoxyuridine (BrdU) was administered i.n. for consecutive four days to assay the *in vivo* proliferation rate of Ag-specific CD8 T cells. (**b**) Representative dot plots showing frequencies of CD8+tet+T cells out of total CD8 T cells in the lung and bronchoalveolar lavage fluid (BAL). Bar graphs comparing absolute numbers of Ag-specific tet+ CD8 T cells in the lung and BAL between isotype control antibody and CD49a mAb treated mice. (**c**) Representative dotplots showing frequencies of CD8+tet+T cells out of total CD8 T cells in the lung parenchymal tissue (LPT) and lung vasculature (LV). Bar graphs comparing absolute numbers of Ag-specific CD8+tet+T cells in the LPT and LV between isotype control antibody and CD49a mAb treated mice. (**d**) Representative histograms comparing frequencies of BrdU+ proliferating Ag-specific CD8 T cells in lung and BAL between isotype control antiobody and CD49a mAb treated mice. Data are presented as mean ± S.E.M. of three mice per group, representative of two independent experiments. *$P < 0.05$, compared to isotype control group.

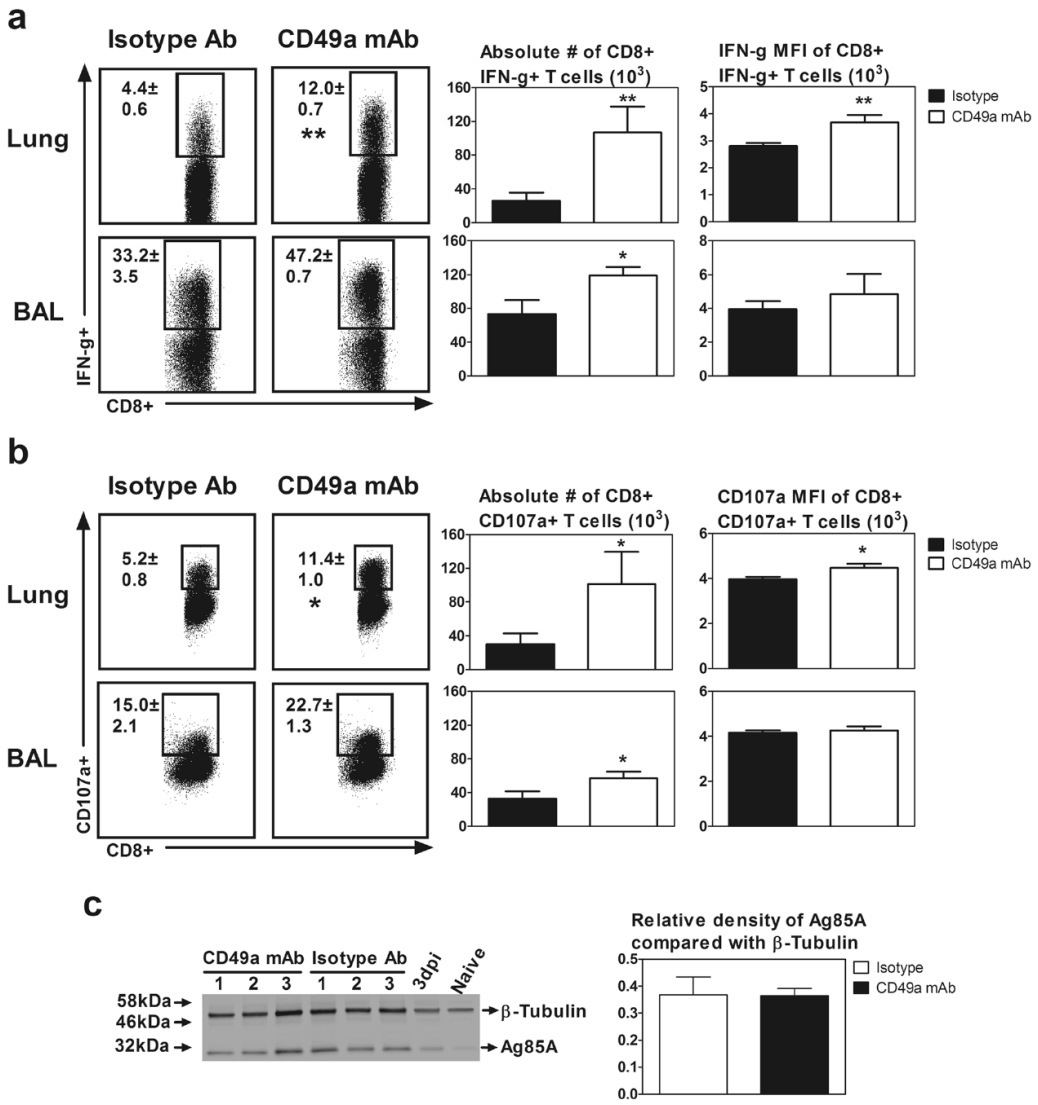


**Figure 6.** Role of VLA-1 in regulation of effector functions of replication-defective viral-vectored respiratory mucosal immunization-induced Ag-specific CD8 T cells in the lung during the contraction phase of T cell responses. Experimental conditions were described in Fig. 5a except that the cells were *ex vivo* re-stimulated with Ag85A antigens. (**a**) Representative dot plots showing frequencies of CD8+ IFN-γ+ T cells out of total CD8 T cells in the lung and BAL. Bar graphs comparing absolute numbers and IFN-γ mean fluorescence intensity (MFI) of CD8+ IFN-γ+ T cells in the lung and bronchoalveolar lavage fluid (BAL) between isotype control antibody and CD49a mAb treated mice. (**b**) Representative dot plots showing frequencies of CD8+ CD107a+ degranulating T cells out of total CD8 T cells in the lung and BAL. Bar graphs comparing absolute numbers and CD107a MFI of CD8+ CD107a+ T cells in the lung and BAL between isotype control antibody and CD49a mAb treated mice. (**c**) Western blot depicts levels of Ag85A protein and β-Tubulin in the total lung of 3 mice (1–3) treated with anti-CD49a mAb, 3 mice (1–3) treated with isotype antibody, a mouse immunized for 3 days (3dpi) and an unimmunized mouse (Naïve). Bar graph shows relative levels of Ag85A calculated in respect to β-Tubulin using Image Studio Lite. Data are presented as mean ± S.E.M. of three mice per group, representative of two independent experiments except western plot data, which is representation of one experiment. *$P < 0.05$, **$P < 0.01$ compared to isotype control group.

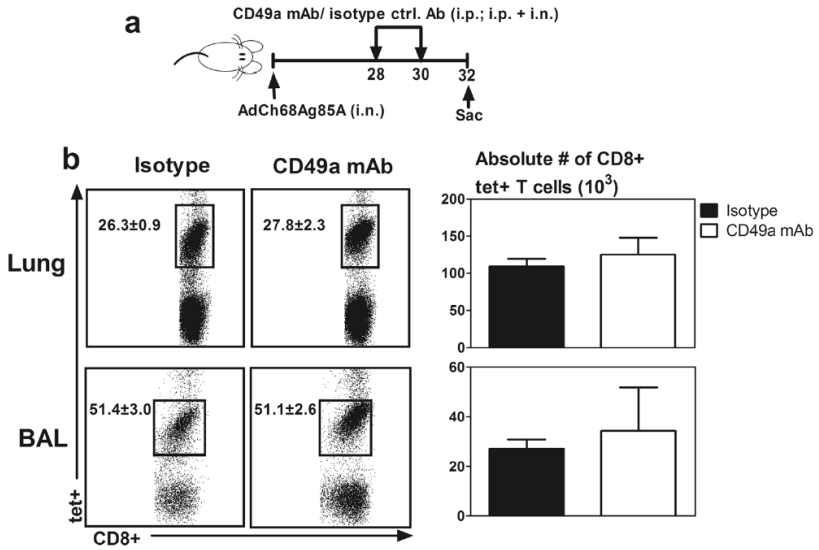


**Figure 7.** Role of VLA-1 in maintenance of replication-defective viral-vectored respiratory mucosal immunization-induced Ag-specific $T_{RM}$ cells in the lung during the memory phase of T cell responses. (**a**) Experimental schema depicting the timing of administration of CD49a blocking mAb and isotype control antibody. Blocking and isotype control antibodies were administered at d28 and d30 post- viral vector respiratory mucosal immunization after the development of Ag-specific memory CD8 T cells in the lung. (**b**) Representative dot plots showing frequencies of Ag-specific tet+ CD8 T cells out of total CD8 T cells in the lung and bronchoalveolar lavage fluid (BAL). Bar graphs comparing absolute numbers of CD8+tet+T cells in the lung and BAL between isotype antibody and CD49a mAb treated mice. Data are presented as mean ± S.E.M. of three mice per group, representative of two independent experiments.

Taken together these data suggest that VLA-1 pathway plays a critical role in negatively regulating Ag-specific CD8 T cell responses during the contraction phase following viral vector based respiratory mucosal TB immunization.

**VLA-1 is not required for maintenance of viral-vectored respiratory mucosal immunization-induced Ag-specific $T_{RM}$ during the memory phase in the lung.** VLA-1 was previously shown to contribute to the retention of Ag-specific CD8 T cells in the lung tissue after influenza infection[12]. We have shown that immune protective Ag-specific CD8 T cells induced by viral vector respiratory mucosal immunization persist in the lung for a long time[34,35] and we have here found these cells to be of $T_{RM}$ phenotype (Fig. 2/3). We thus next determined whether VLA-1 also played a role in maintenance of viral vector vaccine-induced $T_{RM}$ cells in the lung. To test this, CD49a receptor was functionally blocked for a total of four days beginning from day 28 of the memory phase following respiratory mucosal immunization and CD8+tet+T cells were examined at day 32 (Fig. 7a). We found that CD49a blockade during the memory phase had no effect on both the frequencies and numbers of CD8+tet+T cells in the lung and airway, compared to the isotype control animals (Fig. 7b). These data suggest that VLA-1 is not required for the retention of Ag-specific tissue resident memory CD8 T cells in the lung following replication-defective viral vector respiratory mucosal TB immunization.

## Discussion

Effective vaccines for infectious diseases such as tuberculosis, the leading cause of global morbidity and mortality, are currently lacking[36]. Protection against these diseases relies on T cell immunity and the induction and maintenance of tissue resident memory T cells ($T_{RM}$) at the site of infection is critical for vaccine-induced protection[2,3,37]. Improved knowledge in the expression and roles of $T_{RM}$ surface integrin molecules following vaccination will help develop effective vaccination strategies against infections at mucosal sites of pathogen entry. Here we show that a replication-defective virus-vectored TB vaccine administered via the respiratory mucosal route induces Ag-specific $T_{RM}$ in the lung characterized by high-level expression of $T_{RM}$ surface markers VLA-1 and CD103. Of importance, our study shows that expression of these surface markers is differentially regulated post-respiratory mucosal vaccination. VLA-1 is initially markedly upregulated on T cells during the effector/expansion phase in the mediastinal lymph node draining the site of mucosal vaccination and remains highly expressed after the arrival of these T cells in the lung through both the contraction and memory phases. In comparison, CD103 expression is acquired by T cells primarily after they were primed in the draining lymph nodes and trafficked into the lung. However, we find that despite its acquisition outside the lung, VLA-1 is not required for the trafficking of

circulating Ag-specific CD8 T cells to the lung mucosa. We further find that VLA-1 plays a role in the contraction phase in the lung by negatively regulating T cell responses while it does not play a significant role in the maintenance of $T_{RM}$ during the memory phase. Our study provides new information on vaccine-inducible $T_{RM}$ in the lung and shall help develop effective respiratory mucosal vaccination strategies against pulmonary TB.

Replication-defective viral vectored vaccines are attractive vaccine carriers, particularly for respiratory mucosal immunization strategies given their potency and safety[18]. We showed previously that replication-defective adenoviral vector tuberculosis vaccine, AdAg85A, a model viral vector vaccine used in the current study, when administered via respiratory mucosal route, induced long lasting, protective Ag-specific CD8 T cells in the lung and airway[21,34,38]. Here we demonstrate that these cells preferentially express classical $T_{RM}$ surface markers VLA-1 and CD103, which is in line with the phenotype of $T_{RM}$ induced by respiratory viral pathogen exposure[12,39,40]. However, in contrast to viral pathogen-specific CD8 $T_{RM}$ counterparts that mostly acquire VLA-1 expression within the local lung microenvironment immediately after peak T cell responses[12], we find VLA-1 to be expressed on Ag-specific CD8 T cells upon their activation in the dLN following replication-defective viral vector respiratory mucosal vaccination. This difference is likely attributed to differential inflammatory signals and antigenic persistence resulting from replicating viral pathogen exposure versus attenuated replication-defective viral vector vaccination. In support of such difference in immunologic sequela, it has been observed that while continuing peripheral T cell supply contributes to the maintenance of influenza-specific CD8 T cells in the lung[41], *in-situ* T cell proliferation alone, in the absence of peripheral supply, maintains Ag-specific CD8 T cells in an Ag-dependent manner following replication-defective viral vector respiratory mucosal vaccination[34].

Our finding that VLA-1 is not involved in the initial trafficking of replication-defective viral vector vaccine-activated circulating CD8 T cells to the lung mucosa during the effector phase of T cell responses is in accord with the previous observation in a model of influenza viral infection[12]. These findings together are at odds with the findings from the models of rheumatoid arthritis, delayed type hypersensitivity (DTH)[33] and cancer[42] where VLA-1 is seen to play a role in T cell recruitment to the peripheral tissue sites. These observations suggest that VLA-1 is differentially required in T cell trafficking, depending on the specific tissue site and immunologic tissue microenvironment.

So far there have not been any studies to examine the role of VLA-1 in the contraction phase of T cell responses. Here we show VLA-1 to negatively regulate replication-defective viral vector vaccine-induced Ag-specific CD8 T cells during the contraction phase in the lung. Blockade of VLA-1 in our model significantly slows down the pace of T cell contraction in the lung following the initial effector/expansion responses of the T cells, resulting in increased numbers and activation of T cells. Although it remains unclear how VLA-1 negatively regulates the contraction of T cells destined to become $T_{RM}$, it is likely accomplished via its roles in regulating the survival and apoptosis[13], resources competition[43,44], and differentiation[45] of Ag-specific T cells. We further show here that although the Ag-specific CD8 $T_{RM}$ following the contraction phase continue to express VLA-1, VLA-1 does not seem to play a significant role in maintaining Ag-specific CD8 $T_{RM}$ during the memory phase. Our finding contrasts the finding from the model of influenza viral exposure where VLA-1 blockade resulted in the loss of Ag-specific memory CD8 T cells in the lung[12]. It is likely that in our replication-defective viral vector model, VLA-1 deficiency may well be compensated for by the function of other $T_{RM}$ surface integrin molecules such as CD103, which remains highly expressed on our replication-defective viral vector vaccine-induced $T_{RM}$. Indeed, CD103 was previously shown, via its interaction with E-cadherin on epithelial cells, to potentiate the retention of CD8 T cells in the lung[46].

Lack of biological correlates of immune protection is a major bottleneck for development of new prophylactic and immunotherapeutic vaccines for infectious diseases such as TB[47,48]. As such, the protective efficacy of a new vaccine in question remains unknown until the completion of costly late-phase clinical efficacy trials. In this regard, generation of $T_{RM}$ in the lung is a reliable biological correlate for protection following respiratory mucosal TB immunization[49]. However, it may be difficult to access and analyze $T_{RM}$ in human lungs. One possible way is to identify biological correlates of successful induction of lung immunity on T cells in the circulation. However, circulating Ag-specific CD8 T cells constitute a small fraction of total CD8 T cells. Our data imply that VLA-1, as opposed to CD103, expressed by the majority of circulating vaccine-induced Ag-specific CD8 T cells, may serve as a surrogate marker to reliably predict $T_{RM}$ generation at the respiratory mucosa. This is in contrast to low number of VLA-1-expressing CD8+tet+T cells found in blood following replication-defective viral vector intramuscular vaccination (Supplementary Fig. 4). It has long been believed that mucosal homing molecules would be potential biological correlates of protective immunity as they are related to the T cell subsets that likely have the ability to populate mucosal sites. As such, integrin molecules CD103[50] and VLA-4[51] were identified as surrogate markers in the blood to predict T cell responses in the female genital tract and respiratory mucosa, respectively. Our data suggest that not only the mucosal homing molecules but also the molecules that are programmed to express on activated T cells during lineage differentiation in draining lymph nodes may also be considered for the screening of biological correlates of protective immunity. However, the finding in murine models needs cautious interpretation in humans due to disparity in subsets of T cells responding to viral vectored vaccines[52,53].

In conclusion, our current study has examined the kinetic expression of classic $T_{RM}$ markers VLA-1 and CD103 and further deciphered the role of VLA-1 integrin, in different phases of CD8 T cell responses following respiratory mucosal vaccination with a replication-defective viral vector TB vaccine. We find VLA-1 to be expressed on viral vector vaccine-induced CD8 T cells before and after they trafficked to the lung and to play a differential role in various phases of T cell responses. These findings hold implications in understanding vaccine-inducible $T_{RM}$ in the lung and developing novel vaccination strategies against respiratory infectious diseases such as TB.

## Material and Methods

**Ethics approval.** All animal experiments in this study were approved by the animal research ethics board of McMaster University, and were performed in accordance with the approved guidelines for animal experimentation at McMaster University.

**Animals.** Female BALB/c 6 to 8 weeks old mice were purchased from Charles River Laboratories (Charles River, St Constant, Quebec, Canada) and housed in specific pathogen-free Level B rooms within the central animal facility at McMaster University.

**Immunization with viral vectored vaccine.** A replication-deficient adenovirus expressing immunodominant *Mycobacterium tuberculosis* antigen Ag85A was used to immunize animals via either respiratory mucosal or parenteral route. Respiratory mucosal route of immunization was carried out by intranasal (i.n.) delivery of $1 \times 10^7$ plaque forming unites (pfu) per mouse in 25 µl of total volume[21]. In some experiments, mice were immunized intramuscularly (i.m.) via quadriceps muscles with the same dose of the vaccine in 100 µl of total volume as previously described[38].

**Bronchoalveolar lavage, lung, blood and lymph node mononuclear cell isolation.** After anesthetizing animals, peripheral blood was collected from abdominal artery in tubes containing 300 µl of Heparin (40 unites/ml) (Sigma-Aldrich, St Louis, MO, USA). Then, mice were sacrificed by exsanguination. Airway luminal cells were collected through bronchoalveolar lavage (BAL)[21]. Lung mononuclear cells were isolated from perfused lungs as previously described[38]. Lymph nodes were crushed using frosted glass slides, then single-cell suspension was obtained by crushing the organ through 40µm basket filter. Heparinized blood samples were treated twice with ACK lysis buffer (Invitrogen, Burlington, ON, Canada) to remove all red blood cells and washed with phosphate-buffered saline. All isolated cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% L-glutamine.

**Sorting of Ag-specific CD8 T cells and PCR-array gene expression analysis.** Ag-specific CD8 T cells were sorted from lung mononuclear cells using flow sorter. Briefly, lung mononuclear cells were immunostained with a tetramer specific for the Ag85A CD8 T cell peptide (MPVGGQSSF) bound to BALB/c MHC class I allele H-2L$^d$ (National Institutes of Health, Tetramer Core, Atlanta, GA, USA) for 1 h in the dark at RT. Immunostained cells were then sorted using BD FACS Aria III cell sorter (BD Pharmingen, San Jose, CA, USA). In addition, CD8 T cells were isolated from naïve lung mononuclear cells using mouse CD8a (Ly-2) microbeads (Miltenyi Biotec Inc., Auburn, CA, USA). Total mRNA of the purified cells was extracted using RNeasy kit (Qiagen, Toronto, ON, Canada) and then converted to cDNA using an RT$^2$ First Strand Kit (Qiagen, Toronto, ON, Canada). cDNAs were added to RT$^2$ qPCR master mix and then the mixture was aliquoted across a custom made mouse RT$^2$ profiler PCR-array profiling the expression of handpicked genes encoding chemokine receptors, adhesion molecules and T cell effector/cell surface molecules (Supplementary Table 1) (Qiagen, Toronto, ON, Canada). PCR reactions were conducted using a 7900HT fast real-time PCR system with fast 96-well block module (Life Technologies Inc., Burlington, ON, Canada). For data analysis, relative gene expressions with at least 2-fold changes ($P < 0.05$) were considered to be significant.

**Cell stimulation, immunostaining, and flow cytometry.** The isolated mononuclear cells were seeded in U-bottom 96-well plates at a concentration of 20 million cells/ml for lungs and lymph nodes, 10 million cells/ml for blood, and 5 million cells/ml for BAL. Ag-specific T cells were identified using a tetramer specific for the Ag85A CD8 T cell peptide (MPVGGQSSF) bound to BALB/c MHC class I allele H-2Ld and Phycoerythrin fluorochrome (National Institutes of Health Tetramer Core, Atlanta, GA, USA) for 1 h in the dark at RT[25,54]. For intracellular cytokine staining and cytotoxicity assay using a degranulation marker CD107a[55], mononuclear cells were incubated at 37 °C in the presence of Golgi plug (5 mg/ml brefeldin A; BD Pharmingen, San Jose, CA, USA), Golgi stop (0.26% (w/w) Monensin; BD Pharmingen, San Jose, CA, USA), and FITC-conjugated CD107a monoclonal antibody (mAb) (clone 1D4B) (1:50) (Biolegend, San Diego, CA, USA) simultaneously with or without stimulation with an immunodominant *M.tb* antigen 85 A (Ag85A) specific CD8 T cell peptide (MPVGGQSSF) at a concentration of 1 µg/well for 5–6 h. Incubation was followed by washing and blocking using CD16/CD32 block Ab (clone 2.4G2) (1:150) (BD Pharmingen, San Jose, CA, USA) in 0.5% bovine serum albumin/PBS for 15 min on ice. Cells were then washed and stained using cell surface mAbs. Then, cells were washed, permeabilized, and stained intracellularly. For some experiments, only tetramer and extracellular staining were carried out without incubation and antigen stimulation, and cells then were fixed using 1% paraformaldehyde/PBS at RT for 10–15 min. The fluorochrome-conjugated mAbs used included CD3-V450 (clone 17A2) (1:200), CD8a-PE-Cy7 (clone 53–6.7) (1:400), CD4-APC-Cy7 (clone RM4–5) (1:400), CD49a (α1 domain of VLA-1)-Alexa Fluor 647 (clone Ha31/8) (1:100), CD103-Biotin (clone 2E7) (1:100) (Qdot-800-Streptavidin (1:500)), IFN-γ-PerCP-Cy5.5 (clone XMG1.2) (1:200) (BD Pharmingen, San Jose, CA, USA), CCR1-APC (clone 643854) (1:50), and CCR6-Alexa Fluor 488 (clone 140706) (1:100) (R&D system, Minneapolis, MN, USA). Immunostained cells were run on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (version 10; Tree Star, Ashland, OR, USA).

**Intravascular immunostaining.** Intravascular immunostaining was carried out as previously described[29]. Briefly monoclonal anti-CD45.2-Alexa Fluor 700 mAb (clone 104) (BD Pharmingen, San Jose, CA, USA) was prepared at 1 µg in 250 µl concentration and injected intravenously via tail vein. Within three minutes after injection, animals were sacrificed, and blood, BAL and lung were obtained for analysis.

***In vivo* VLA-1 blockade.** To investigate the role of VLA-1 *in vivo*, we used the function blocking hamster mAb (clone Ha31/8) against CD49a and the hamster isotype-matched control Ab (clone Ha4/8) (BD Pharmingen, San Jose, CA, USA) for blocking experiments. For each injection, the antibodies were administered intraperitoneally (i.p.) (150 µg or 200 µg/mouse) or intranasally (i.n.) (50 µg/mouse)[33].

**5-Bromo-2′-deoxyuridine (BrdU) administration for evaluation of *in vivo* T cell proliferation.** Frequency of proliferating Ag–specific CD8 T cells in the lung was determined by *in vivo* Brdu incorporation assay as previously described[34].

**Western blot analysis of Ag85A protein in the lung.** The lungs from naïve mice or i.n immunized mice with or without treatment with blocking or isotype control antibodies were collected and perfused by injecting cold Hank's buffer through the right ventricle in order to remove intravascular mononuclear cells, and kept in cold Hank's buffer. Total lung protein was subjected to western blotting with an anti-Ag85A monoclonal antibody (clone TD-17) and anti-β-Tubulin monoclonal antibody (clone TUB2.1) (Sigma-Aldrich, St. Louis, MO, USA) as a control. Densitometric quantitation of western blot analysis was conducted using Image Studio Lite (LI-COR Biosciences, Lincoln, NE, USA).

**Statistical analysis.** All data were analyzed using Graph Pad Prism software (GraphPad Software, San Diego, CA, USA). The differences considered statistically significant were indicated as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. A two-tailed Student t test was used for pairwise comparisons.

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## Author Contributions

S.H., M.J., and Z.X. conceived and designed the study. S.H., N.T., S.A., and A.K. performed the experiments. S.H. and M.J. analyzed the data. S.H., M.J., and Z.X. wrote the manuscript. All the authors reviewed the manuscript.

## Additional Information

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# CHAPTER 3: Respiratory mucosal modulation in parenteral TB vaccine immunized hosts induces helper CD4 T cell-dependent resident memory CD8 T cells

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**Respiratory mucosal modulation in parenteral TB vaccine immunized hosts induces helper CD4 T cell-dependent resident memory CD8 T cells**

In our previous study we have demonstrated that RM replication-deficient viral vector-based TB vaccination leads to induction of CD8 $T_{RM}$ at the mucosal sites of the lung. To date, parenteral route of vaccination is being used for BCG and most of other vaccines in humans, while safe and convenient, unfortunately it fails to elicit non-circulating, long-lasting, and protective T cell immunity at the restricted-entry sites of the lung. Recently, the concept of $T_{RM}$ generation at the lung mucosal sites through RM manipulation of parenterally viral pathogen-infected hosts (prime-pull strategy) has emerged, which requires local lung inflammatory signals as well as specific-Ag. In this regard, it remains unknown whether utilization of such prime-pull strategy in the context of parenteral replication-deficient viral vector-based TB vaccination also generates lung $T_{RM}$, and if so, what are the inductive cellular and molecular mechanisms.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

**Respiratory mucosal modulation in parenteral TB vaccine immunized hosts induces helper CD4 T cell-dependent resident memory CD8 T cells**

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**Abstract**

Lung tissue-resident memory CD8 T cells ($T_{RM}$) characterized by the expression of CD69 (early activation marker), CD103 ($\alpha_E\beta7$ integrin) and VLA-1 ($\alpha1\beta1$ integrin), are induced at the lung mucosa upon viral respiratory infection/vaccination and constitute the first line of defense against reinfection. However, parenteral vaccination is incapable of generating such cells at mucosal sites, leaving most of parenterally vaccinated hosts potentially susceptible to mucosal intracellular infections. In this regard, the strategies empowering parenteral vaccination through eliciting CD8 $T_{RM}$ at the lung mucosa have remained to be developed. Here by using a mouse model of parenteral vaccination with a replication-deficient viral vector TB vaccine expressing immunodominant Ag (Ag85A) of *Mycobacterium tuberculosis* (*M.tb*), we show that RM delivery of a soluble *M.tb* protein Ag (Ag85 complex), but not a nonspecific inflammatory agonist CpG to parenterally immunized hosts induces lung Ag-specific CD8 T cells selectively expressing $T_{RM}$ signatures including CD69 and CD103. We further show that RM modulation during the memory, but not effector, phase of T cell responses has a far greater capability of generating such cells in the lung. Of importance, this strategy induces a balanced generation of Ag-specific CD4 and CD8 T cells in the lung, as well as a major subset of Ag-specific CD8 T cells expressing CD69 and CD103 in dMLNs. Using a CD4 depleting mAb, we demonstrate that CD4 T cell deficiency diminishes establishment of Ag-specific CD8 $T_{RM}$ cells in the lung restricted entry sites by reducing cell survival and proliferation. Our study provides new information on vaccine-inducible lung $T_{RM}$ and shall help develop effective

parenteral vaccination strategies against mucosal intracellular infectious diseases such as TB.

**Introduction**

Memory T cells are a major component of the adaptive immune response. While the circulating T cell subsets including effector ($T_{EM}$) and central ($T_{CM}$) memory T cells play a critical role in host defense against systemic infections (1), the non-circulating subset known as tissue-resident memory T cells ($T_{RM}$) is uniquely positioned to control mucosal infections at the site of pathogen entry (2-6).

Lung tissue-resident memory CD8 T cells ($T_{RM}$) expressing CD69 (early activation marker), $\alpha_E$(CD103)β7 integrin, and α1(CD49a)β1 integrin (VLA-1) are induced at the lung mucosal sites upon local natural viral infection and constitute the first line of defense against a subsequent reinfection (4, 5). While most currently successful human vaccines have been administered via the parenteral route, they fail to elicit $T_{RM}$ at mucosal sites, leaving the majority of such vaccinees potentially susceptible to mucosal intracellular infectious diseases such as TB (7). Until now, vaccination strategies to empower parenteral vaccination to elicit $T_{RM}$ at mucosal sites, and the underlying mechanisms have remained to be addressed. Enhanced knowledge in this regard is pivotal to developing effective parenteral vaccination strategies for mucosal intracellular infectious diseases.

Recently, intraperitoneal (i.p.) infection with influenza virus (prime), followed by respiratory inoculation of a nonspecific inflammatory agonist (e.g., CpG) with a specific-Ag (pull) was shown to enable establishment of CD8 $T_{RM}$ in the mucosal sites of the lung

(e.g., airway and parenchyma) in an Ag-dependent manner (8). Such prime-pull strategies hold implications in the field of TB vaccine development. In this regard, when i.m. vaccination with a recombinant replication-deficient human adenovirus or DNA plasmid vaccine expressing *Mycobacterium tuberculosis* (*M.tb*) immunodominant Ag (Ag85A) is followed by local administration of a soluble *M.tb* protein Ag (Ag85 complex), long-lasting and protective Ag-specific CD8 T cells were induced in the lung mucosa (9, 10). Since many factors including the immune responses induced to various viral species, replication effective (wild type) vs replication-deficient viral infections, differential innate immune activation, and antigenic expression can all influence $T_{RM}$ generation (11), a critical issue that remains to be clarified is whether mucosal luminal modulation following replication-deficient viral vector parenteral vaccination induces immune protective $T_{RM}$ in the lung, and if so, what would be the underlying mechanisms.

Here to address this question, we have used a mouse model of parenteral vaccination with a replication-deficient chimpanzee Ad-vectored TB vaccine expressing immunodominant Ag of *M.tb* (AdCh68Ag85A). We show that airway delivery of *M.tb* protein Ags (Ag85 complex), but not nonspecific inflammatory agonist CpG, to parenterally immunized mice elicits lung CD8 $T_{RM}$ which selectively express CD69 and CD103. RM modulation with *M.tb* Ags during the memory, but not effector, phase of T cell responses has a far greater capability of generating such cells in the lung. Of note, RM modulation during the memory phase induces a balanced generation of Ag-specific CD4 and CD8 T cells in the lung. It also induces a major subset of Ag-specific CD8 T cells expressing CD69 and CD103 in dMLNs. Using a CD4 T cell depleting mAb, we find CD4

T cells to play a role in induction Ag-specific CD8 T cells in the airways by enhancing both cell survival and proliferation.

## Material and methods

### Ethics approval

All animal experiments in this study were approved by the animal research ethics board of McMaster University, and were performed in accordance with the approved guidelines for animal experimentation at McMaster University.

### Animals

Female BALB/c 6 to 8 weeks old mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in specific pathogen-free Level B rooms within the central animal facility at McMaster University.

### Vaccination with viral vectored vaccine

A recombinant replication-deficient chimpanzee Ad-based vaccine expressing immunodominant *Mycobacterium tuberculosis* Ag Ag85A (AdCh68Ag85A) (12) was used to immunize animals via parenteral route. Parenteral route of vaccination was carried out intramuscularly (i.m.) via quadriceps muscles with $1\times10^7$ plaque forming unites (pfu) per mouse of the vaccine in 100 μl of total volume as previously described (13).

### Bronchoalveolar lavage, lung, blood, spleen and lymph node mononuclear cell isolation

After anesthetizing animals, peripheral blood was collected from abdominal artery in tubes containing 300 µl of Heparin (40 unites/ml) (Sigma-Aldrich, St Louis, MO, USA). Then, mice were sacrificed by exsanguination. Airway luminal cells were collected through bronchoalveolar lavage (BAL) (14). Lung mononuclear cells were isolated as previously described (13). Heparinized blood samples were treated twice with ACK lysis buffer (Invitrogen, Burlington, ON, Canada) to remove all red blood cells and washed with phosphate-buffered saline. Spleens were crushed using frosted glass slides, samples were treated once with ACK lysis buffer, and splenocytes were isolated through 40 µm basket filter (15, 16). Lymph nodes were crushed using frosted glass slides, then single-cell suspension was obtained by crushing the organ through 40µm basket filter. All isolated cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% L-glutamine.

**Cell stimulation, immunostaining, and flow cytometry**

The isolated mononuclear cells were seeded in U-bottom 96-well plates at a concentration of 20 million cells/ml for lungs and lymph nodes, 10 million cells/ml for blood, and 5 million cells/ml for BAL. LIVE/DEAD Fixable Aqua Stain was used to discriminate live and dead cells (Invitrogen, Burlington, ON, Canada). Ag-specific T cells were identified using a tetramer specific for the Ag85A CD8 T cell peptide (MPVGGQSSF) bound to BALB/c MHC class I allele H-2Ld and Phycoerythrin fluorochrome (National Institutes of Health Tetramer Core, Atlanta, GA, USA) for 1 h in the dark at RT (17, 18). For intracellular cytokine staining, mononuclear cells were incubated at 37°C in the presence of Golgi plug (5 mg/ml brefeldin A; BD Pharmingen,

San Jose, CA, USA), Golgi stop (0.26% (w/w) Monensin; BD Pharmingen, San Jose, CA, USA), with or without stimulation with an immunodominant *M.tb* protein Ag85A specific CD4 (LTSELPGWLQANRHVKPTGS) or CD8 (MPVGGQSSF) T cell peptide at a concentration of 1µg/well for 5–6 h. Incubation was followed by washing and blocking using CD16/CD32 block Ab (clone 2.4G2) (1:150) (BD Pharmingen, San Jose, CA, USA) in 0.5% bovine serum albumin/PBS for 15 min on ice. Cells were then washed and stained using cell surface mAbs. After this stage, in some experiments for identification of apoptotic cells, cells were resuspended in 1× annexin V buffer and stained with annexin V-APC (1:50) (Invitrogen, Burlington, ON, Canada) for 15 min at room temperature. Following surface staining, cells were washed, permeabilized, and stained intracellularly. For some experiments, only tetramer and extracellular staining were carried out without incubation and Ag stimulation, and cells then were fixed using 1% paraformaldehyde/PBS at RT for 10-15 min. The fluorochrome-conjugated mAbs used included CD3-PerCP-Cy5.5 (clone 145-2C11) (1:200), CD3-V450 (clone 17A2) (1:200), CD8a-PE-Cy7 (clone 53-6.7) (1:400), CD4-APC-Cy7 (clone RM4-5) (1:400), CD49a (α1 domain of VLA-1)-Alexa Fluor 647 (clone Ha31/8) (1:100), CD103-Biotin (clone 2E7) (1:100) (Qdot-800-Streptavidin (1:500), IFN-γ-PerCP-Cy5.5 (clone XMG1.2) (1:200) (BD Pharmingen, San Jose, CA, USA), CD69-BV605 (clone H1.2F3) (1:100), CD44-BV650 (clone IM7) (1:100), Ki67-BV421 (clone 16A8) (1:50), and CD62L-PerCP-Cy5.5 (clone MEL-14) (1:300) (Biolegend, Minneapolis, MN, USA). Immunostained cells were run on an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (version 10; Tree Star, Ashland, OR, USA).

**Intravascular immunostaining**

Intravascular immunostaining was carried out as previously described (19). Briefly monoclonal anti-CD45.2-Alexa Fluor 700 mAb (clone 104) (BD Pharmingen, San Jose, CA, USA) was prepared at 1µg in 250 µl concentration and injected intravenously (i.v.) via tail vein. Within three minutes after injection, animals were sacrificed, and blood, spleen, lymph node, BAL and lung were obtained for analysis.

***In vivo* CD4 T cell depletion**

Mice were inoculated i.p. with 200 µg of anti-CD4 mAb (clone GK1.5) (ATCC TIB-207; ATCC, Manassas, Virginia) one day prior to administration of specific-Ag to i.m. AdCh68Ag85A immunized hosts, thereafter by injection of 100 µg of anti-CD4 mAb on day 1 after specific-Ag administration. CD4 T cell deficiency was maintained by weekly inoculation of 100 µg of anti-CD4 mAb until the end of experiment.

**5-Bromo-2'-deoxyuridine (BrdU) administration for evaluation of *in vivo* T cell proliferation**

Frequency of proliferating Ag–specific CD8 T cells in the lung was determined by *in vivo* Brdu incorporation assay as previously described (20).

**Statistical analysis**

All data were analyzed using Graph Pad Prism software (GraphPad Software, San Diego, CA, USA). The differences considered statistically significant were indicated as *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. A two-tailed Student t test was used for pairwise comparisons.

## Results

### Respiratory delivery of specific-Ag during the effector phase of T cell responses in i.m. AdCh68Ag85A immunized hosts modestly induces lung CD8 $T_{RM}$

It has been previously demonstrated that parenteral viral infection/ vaccination fails to elicit CD8 $T_{RM}$ in the restricted entry sites of the lung including lung airway (referred to as BAL) and lung parenchyma (LPT) (7, 8, 21). Given that RM delivery of an Ag-dependent inflammatory agonist in parenteral replication-efficient influenza virus infected hosts enables establishment of lung CD8 $T_{RM}$ (8), we sought to examine whether such a prime-pull strategy could also induce lung CD8 $T_{RM}$ upon parenteral replication-deficient viral vector vaccine vaccination. To this end, we immunized BALB/c mice intramuscularly (i.m.) with AdCh68Ag85A, and 7 days later, characterized as the effector phase of T cell responses, the mice underwent RM delivery of an Ag-independent inflammatory agonist CpG ODN 'B' (AdCh/CpG group), or an Ag-dependent inflammatory agonist, a soluble *M.tb* protein Ag (Ag85 complex) (AdCh/Ag85 group) (Figure 1A). Then, we used intravascular immunostaining (i.v.) approach to determine cell distribution in different lung compartments including airway (BAL), lung parenchymal tissue (LPT), and lung vasculature (LV) (19, 22). In this regard, cells positioned in BAL and LPT are i.v.-, but their counterparts in LV are i.v.+. We noticed that i.m. vaccination alone (AdCh group) failed to induce Ag-specific CD8 T cell responses in both BAL and LPT, which is consistent with our previous study conducted at various phases of T cell responses (effector/expansion, contraction, and memory phases) (21, 23). Of note, 5 days post-RM delivery of CpG or specific-Ag comparably led to en masse induction of CD8 tet+ and CD8

tet-T cells in BAL (Figure 1B) and LPT (Figure 2A). By 14 days post-RM delivery, while the number of CD8 tet+ and CD8 tet- T cells in both BAL and LPT significantly decreased from the 5 days post-RM delivery, the number and frequency of CD8 tet+ T cells in LPT, but not BAL, were temporarily higher in AdCh/Ag85 group compared to AdCh/CpG group (Figures 1B/2A). However, by 28 days post-RM delivery, the number of CD8 tet+ T cells in both BAL and LPT was very low, but stable, hence being in the memory phase of T cell responses (Figures 1B/2A). Of importance, compared to i.m. vaccination alone, at all different phases after RM delivery of inflammatory agonists, we identified comparable and stable frequencies and numbers of CD8 tet+, or CD8 tet-, T cells in LV (Supplementary Figure 1).

Apart from tetramer specificity of Ag-specific CD8 T cells, we further evaluated IFN-γ production by CD4 and CD8 T cells in the lung upon *ex vivo* Ag re-stimulation. In contrast to i.m. vaccination alone, 5 days post-RM delivery of CpG or specific-Ag comparably led to modestly increased frequencies and numbers of Ag-specific CD4 T cells capable of IFN-γ production (Supplementary Figure 2), but that was in parallel with significantly increased frequencies and numbers of Ag-specific CD8 T cells capable of IFN-γ production (Supplementary Figure 3). By 14 days post-RM delivery, the frequencies and numbers of these cells, particularly IFN-γ-producing Ag-specific CD8 T cells significantly decreased (Supplementary Figures 2/3). By 28 days post-RM delivery, frequencies and numbers of Ag-specific CD4 or CD8 T cells capable of IFN-γ production remained stable, but it was very low (Supplementary Figures 2/3).

Having established that effector T cells seeding non-lymphoid tissues are considered as potential $T_{RM}$ precursors (6), in the same set of experiments we also examined expression of typical $T_{RM}$ surface markers CD69, CD103, and CD49a on recruited effector CD8 T cells, as gated from CD44+CD62L- T cells, in the lung. Compared to AdCh/CpG group, by 5 days post-RM delivery of specific-Ag, higher frequencies of effector CD8 tet+ T cells, but not effector CD8 tet- T cells, in BAL acquired $T_{RM}$ surface markers CD69, CD103, and CD49a (Figure 1C). Moreover, only higher frequencies of effector CD8 tet+ T cells, but not effector CD8 tet- T cells, in LPT acquired CD103 (Figure 2B). By 14 days post-RM delivery of specific-Ag, only higher frequencies of effector CD8 tet+ T cells, but not effector CD8 tet- T cells, in BAL and LPT expressed CD103 (Figures 1C/2B). By 28 days post-RM delivery of specific-Ag, while only higher frequencies of effector CD8 tet+ T cells, but not CD8 tet- T cells, in BAL and LPT expressed CD69 (Figures 1C/2B), only higher frequencies of effector CD8tet+ T cells, but not CD8 tet- T cells, in BAL stably expressed CD103 (Figures 1C/2B).

Taken together these data demonstrate that RM delivery of specific-Ag, but not CpG, during the effector phase of T cell responses in parenterally i.m. immunized hosts induces limited CD8 $T_{RM}$ in the BAL.

**Respiratory mucosal delivery of specific-Ag during the memory phase of T cell responses in i.m. AdCh68Ag85A immunized hosts robustly induces lung CD8 $T_{RM}$**

We further examined whether the RM modulation in parenteral replication-deficient viral vector vaccine immunized hosts could be applied after establishment of

memory CD8 T cells to induce CD8 $T_{RM}$ in the lung. To this end, we immunized BALB/c mice with AdCh68Ag85A i.m., and 28 days later, considered as memory phase of T cell responses, the mice underwent RM delivery of CpG or specific-Ag (Figure 3A). Of importance, 5 days post-RM delivery, compared to CpG, specific-Ag inoculation led to comparable numbers of CD8 tet+ T cell, but higher numbers of CD8 tet- T cell, responses in BAL (Figure 3B). However, RM delivery of specific-Ag resulted in higher numbers of CD8 tet+ T cell, but comparable numbers of CD8 tet- T cell, responses in LPT (Figure 4A). By 14 days post-RM modulation, while the number of CD8 tet- T cells significantly contracted in both BAL and LPT from the 5 days post-RM delivery, compared to CpG, specific-Ag-induced CD8 tet+ T cells underwent less contraction particularly in BAL, as demonstrated by significantly higher frequencies and numbers of these cells in both BAL and LPT (Figures 3B/4A). Interestingly, compared to i.m. vaccination alone, at all different phases after RM delivery of inflammatory agonists, we identified comparable and stable frequencies and numbers of CD8 tet+, or CD8 tet-, T cells in LV (Supplementary Figure 4).

Apart from tetramer specificity of Ag-specific CD8 T cells, we also examined IFN-γ production by CD4 and CD8 T cells in the lung upon *ex vivo* Ag re-stimulation. 5 days post-RM delivery, while CpG delivery only modestly induced Ag-specific CD4 T cells capable of IFN-γ production in both BAL and lung, specific-Ag elicited markedly higher frequencies and numbers of these cells (Figures 5A/6A). Furthermore, 5 days post-RM modulation, compared to CpG, while specific-Ag resulted in similar frequencies and numbers of Ag-specific CD8 T cells capable of IFN-γ production in BAL, its RM delivery

led to significantly higher frequencies and numbers of Ag-specific CD8 T cells capable of IFN-γ production in the lung (Figures 5B/6B). By 14 days post-RM delivery, while the CpG-induced IFN-γ producing Ag-specific CD4 T cells underwent more contraction leading to minuscule numbers in both BAL and lung, their specific-Ag induced counterparts modestly contracted and stayed in higher frequencies and numbers in both BAL and lung (Figures 5A/6A). Furthermore, 14 days post-RM modulation, while the CpG-induced IFN-γ producing Ag-specific CD8 T cells contracted significantly in both BAL and lung, their specific-Ag induced counterparts demonstrated markedly higher frequencies and numbers in both BAL and lung (Figures 5B/6B).

To investigate the effect of RM-delivery of specific-Ag during memory phase in parenterally immunized hosts on $T_{RM}$ induction, in the same set of experiments we also examined expression of typical $T_{RM}$ surface markers CD69, CD103, and CD49a on CD8 $T_{EM}$, as gated from CD44+CD62L- T cells, after being recruited to the lung. Compared to AdCh/CpG group, by 5 days post-RM delivery of specific-Ag, higher frequencies of CD8 tet+ $T_{EM}$, but not CD8 tet- $T_{EM}$, in both BAL and LPT acquired $T_{RM}$ surface markers CD69 and CD103 (Figures 3C/4B). By 14 days post-RM delivery of specific-Ag, higher frequencies of CD8 tet+ $T_{EM}$, but not CD8 tet- $T_{EM}$, in both BAL and LPT stably expressed CD69 and CD103 (Figures 3C/4B). In this regard, in contrast to AdCh/CpG group, the majority of CD8 tet+ T cells in the lung restricted sites including BAL and LPT co-expressed CD103 or CD49a with CD69 upon RM delivery of specific-Ag (Figure 4B).

Taken together, these data suggest that RM delivery of specific-Ag, but not CpG, during the memory phase of T cell responses in parenterally i.m. immunized hosts gives

rise to balanced induction of Ag-specific CD4 and CD8 T cell responses in the lung where CD8 T cells demonstrate $T_{RM}$ phenotype.

**Ag-specific CD8 T cells induced upon RM modulation in i.m. immunized hosts acquire $T_{RM}$ markers expression in local dLNs**

Having established that the majority of recruited Ag-specific memory CD8 T cells in the lung by RM inoculation of an Ag-dependent inflammatory agonist in parenterally immunized hosts express typical $T_{RM}$ surface markers, CD69, CD103, and CD49a (24), we sought to determine the geographical origin of these markers on Ag-specific CD8 T cells. To this end, we examined $T_{RM}$ surface marker expression on CD8 tet+ memory T cells with an effector phenotype (CD44+CD62L-) ($T_{EM}$), in extrapulmonary sites including blood, spleen, and dMLNs, upon RM delivery of specific-Ag during the memory phase of T cell responses in i.m. immunized hosts. By 5 and 14 days following RM modulation, we observed RM modulation results in very low frequencies of circulating CD8 tet+ T cells expressing CD69 and CD103, while around 40% of them expressed CD49a (Figure 7). Moreover, while high frequencies of CD8 tet+ T cells in spleen only transiently expressed CD69 by 5 days post-RM modulation, high levels of their counterparts in dMLNs stably expressed CD69 and CD103 by 5 and 14 days post-RM delivery of specific-Ag (Figure 7). Still moderate levels of such cells continuously express CD49a in both spleen and dMLNs (Figure 7). These data suggest acquisition of $T_{RM}$ surface markers by Ag-specific CD8 T cells induced upon RM modulation in parenterally immunized hosts in extrapulmonary sites as demonstrated by highly expressed CD69 and CD103, and moderately expressed CD49a in dMLNs.

**CD4 T cell depletion upon respiratory delivery of specific-Ag in i.m. AdCh68Ag85A immunized hosts diminishes induction of Ag-specific CD8 $T_{RM}$ in the lung**

It has been shown that CD4 T cell help is required for the formation of CD8 $T_{RM}$ in the lung upon primary respiratory influenza virus infection (25). Given that RM delivery of specific-Ag during the memory phase of T cell responses in parenterally replication-deficient viral vector TB vaccine immunized hosts induces balanced formation of Ag-specific CD4 T cells and CD8 $T_{RM}$ in the lung, next we asked whether CD4 T cells also played a role in the formation of Ag-specific CD8 $T_{RM}$ upon the prime-pull vaccination strategy. In this regard, we used a murine model of CD4 T cell deficiency by continuous depletion of CD4 T cells with i.p. injection of an anti-CD4 GK1.5 mAb (26). This model allows us to maintain a wild-type immunological microenvironment without developing high levels of MHC class II-restricted CD8 T cells as do CD4 knockout mice (27). To this end, we started to deplete CD4 T cells one day prior to RM delivery of specific-Ag during memory phase (28-days post vaccination) to i.m. AdCh68Ag85A immunized hosts and subsequently on days 1 and 8 after RM delivery and sacrificed them on day 14 post RM delivery (Adch/Ag85/αCD4 group) (Figure 8A). Our experiments showed that the CD4 depletion significantly led to over 97% CD4 T cell reduction in the lung interstitium and lung airways (BAL), and over 85% CD4 T cell depletion efficiency in dMLNs (Supplementary Figure 5). One group of mice underwent RM introduction of specific-Ag, 28 days after i.m. AdCh68Ag85A vaccination but left untreated with antiCD4 mAb (AdCh/Ag85 group) as control. Compared to AdCh/Ag85 group, the lack of CD4 T cells resulted in markedly reduced frequencies and numbers of both CD8 tet+ and CD8 tet- T

cells in the lung restricted entry sites, particularly in BAL, while we observed comparable levels of such cells in LV and dMLNs (Figure 8B). Apart from tetramer specificity of Ag-specific memory CD8 T cells, we also examined IFN-γ production by CD8 T cells in BAL and lung upon *ex vivo* Ag re-stimulation. Compared to AdCh/Ag85 group, the absence of CD4 T cells during the RM delivery of specific-Ag, markedly dampened frequencies and numbers of Ag-specific CD8 T cells capable of IFN-γ production in both BAL and lung (Figure 9).

Having demonstrated that $T_{RM}$ surface markers CD69 and CD103 are selectively acquired by high levels of Ag-specific CD8 T cells in the lung restricted sites upon RM delivery of specific-Ag during memory phase in parenterally immunized hosts, in the same set of experiments we also examined expression of these $T_{RM}$ markers on CD8 T cells with an effector phenotype (CD44+CD62L-) in the lung and dMLNs. Compared to AdCh/Ag85 group, we found while CD4 T cell depletion has no impact on the frequencies of CD8 tet+, or CD8 tet-, T cells expressing CD69 or CD103 in LPT, LV, and dMLNs (Figure 8C), there was a significant reduction in the mean fluorescent intensity (MFI) of CD103 expression on CD8 tet+ T cells in LPT (Figure 8D). These data suggest that the absence of CD4 T cells results in not only diminished induction of lung Ag-specific CD8 T cells, but also reduced the level of CD103 expression on these cells upon RM delivery of specific-Ag during memory phase in parenterally i.m. immunized hosts.

**CD4 T cell help enhances the survival and proliferation of Ag-specific CD8 $T_{RM}$ upon respiratory delivery of specific-Ag in i.m. AdCh68Ag85A immunized hosts**

Given that CD4 T cell deficiency results in markedly reduced induction of CD8 tet+ T cells in the lung at day 14 after RM delivery of specific-Ag, we began to investigate the immunological mechanisms of such scenario. In this regard, to examine whether CD4 T cell deficiency affects recruitment of systemically induced CD8 tet+ T cells to the lung restricted sites (BAL and LPT), we started with the same set of experiments as described in the previous section including AdCh/Ag85 and AdCh/Ag85/αCD4 groups, as well as a control group of i.m. immunized hosts with RM delivery of CpG (AdCh/CpG group). However, all animals were sacrificed earlier at day 5 after the last RM delivery of inflammatory agonists (Figure 10A). Compared to AdCh/CpG group, we observed comparable frequencies of CD8 tet+ T cells recruited to LPT and BAL in the presence or absence of CD4 T cells, but there were higher numbers of these cells in the presence, but not absence, of CD4 T cells in such mucosal sites (Figure 10B). These data suggest that while in the absence of CD4 T cell help, CD8 tet+ T cells are recruited to the lung restricted sites upon RM delivery of specific-Ag, there are higher magnitude of such cells in the presence of CD4 T cells.

That RM delivery of specific-Ag in the presence of CD4 T cells increases the number of CD8 tet+ T cells in the lung raised the question whether CD4 T cell help affects induction of such cells via regulating T cell survival. To test this possibility, in the same set of experiments we examined the rate of apoptosis/necrosis in CD8 tet+ T cells by using *ex vivo* annexin V (28) and LIVE/DEAD Fixable Aqua (29) labelling approaches, respectively (Figure 10A). We found minuscule levels of CD8 tet+ T cells from the lungs of all the experimental groups were in the early stage of apoptosis (annexin V+ aqua-)

(Figure 10C). In this regard, the rates of such cells in the LPT, but not BAL and LV, of AdCh/Ag85 group were higher than those in other groups (Figure 10C). However, we noticed high rates of CD8 tet+ T cells from the lungs of all the experimental groups were in the late stage of apoptosis (annexin V+ aqua+) (Figure 10D). Of importance, the rates of such cells in the restricted sites of the lung particularly LPT of AdCh/Ag85 group were markedly less than those in other groups (Figure 10D). However, we observed no difference in the rate of such cells from LV of all the experimental groups (Figure 10D). These data suggest that CD4 T cell help enhances the survival of CD8 tet+ T cells recruited to the lung upon RM delivery of specific-Ag in parenterally i.m. immunized hosts.

To further examine the possibility whether CD4 T cell depletion diminishes induction of CD8 tet+ T cells in the lung via altering T cell proliferation, in the same set of experiments we examined the proliferation rate of CD8 tet+ T cells by using assays including *ex vivo* Ki67 labelling and *in vivo* BrdU incorporation. Ki67 is a nuclear Ag that is expressed only in cells in late G1, S, G2 and M phases of the cell cycle (30), demonstrating activated or proliferating cells. We found high rates of CD8 tet+ T cells from the BAL and LPT of all the experimental groups comparably expressed Ki67 (Figure 10E). Moreover, high rates of CD8 tet+ T cells from the LV expressed Ki67, but the rate of such cells in AdCh/Ag85/αCD4 group were slightly higher than those in other experimental groups. T cell BrdU labeling was conducted via repeated intranasal (i.n.) deliveries of BrdU (Figure 10A), which was demonstrated to more effectively label proliferating cells in the lung compared to i.p. BrdU administration (31, 32). Compared to AdCh/CpG group, we noticed similarly higher rates of CD8 tet+ T cell proliferation in the presence or absence of

CD4 T cells (Figure 10F). However, compared to AdCh/CpG and AdCh/Ag85/αCD4 groups, we found significantly higher rates of CD8 tet+ T cell proliferation in the LPT of AdCh/Ag85 group (Figure 10F). Of note, we found no difference between the rates of CD8 tet+ T cell proliferation in the LV of all the experimental groups (Figure 10F). These data suggest that CD4 T cell help enhances proliferation of CD8 tet+ T cells recruited to the lung restricted sites upon RM delivery of specific-Ag in parenterally i.m. immunized hosts.

Taken together, these data demonstrate that while CD4 T cell help is not critical for CD8 tet+ T cell recruitment to the lung, it enhances the survival and proliferation of such cells after arrival to the lung upon RM delivery of specific-Ag during memory phase in parenterally i.m. immunized hosts.

**Discussion**

Tissue resident memory T cells ($T_{RM}$) considered as non-recirculating sentinels persistently reside in the site of pathogen entry, constitute the first line of defense against subsequent reinfection with mucosal intracellular pathogens such as HIV, *M.tb*, and Herpes viruses (4, 6, 33). Currently, the majority of successful vaccines in humans has been administered via the parenteral route, but they unlikely elicit such cells at the mucosal sites, thus leaving the majority of vaccinees potentially susceptible to mucosal infectious diseases caused by such pathogens (7). Until now, there is an urgent need to develop effective strategies to empower parenteral vaccination through generation of long-lasting $T_{RM}$ at mucosal points. Improved knowledge in this regard not only opens new avenues to develop effective parenteral vaccination strategies to successfully combat mucosal intracellular

infectious diseases, but also enhances the current understanding about $T_{RM}$ immunobiology.

We have shown that while parenteral TB vaccine vaccination only induces activated Ag-specific T cells systemically and is unable to confer effective protection against pulmonary TB (9, 10, 34), RM delivery of an Ag-dependent, but not an Ag-independent inflammatory agonist to parenterally genetic TB vaccine-immunized hosts, elicits long-lasting and protective Ag-specific CD8 T cells in the lung airways towards pulmonary TB (9, 10). Furthermore, we have demonstrated that respiratory introduction of an Ag-dependent inflammatory agonist to parenterally BCG immunized hosts induces long-lasting and protective Ag-specific CD4 T cells in the lung airways towards pulmonary TB (34). It has been demonstrated that both local inflammatory cues and specific-Ag are required for $T_{RM}$ generation in the lung (8). Here we applied RM modulation in a murine model of parenteral AdCh68Ag85A vaccination. We found that RM delivery of an inflammatory specific-Ag during memory, but not effector phase of T cell responses, leads to robust induction of lung Ag-specific CD8 $T_{RM}$ cells. Such cells are characterized by preferential expression of $T_{RM}$ surface markers including CD69 and CD103 (4, 6). Of interest, RM introduction of an Ag-independent inflammatory agonist, CpG leads to massive recruitment of T cells into the lung restricted sites including parenchyma (LPT) and airways (BAL), such increments in the numbers are seen only for a limited period of time and fail to differentiate into $T_{RM}$ cells. A previous study demonstrated that lung Ag-specific CD8 $T_{RM}$ are also established through RM introduction of an Ag-independent inflammatory agonist, CpG plus influenza virus specific peptide during effector or memory

phase in parenterally influenza virus infected hosts (8). While this report is consistent with our finding that RM delivery of Ag-dependent inflammatory signals are critical for $T_{RM}$ formation in the lung, it disagrees with our study in that we have seen generation of lung $T_{RM}$ upon RM modulation only during the memory, but not effector phase of T cell responses. In this regard, the difference observed between these two studies is likely attributed to distinct quality of cells with effector phenotype to those with effector memory phenotype induced by different viral species (35), replication effective vs replication-deficient viruses (36), and distinct innate immune activation (37), as well as differences in local lung inflammatory signals and antigenic persistence (38).

Our finding that both local lung inflammatory signals and specific-Ag are pivotal for $T_{RM}$ formation in the lung contradicts previous studies showing conversion of $T_{RM}$ in other NLTs including skin or female reproductive tract upon adoptive transfer of activated T cells in the presence of local non-specific inflammatory agonists in an Ag-independent manner (39, 40). This discrepancy is likely due to various tissue-specific requirements for formation of $T_{RM}$ as demonstrated by distinct transcriptional profiling between $T_{RM}$ cells from various tissues, in which unique tissue-specific gene expression profile in $T_{RM}$ cells suggests that tissue-derived cues are critical for defining $T_{RM}$ cell generation in specific tissues (41, 42).

We find that RM modulation by using CpG during the memory phase of T cell responses in parenterally TB vaccinated hosts, elicits VLA-1 expression on lung Ag-specific CD8 T cells in an Ag-independent manner. This finding is at odds with a previous study demonstrating Ag-dependent expression of VLA-1 on influenza virus-specific CD8

$T_{RM}$ in the lung following RM modulation of parenterally influenza virus infected hosts (8). However, our finding is consistent with findings from the Ag-independent inflammatory models of rheumatoid arthritis, delayed type hypersensitivity (DTH) (43) and cancer (44), where VLA-1 is non-specifically upregulated on T cells during inflammation. These observations suggest that VLA-1 is differentially acquired by T cells depending on the model and immunologic tissue microenvironment.

This is the first study demonstrating RM modulation using specific-Ag in parenterally immunized hosts to elicit a subset of Ag-specific CD8 T cells in dMLNs exhibiting phenotypic signatures associated with $T_{RM}$ including CD69 and CD103. This finding is consistent with the models of parenteral acute lymphocytic choriomeningitis virus infection where Ag-specific, CD69-expressing CD8 $T_{RM}$ cells were seen in spleen and dLNs of the intestine (45). It remains to be understood whether such cells have a transcriptional profile comparable to $T_{RM}$ cells in non-lymphoid tissues or whether they are bona fide $T_{RM}$ cells, and how they relate to host defence against intracellular pathogens such as *M.tb*.

One of the major goals of TB vaccinology is to develop vaccine strategies leading to balanced induction of TB-specific CD4 and CD8 T cells in the lung restricted sites, particularly the airways. Here for the first time we show that RM modulation by using specific-Ag during the memory, but not effector, phase of T cell responses in parenteral viral vector-based TB vaccine immunized hosts, elicits Ag-specific CD4 and CD8 T cells in the lung restricted sites. In this regard, CD4 T cell help is required for induction of such CD8 T cells exhibiting $T_{RM}$ phenotype. Our finding is consistent with a previous study

demonstrating a critical role for CD4 T cells in formation of airway luminal CD8 $T_{RM}$ cells during primary respiratory influenza virus infection (25). However, different from this study, we show the unaltered magnitude of Ag-specific CD8 T cells in the lung parenchyma in the absence of CD4 T cells (25). This difference is likely because of the difference in models (RM replication-efficient viral infection vs RM modulation upon parenteral replication-deficient vaccination), the formula of RM delivered specific-Ag (viral infection vs protein Ag85 complex), and lung microenvironment. Furthermore, while our finding is in line with the current dogma of CD4 T cell-dependent CD8 T cell priming, expansion, and memory generation during the effector phase of T cell responses (46), it suggests an important role of CD4 T cells in conversion of CD8 $T_{EM}$ to CD8 $T_{RM}$ cells in the lung. Of note, our current finding that CD4 T cell help is required for the establishment of Ag-specific memory CD8 T cells in the lung upon the prime-pull TB vaccination strategy, is contrary to our previous report showing CD4 T cell-independent establishment of fully immune protective Ag-specific memory CD8 T cells post-primary RM vaccination (47). This difference is probably attributed to differences in models (RM vaccination vs RM modulation of parenteral vaccination), the formula of delivered specific-Ag (recombinant viral vector expressing Ag85A vs protein Ag85 complex), and lung microenvironment. Our finding that CD4 helped Ag-specific CD8 T cells in the lung restricted sites reveal enhanced proliferation agrees with previous studies showing high capacity of CD4-helped CD8 T cells for autonomous secondary proliferation upon re-encounter with Ag (48-51). In addition, our result that CD4 helped Ag-specific CD8 T cells in the lung restricted sites show improved survival, agrees with a previous study demonstrating increased apoptosis

in helpless CD8 T cells upon secondary stimulation which is mediated by TNF-related apoptosis-inducing ligand (TRAIL) (52). It still remains to be determined whether the $T_{RM}$ induced by specific Ags in parenteral vaccinated animals translate into much improved immune protection against *M.tb* challenge.

In summary, in the present study we have established a novel prime-pull TB vaccination strategy by which RM modulation using an inflammatory specific-Ag preparation during the memory phase in parenterally immunized hosts with a viral vectored TB vaccine leads to balanced induction of Ag-specific CD4 and CD8 T cells in the lung, where Ag-specific CD8 T cells display a $T_{RM}$-associated phenotype. Such Ag-specific CD8 $T_{RM}$ are also induced in dMLNs. Furthermore, CD4 T cell help is required for local establishment of such Ag-specific CD8 T cells in the lung as demonstrated by enhanced cell survival and proliferation. Our study shall help develop effective parenteral vaccination strategies against mucosal intracellular infectious diseases such as TB.

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**Author contribution**

SH, MJ, and ZX conceived and designed the study. SH, YY, MVS and SA performed the experiments. SH analyzed the data. SH wrote the manuscript. ZX edited and revised the manuscript. All the authors reviewed the manuscript.

**Disclosures**

The authors declare no conflicts of interest.

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## Figures and legends

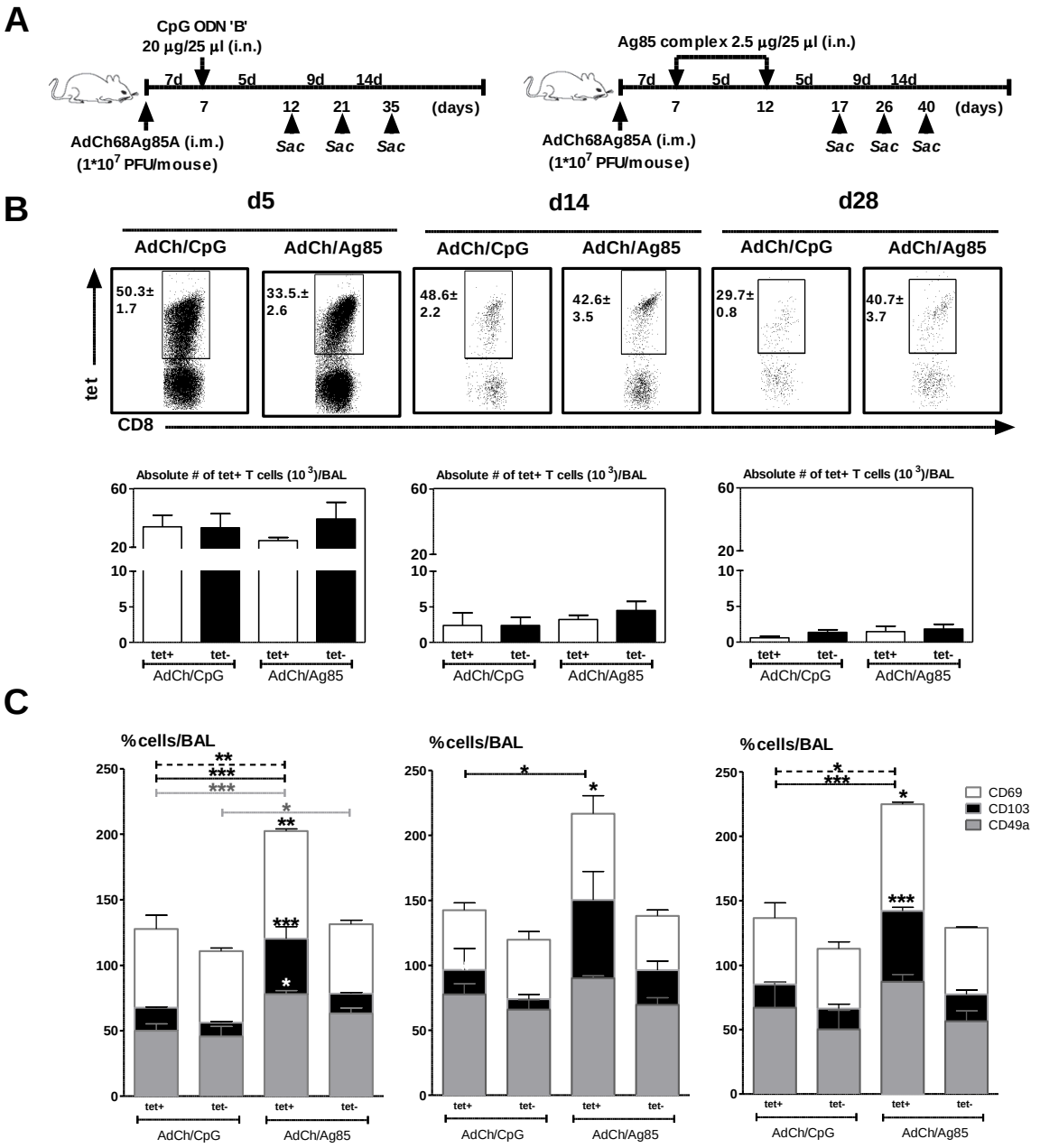



**Figure 1. Modest induction of airway luminal CD8 $T_{RM}$ upon RM administration of specific-Ag during effector phase in i.m. immunized mice.** A) Experimental schema: BALB/c mice were inoculated via i.m. route with $1\times10^7$ PFU of AdCh68Ag85A. Then soluble specific-Ag (Ag85 complex) was delivered i.n. at 7 & 12 days post vaccination (effector phase). As control, CpG was delivered to some other immunized mice 12 days post vaccination. At 5, 14, and 28 days after the last delivery of these inflammatory agonists, mice

were sacrificed 3 min after i.v. injection of fluorochrome conjugated CD45.2 mAb. BAL was collected for mononuclear cell isolation. CD8 T cells were analyzed for Ag-specificity (tetramer), CD103, CD69, and VLA-1 using flow cytometry. B) Representative dot plots showing frequencies of Ag-specific CD8 tet+/- T cells out of total CD8 T cells in bronchoalveolar lavage (BAL). Bar graphs comparing absolute numbers of CD8 tet+ T cells in BAL between specific-Ag and CpG treated mice. C) Bar graphs comparing frequencies of CD8 tet+/-CD69+, CD103+, or CD49a+ T cells with effector phenotype (CD44+CD62L-) in BAL between specific-Ag and CpG treated mice. Data are presented as mean ± S.E.M. of three mice per group representative of one experiment. *$P<0.05$, **$P<0.01$, ***$P<0.001$ were considered significant difference.

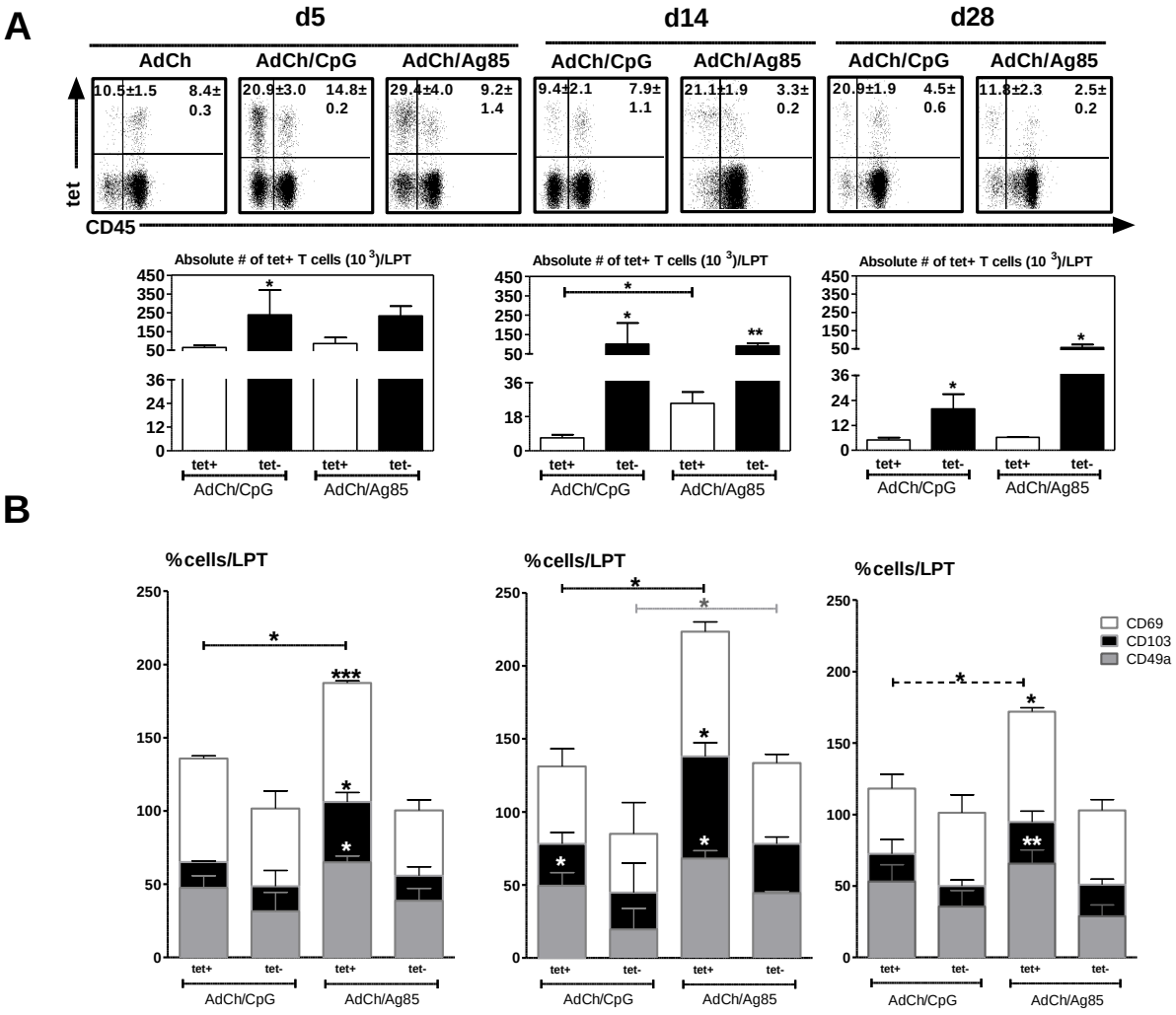



**Figure 2. Modest induction of lung parenchymal CD8 $T_{RM}$ upon RM administration of specific-Ag during effector phase in i.m. immunized mice.** Experimental schema was described in Figure 1A except that lungs were collected and processed for mononuclear cell isolation. CD8 T cells were analyzed for Ag-specificity (tetramer), CD103, CD69, and VLA-1 using flow cytometry. A) Representative dot plots showing frequencies of Ag-specific CD8 tet+/- T cells out of total CD8 T cells in the lung parenchyma (LPT) (i.v.-) & lung vasculature (i.v.+). Bar graphs comparing absolute numbers of CD8 tet+ T cells in LPT between specific-Ag and CpG treated mice. B) Bar graphs comparing frequencies of CD8 tet+/-CD69+, CD103+, or CD49a+ T cells with effector phenotype (CD44+CD62L-) in LPT between specific-Ag and CpG treated mice. Data are presented as mean ± S.E.M. of three mice per group representative of one experiment. *P<0.05 and **P<0.01, were considered significant difference.

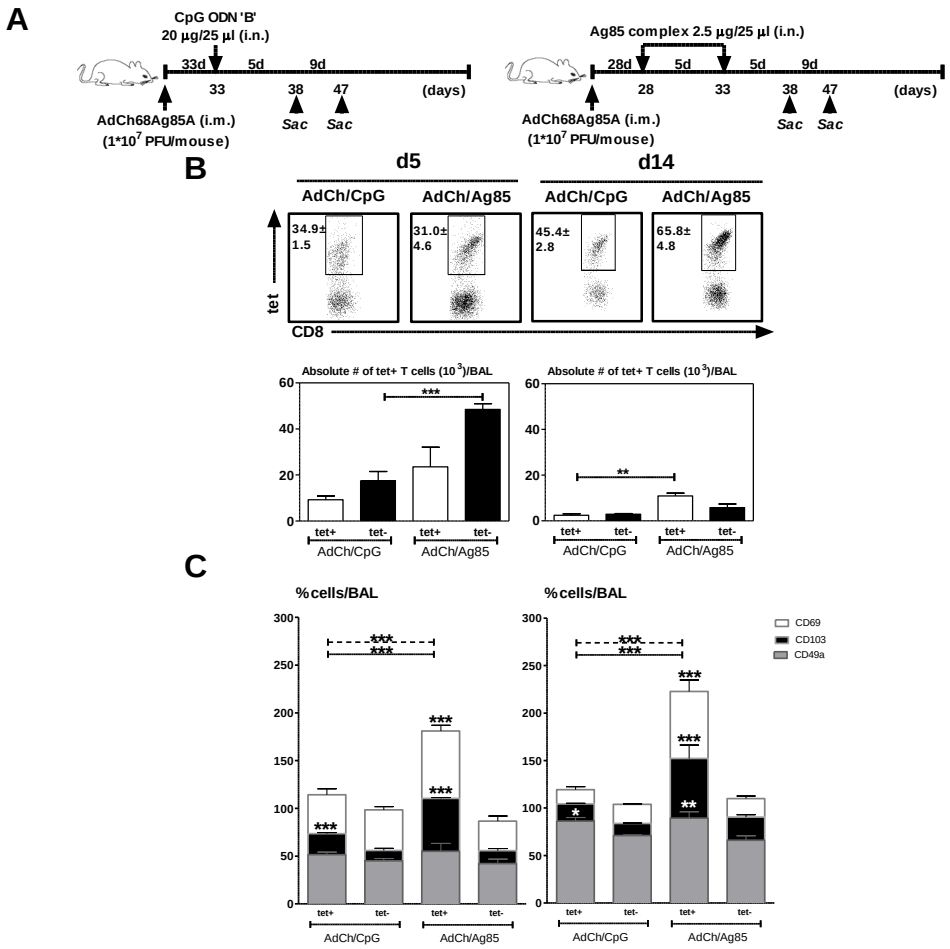



**Figure 3. Robust induction of airway luminal CD8 $T_{RM}$ upon RM administration of specific-Ag during memory phase in i.m. immunized mice.** A) Experimental schema: BALB/c mice were inoculated via i.m. route with $1\times10^7$ PFU of AdCh68Ag85A. Then soluble specific-Ag (Ag85 complex) was delivered i.n. at 28 & 33 days post vaccination (memory phase). As control, CpG was delivered to some other immunized mice 33 days post vaccination. At 5 and 14 days after the last delivery of these inflammatory agonists, mice were sacrificed 3 min after i.v. injection of fluorochrome conjugated CD45.2 mAb. BAL was collected for mononuclear cell isolation. CD8 T cells were analyzed for Ag-specificity (tetramer), CD103, CD69, and VLA-1 using flow cytometry. B) Representative dot plots showing frequencies of Ag-specific CD8 tet+/- T cells out of total CD8 T cells in bronchoalveolar lavage (BAL). Bar graphs comparing absolute numbers of CD8 tet+ T cells in BAL between specific-Ag and CpG treated mice. C) Bar graphs comparing frequencies of CD8 tet+/-CD69+, CD103+, or CD49a+ T cells with effector phenotype (CD44+CD62L-) in BAL between specific-Ag and CpG treated mice. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments. *P<0.05, **P<0.01, ***P<0.001 were considered significant difference.

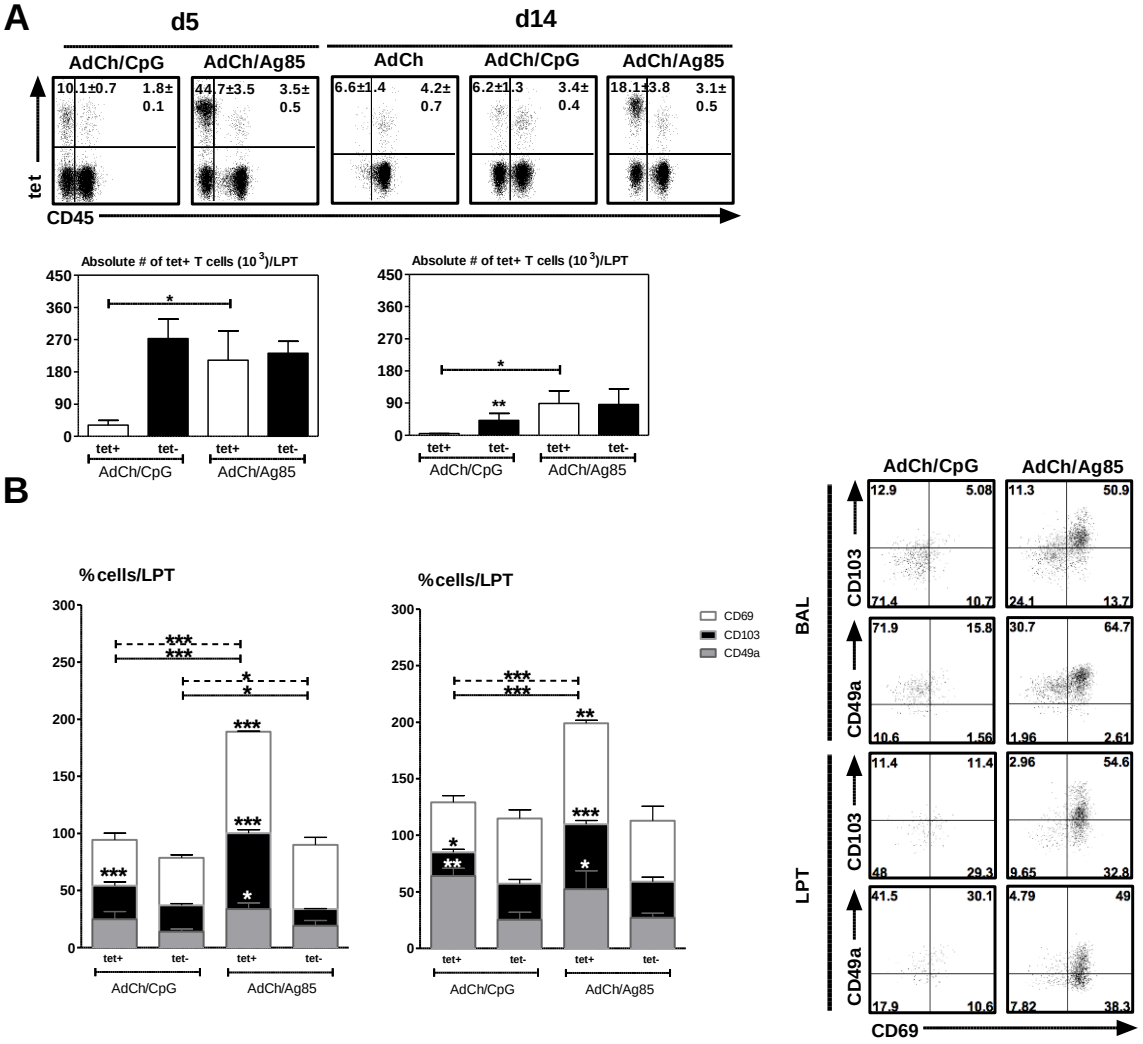



**Figure 4. Robust induction of lung parenchymal CD8 $T_{RM}$ upon RM administration of specific-Ag during memory phase in i.m. immunized mice.** Experimental schema was described in Figure 3A except that lungs were collected and processed for mononuclear cell isolation. CD8 T cells were analyzed for Ag-specificity (tetramer), CD103, CD69, and VLA-1 using flow cytometry. A) Representative dot plots showing frequencies of Ag-specific CD8 tet+/- T cells out of total CD8 T cells in the lung parenchyma (LPT) (i.v.-) & lung vasculature (i.v.+). Bar graphs comparing absolute numbers of CD8 tet+ T cells in LPT between specific-Ag and CpG treated mice. B) Bar graphs comparing frequencies of CD8 tet+/-CD69+, CD103+, or CD49a+ T cells with effector phenotype (CD44+CD62L-) in LPT between specific-Ag and CpG treated mice. Representative dot plots showing frequencies of Ag-specific CD8 tet+ T cells with effector phenotype co-expressing CD103 or CD49a with CD69 at 14 days after RM modulation. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments. *P<0.05, **P<0.01, ***P<0.001 were considered significant difference

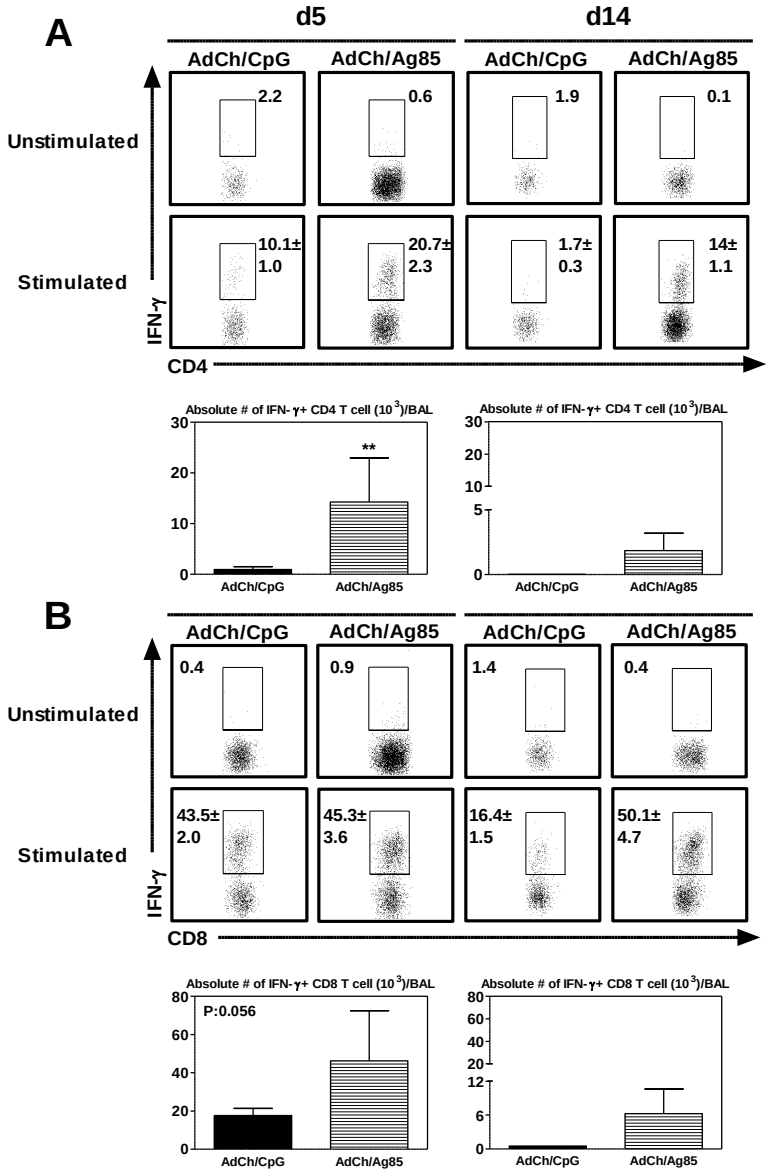



**Figure 5. Robust induction of IFN-γ-producing CD4 and CD8 T cells in airway lumen upon RM administration of specific-Ag during memory phase in i.m. immunized mice.** Experimental schema was described in Figure 3A except that BAL cells were *ex vivo* re-stimulated with Ag85A CD4 or CD8 peptide in BALB/c. Representative dot plots showing frequencies of CD4 or CD8 IFN-γ+ T cells out of total CD4 or CD8 T cells in the BAL. Bar graphs comparing absolute numbers CD4 or CD8 IFN-γ+ T cells in the lung. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments. *P<0.05 and **P<0.01 were considered significant difference.

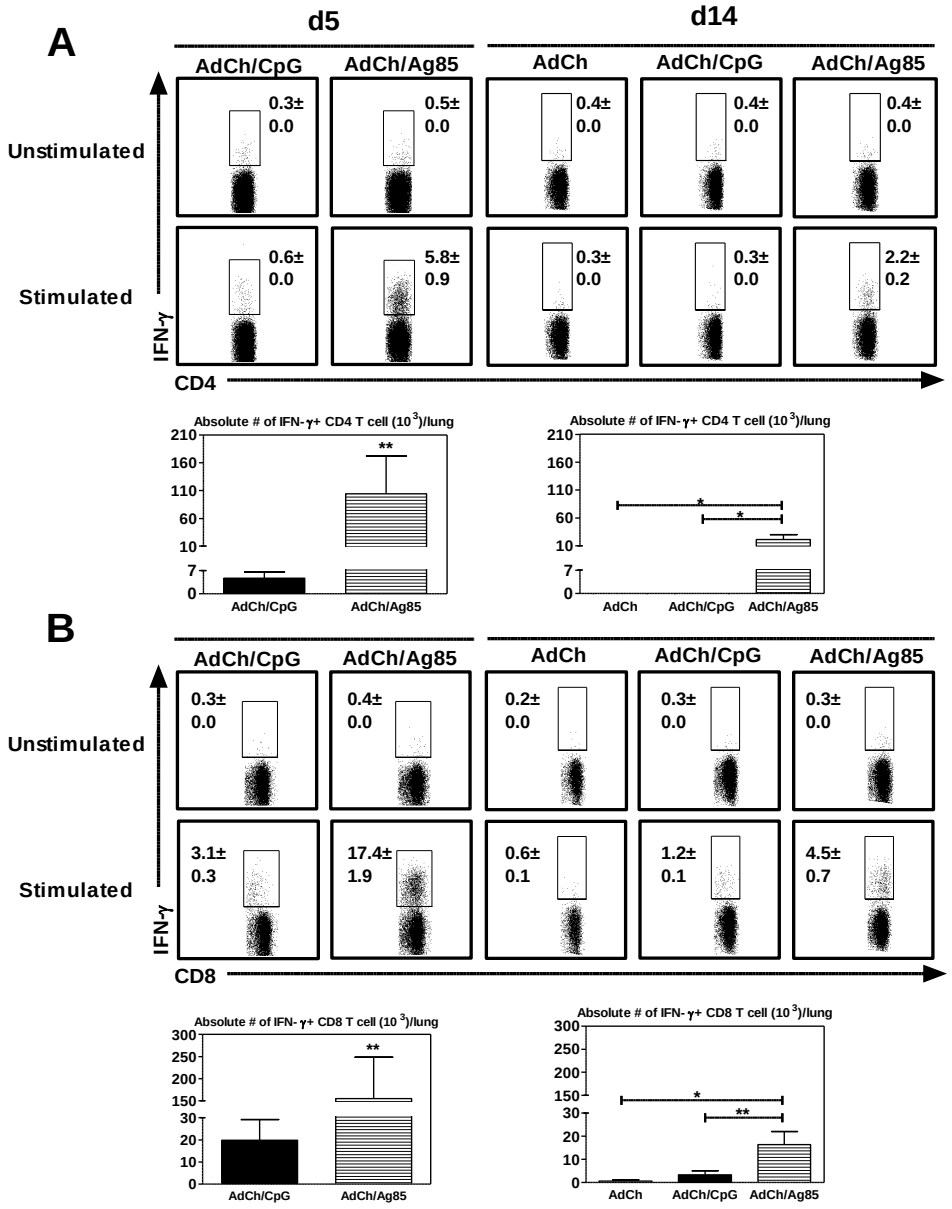



**Figure 6. Robust induction of IFN-γ-producing CD4 and CD8 T cells in the lung upon RM administration of specific-Ag during memory phase in i.m. immunized mice.** Experimental schema was described in Figure 3A. except that lungs were collected and processed for mononuclear cell isolation. Then, the cells were *ex vivo* re-stimulated with Ag85A CD4 or CD8 peptide in BALB/c. Representative dot plots showing frequencies of CD4 or CD8 IFN-γ+ T cells out of total CD4 or CD8 T cells in the lung. Bar graphs comparing absolute numbers CD4 or CD8 IFN-γ+ T cells in the lung. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments. *P<0.05 and **P<0.01 were considered significant difference.

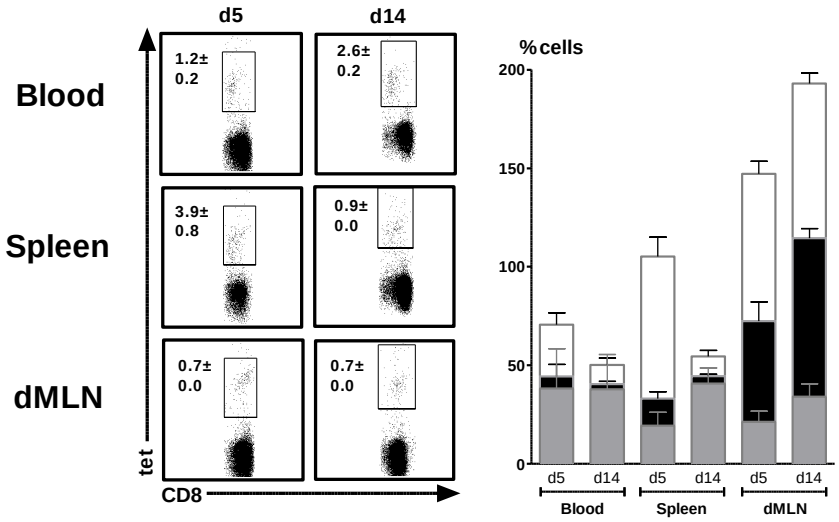



**Figure 7. Acquisition of $T_{RM}$ surface markers by Ag-specific CD8 T cells induced upon RM modulation in parenterally immunized hosts in local dLNs**

Experimental schema was described in Figure 3A. except that blood, spleen, and dMLNs were collected and processed for mononuclear cell isolation. Ag-specific CD8 T cells were analyzed using flow cytometry. Representative dot plots showing frequencies of Ag-specific CD8 tet+ T cells out of total CD8 T cells. Bar graphs demonstrating frequencies of CD8 tet+ CD69+, CD103+, or CD49a+ T cells with effector phenotype (CD44+CD62L-) in specific-Ag treated mice. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments.

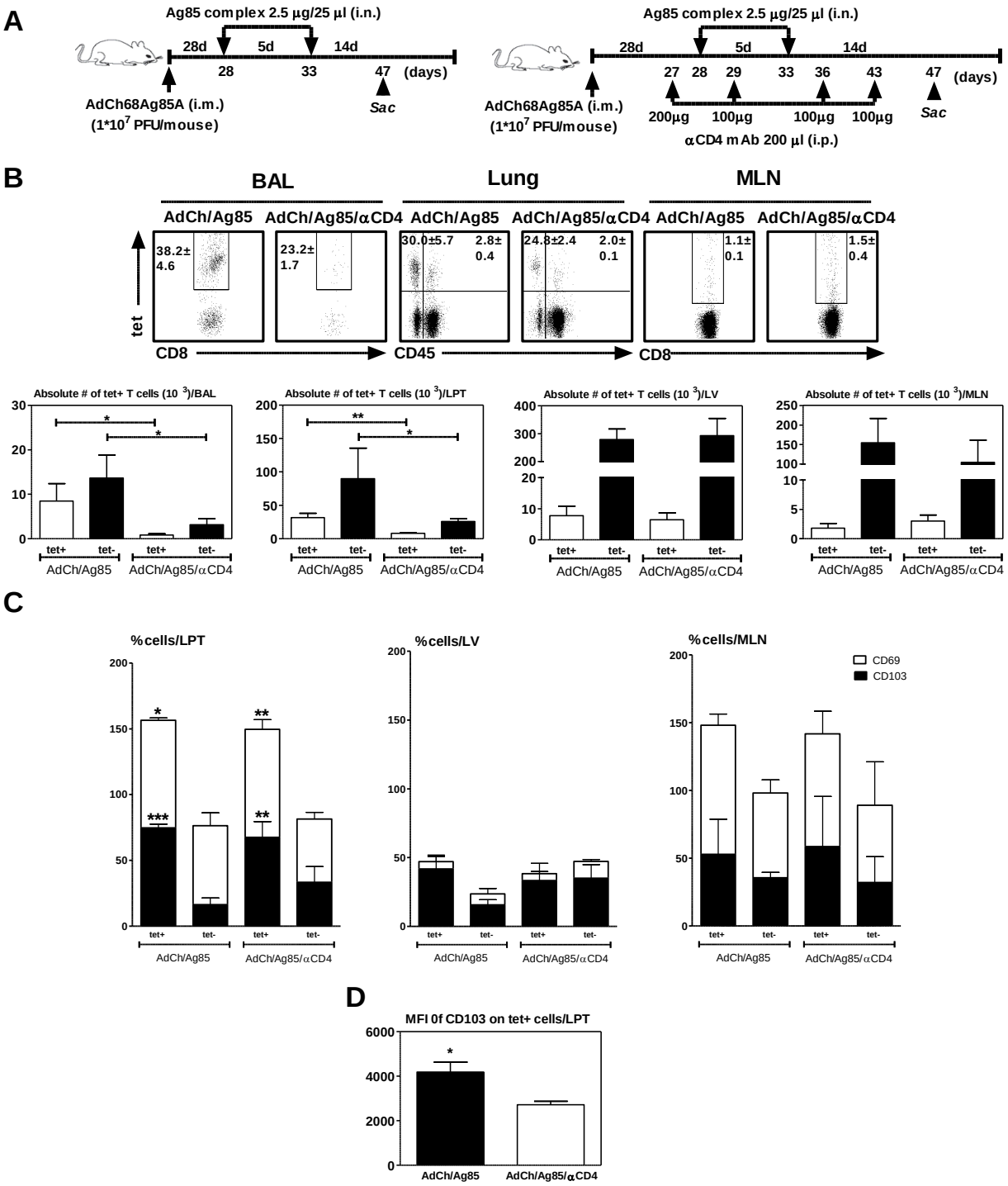



**Figure 8. CD4 T cell-dependent induction of Ag-specific CD8 $T_{RM}$ in the lung upon respiratory delivery of specific-Ag during memory phase in i.m. AdCh68Ag85A immunized hosts.** A) Experimental schema: BALB/c mice were inoculated via i.m. route

with $1\times10^7$ PFU of AdCh68Ag85A. Then soluble specific-Ag (Ag85 complex) was delivered i.n. at 28 & 33 days post vaccination (memory phase). For one group of mice, we started to deplete CD4 T cells one day prior to administration of specific-Ag to 28-days i.m. AdCh68Ag85A immunized hosts and subsequently on days 1 and 8 after specific-Ag inoculation. At 14 days after the last delivery of specific-Ag, mice were sacrificed 3 min after i.v. injection of fluorochrome conjugated CD45.2 mAb. Mononuclear cells were isolated from BAL, lung, and mediastinal lymph nodes (dMLNs). CD8 T cells were analyzed for Ag-specificity (tetramer), CD103, CD69, and VLA-1 using flow cytometry. B) Representative dot plots showing frequencies of Ag-specific CD8 tet+/- T cells out of total CD8 T cells in the BAL, lung parenchyma (LPT) (i.v.-), lung vasculature (i.v.+), and dMLNs. Bar graphs comparing absolute numbers of CD8 tet+/- T cells between CD4 T cell undepleted and depleted mice. C) Bar graphs comparing frequencies of CD8 tet+/-CD69+, CD103+, or CD49a+ T cells with effector phenotype (CD44+CD62L-) between CD4 T cell undepleted and depleted mice. D) Bar graph comparing mean fluorescence intensity (MFI) of CD103 on CD8 tet+ T cells with effector phenotype (CD44+CD62L-) between CD4 T cell undepleted and depleted mice. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ were considered significant difference.

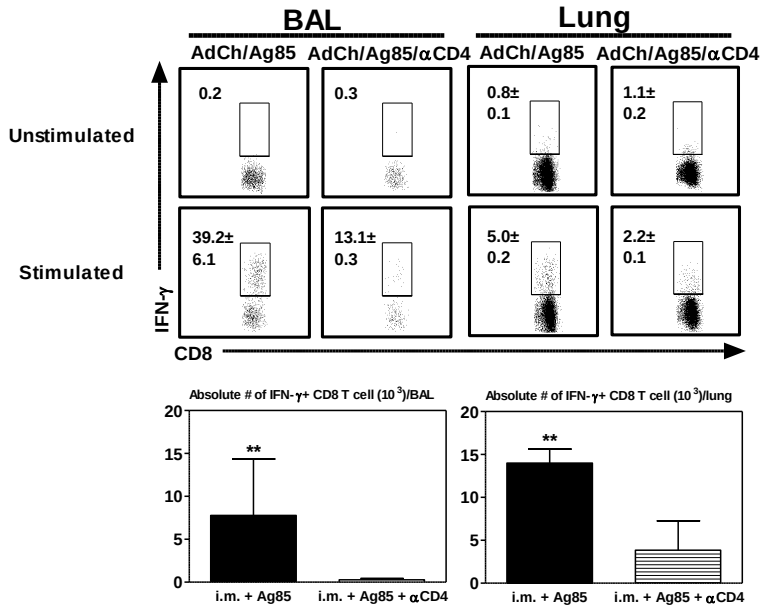



**Figure 9. CD4 T cell-dependent induction of IFN-γ-producing CD8 T cells in the lung upon RM administration of specific-Ag during memory phase in i.m. immunized mice.** Experimental schema was described in Figure 8A except that mononuclear cells were isolated from BAL and lung. Then, the cells were *ex vivo* re-stimulated with Ag85A CD8 peptide in BALB/c. Representative dot plots showing frequencies of CD8 IFN-γ+ T cells out of total CD8 T cells in the BAL. Bar graphs comparing absolute numbers CD8 IFN-γ+ T cells in the lung. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments. *P<0.05 and **P<0.01 were considered significant difference.

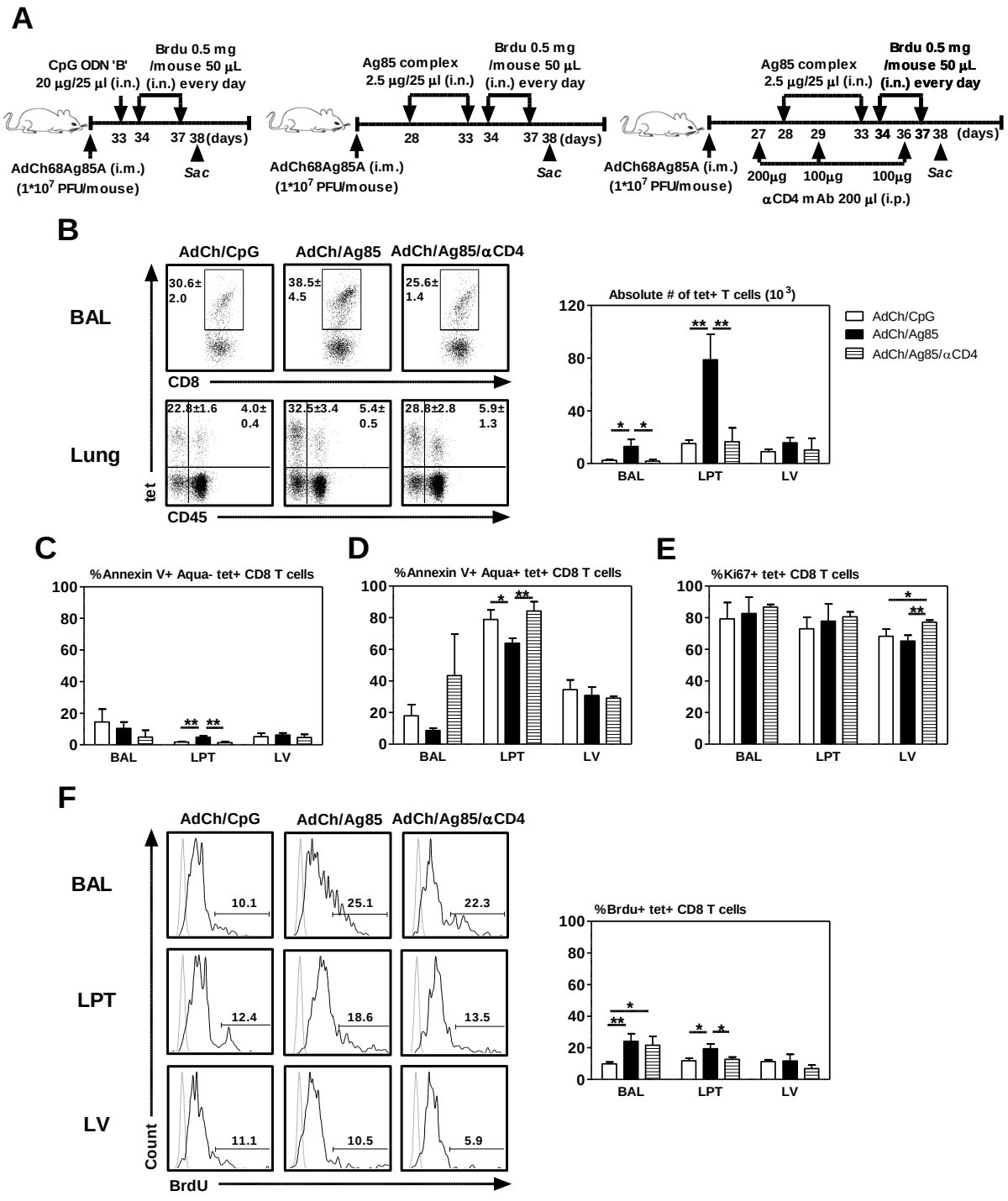
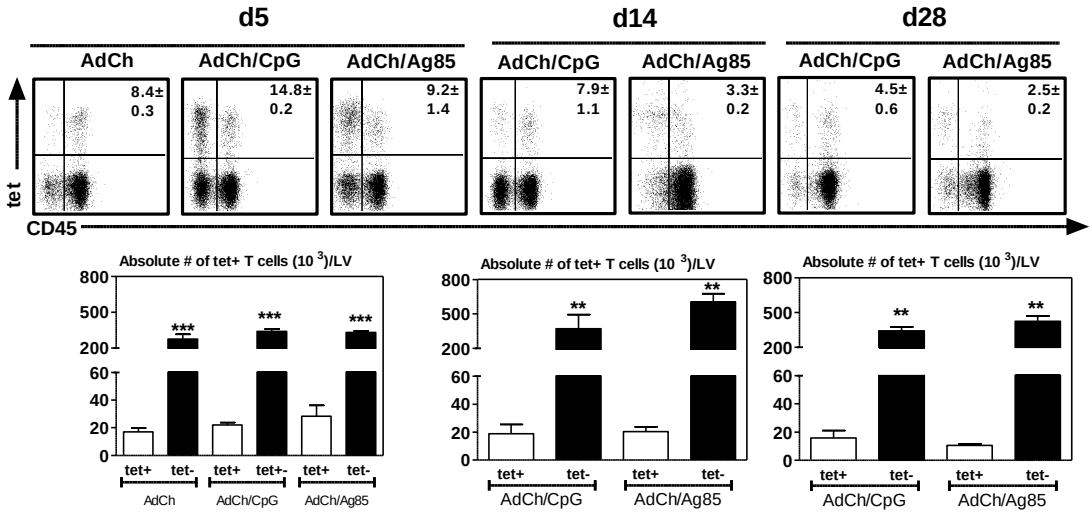
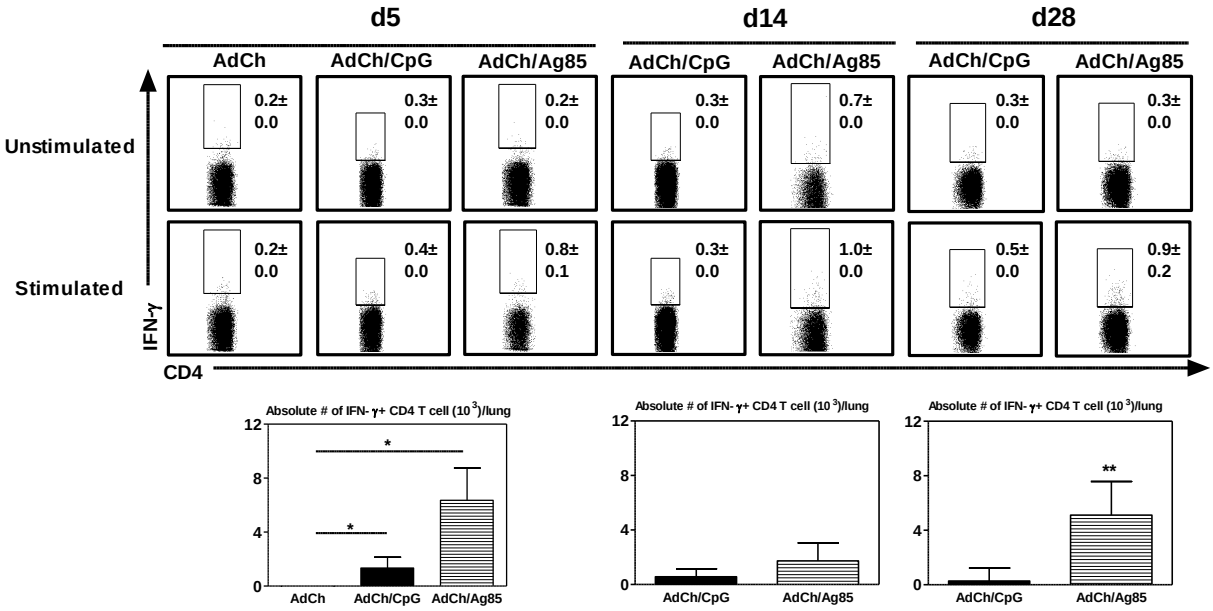



**Figure 10. CD4 T cell-dependent enhancement of the survival and proliferation of Ag-specific CD8 T cells induced upon respiratory delivery of specific-Ag during memory phase in i.m. AdCh68Ag85A immunized hosts.** A) Experimental schema is like what was described in Figure 8A except that after the last RM delivery of

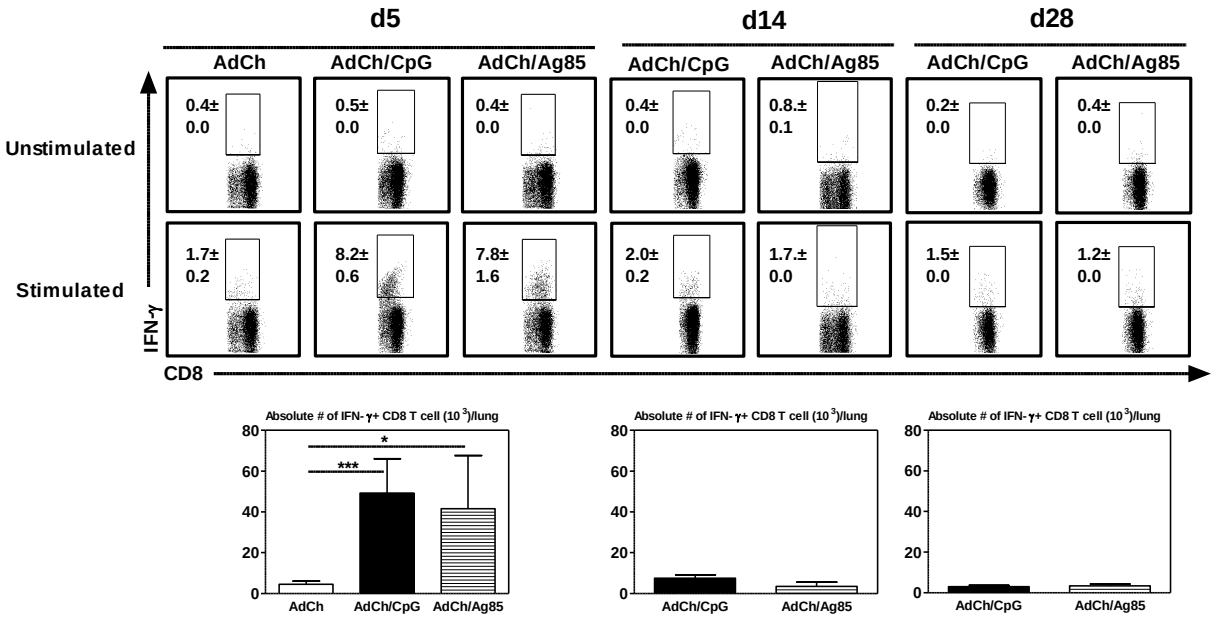
inflammatory agonists, bromodeoxyuridine (BrdU) was administered i.n. for consecutive four days to assay the *in vivo* proliferation rate of Ag-specific CD8 T cells and all animals were sacrificed at day 5 after the last RM delivery of inflammatory agonists. A group of i.m. immunized hosts with RM delivery of CpG was considered as control (AdCh/CpG group). Mice were sacrificed 3 min after i.v. injection of fluorochrome conjugated CD45.2 mAb. Mononuclear cells were isolated from BAL and lung. CD8 T cells were analyzed for Ag-specificity (tetramer), annexin V, Ki67, and BrdU using flow cytometry. B) Representative dot plots showing frequencies of Ag-specific CD8 tet+ T cells out of total CD8 T cells in the BAL, lung parenchyma (LPT) (i.v.-), and lung vasculature (i.v.+). Bar graphs comparing absolute numbers of CD8 tet+ T cells in BAL, LPT, and LV between AdCh/CpG, AdCh/Ag85, and AdCh/Ag85/αCD4 groups. C-E) Bar graphs comparing frequencies of C) CD8 tet+annexinV+aqua-, D) CD8 tet+annexinV+aqua+, and E) CD8 tet+Ki67+ T cells between AdCh/CpG, AdCh/Ag85, and AdCh/Ag85/αCD4 groups. F) Representative histograms showing frequencies of CD8 tet+BrdU+ T cells in BAL, LPT, and LV between AdCh/CpG, AdCh/Ag85, and AdCh/Ag85/αCD4 groups. Bar graph comparing frequencies of such cells in BAL, LPT, and LV between AdCh/CpG, AdCh/Ag85, and AdCh/Ag85/αCD4 groups. Data are presented as mean ± S.E.M. of three mice per group representative of one experiment. *$P<0.05$ and **$P<0.01$were considered significant difference.



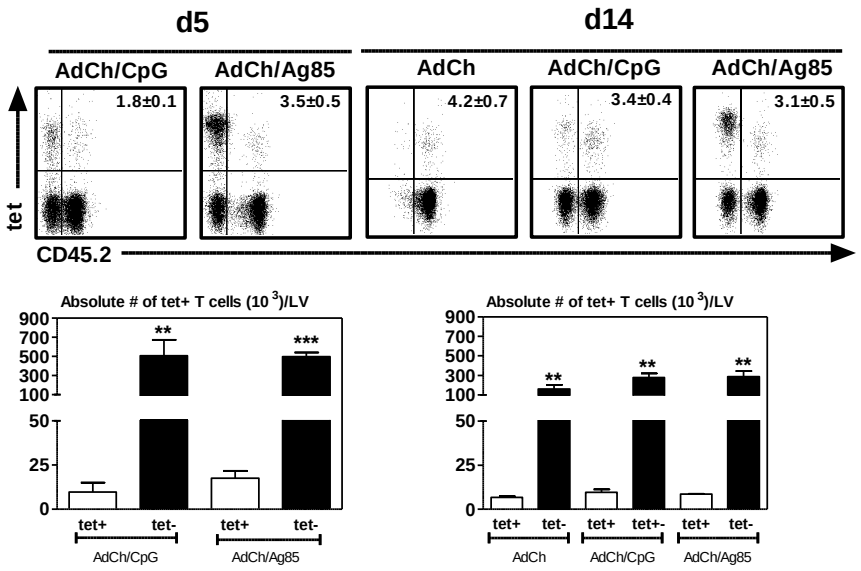

**Supplementary Figure 1. Kinetics of T cell responses in the lung vasculature upon RM administration of inflammatory agonists during effector phase in i.m. immunized mice.** Experimental schema was described in Figure 1A except that lungs were collected and processed for mononuclear cell isolation. CD8 T cells were analyzed for Ag-specificity (tetramer), CD103, CD69, and VLA-1 using flow cytometry. A) Representative dot plots showing frequencies of Ag-specific CD8 tet+/- T cells out of total CD8 T cells in the lung parenchyma (LPT) (i.v.-) & lung vasculature (i.v.+). Bar graphs comparing absolute numbers of CD8 tet+ T cells in LPT between specific-Ag and CpG treated mice. B) Bar graphs comparing frequencies of CD8 tet+/-CD69+, CD103+, or CD49a+ T cells with effector phenotype (CD44+CD62L-) in LPT between specific-Ag and CpG treated mice. Data are presented as mean ± S.E.M. of three mice per group representative of one experiment. *P<0.05 and **P<0.01, were considered significant difference.



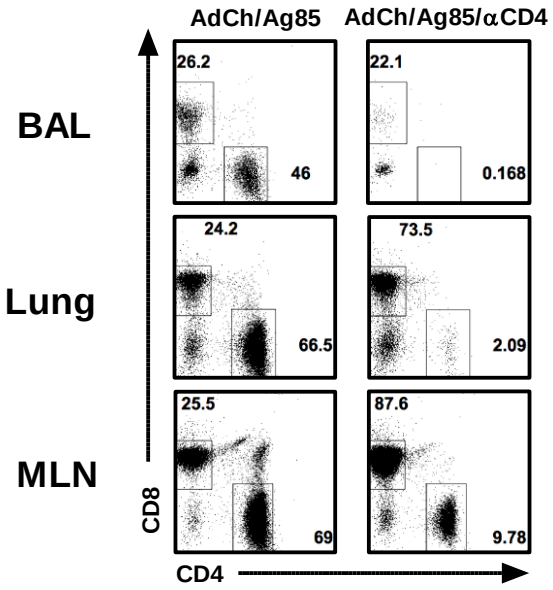

**Supplementary Figure 2. Negligible generation of IFN-γ producing CD4 T cells in the lung upon RM administration of specific-Ag during effector phase in i.m. immunized mice.** Experimental schema was described in Figure 1A except that cells were ex vivo re-stimulated with Ag85A CD4 peptide in BALB/c. Representative dot plots showing frequencies of CD4 IFN-γ+ T cells out of total CD4 T cells in the lung. Bar graphs comparing absolute numbers CD4 IFN-γ+ T cells in the lung. Data are presented as mean ± S.E.M. of three mice per group representative of one experiment. *P<0.05, **P<0.01 considered significant difference.




**Supplementary Figure 3. Modest induction of IFN-γ producing CD8 T cells in the lung upon RM administration of specific-Ag during effector phase in i.m. immunized mice.** BALB/c Experimental schema was described in Figure 1A except that cells were ex vivo re-stimulated with Ag85A CD8 peptide in BALB/c. Representative dot plots showing frequencies of CD8 IFN-γ+ T cells out of total CD8 T cells in the lung. Bar graphs comparing absolute numbers CD8 IFN-γ+ T cells in the lung. Data are presented as mean ± S.E.M. of three mice per group representative of one experiment. *P<0.05, **P<0.01, ***P<0.001 were considered significant difference.




**Supplementary Figure 4. Kinetics of T cell responses in the lung vasculature upon RM administration of inflammatory agonists during memory phase in i.m. immunized mice.** Experimental schema was described in Figure 3A except that lungs were collected and processed for mononuclear cell isolation. Ag-specific CD8 T cells were analyzed using flow cytometry. Representative dot plots showing frequencies of Ag-specific CD8 tet+T cells out of total CD8 T cells in the lung vasculature (i.v.+). Bar graphs comparing absolute numbers of CD8 tet+ T cells in LV between, only immunized, specific-Ag, and CpG treated mice. Data are presented as mean ± S.E.M. of three mice per group representative of two experiments. *P<0.05, **P<0.01, ***P<0.001 were considered significant difference.




**Supplementary Figure 5. Efficiency of CD4 T cell depletion following *in vivo* anti-CD4 depleting mAb treatment.** BALB/c mice were inoculated via i.m. route with $1\times10^7$ PFU of AdCh68Ag85A. Then soluble specific-Ag (Ag85 complex) was delivered i.n. at 28 & 33 days post vaccination (memory phase). For one group of mice, we started to deplete CD4 T cells one day prior to administration of specific-Ag to 28-days i.m. AdCh68Ag85A immunized hosts and subsequently on days 1 and 8 after specific-Ag inoculation. At 14 days after the last delivery of specific-Ag, mice were sacrificed. Mononuclear cells were isolated from BAL, lung, and mediastinal lymph nodes (MLN). Cells were stained for CD3, CD4, and CD8, and analyzed using flow cytometry. Dot plots showing frequencies of CD4 or CD8 T cells out of total T cells. Data are representative of three mice per group from one experiment.

# CHAPTER 4: DISCUSSION

## 4.1 Summary of major findings and contributions to the field

Throughout my Ph.D., I have made significant contributions to mechanistic understanding of the regulation of lung $T_{RM}$ cells induced by TB vaccination strategies. Specifically, I have furthered the notion that lung $T_{RM}$ cells can be induced upon RM vaccination, or RM modulation after parenteral vaccination, with a replication deficient viral vector-based TB vaccine. In **Chapter 2**, we have documented the expression and role of VLA-1 in lung resident memory CD8 T cell responses to RM vaccination, noting establishment of lung Ag-specific CD8 T cells expressing $T_{RM}$-associated molecules VLA-1 and CD103 after RM vaccination. While VLA-1 expression occurs during T cell priming in dMLNs, CD103 expression is acquired after T cell trafficking into the lung. After arrival to the lung, Ag-specific CD8 T cells persistently express VLA-1 at high levels during the effector/expansion, contraction, and memory phases of T cell responses. Although VLA-1 does not play a significant role in T cell homing to the lung, it negatively regulates them in the contraction phase. Moreover, VLA-1 plays a negligible role in the maintenance of these cells in the lung.

At the other end of the spectrum, in **Chapter 3**, we have demonstrated that RM modulation in parenterally vaccinated hosts induces helper CD4 T cell-dependent CD8 $T_{RM}$ in the lung. While parenteral vaccination is incapable of generating lung CD8 $T_{RM}$, RM delivery of an Ag-dependent agent, but not an Ag-independent inflammatory molecule, to parenterally vaccinated hosts, elicits lung Ag-specific CD8 T cells preferentially expressing $T_{RM}$-associated molecules such as CD69 and CD103. We have observed that RM modulation during the memory, but not effector, phase of T cell responses has a greater

capability of inducing lung CD8 $T_{RM}$. Of importance, the former leads to a balanced establishment of Ag-specific CD4 and CD8 T cells in the lung. Such vaccination strategy also leads to induction of Ag-specific CD8 $T_{RM}$ in the dMLNs. We further show that CD4 T cells play a role in regulating Ag-specific CD8 $T_{RM}$ in the lung mucosa via inhibiting T cell apoptosis and increasing T cell proliferation. In summary, my Ph.D. work has provided new knowledge in the induction and mechanisms of lung $T_{RM}$ by different TB vaccination strategies.

### 4.2 Generation of lung $T_{RM}$ following distinct TB vaccination strategies

Effective vaccines for mucosal infectious diseases such as TB, the most deadly health threat to humankind, are currently lacking (5). Long term protection towards such diseases depends on T cell immunity, and the generation and retention of $T_{RM}$ at the site of infection is pivotal for vaccine-induced protection (187-189). Lung CD8 $T_{RM}$ characterized by the expression of CD69, CD103, and VLA-1 are elicited at the restricted entry sites of the lung following respiratory viral infections and constitute the first line of defense to a subsequent reinfection (190, 191). It has been demonstrated that RM replication-deficient viral vector-based TB vaccination induces long-lasting lung Ag-specific CD8 T cells in an Ag-dependent manner and maintains their protective potential like lung CD8 $T_{RM}$ cells generated upon respiratory viral infections (99, 114). Currently, it remains unknown whether RM viral vector-based TB vaccination also induces lung CD8 T cells exhibiting $T_{RM}$-associated molecules. On the other hand, the parenteral vaccination is being applied for most of TB vaccine candidates in clinical trials, but it fails to induce lung CD8 $T_{RM}$. Recently, it has been shown that lung CD8 $T_{RM}$ can be generated through RM manipulation

of parenterally viral infected hosts (prime-pull strategy). Such lung CD8 $T_{RM}$ generation requires local lung inflammatory cues and specific-Ag (180). To this end, we have previously demonstrated that parenteral replication-deficient viral vector-based TB vaccination followed by RM introduction of inflammatory specific-Ag, but not non-specific inflammatory agonists, generates persisting and protective CD8 T cell-mediated immunity in the lung against pulmonary TB (114). However, it has remained unclear whether systemically activated T cells by parenteral viral vectored TB vaccination can be mobilized into the lung and become $T_{RM}$.

In our first study (Chapter 2), we demonstrated that a replication deficient viral vector-based TB vaccine administered via the RM route leads to Ag-specific CD8 $T_{RM}$ cells in the lung characterized by high level expression of $T_{RM}$-associated molecules CD69, CD103, and VLA-1, similar to lung $T_{RM}$ cells induced upon respiratory viral infection (192-194). While VLA-1 expression is mainly acquired by respiratory viral infection-induced Ag-specific CD8 T cells after arrival to the lung (192), we have shown VLA-1 expression is mostly acquired by vaccine-induced Ag-specific CD8 T cells during their priming in the dMLNs. Comparable to the respiratory viral infection-induced Ag-specific CD8 T cells, CD103 expression is acquired by CD8 T cells mostly within the lung microenvironment. This discrepancy is most likely related to differences in inflammatory cues and antigenic persistence caused by replication-efficient viral pathogen versus attenuated replication-deficient viral vector (195). Indeed, continuous peripheral T cell provision was found to play a role in the persistence of influenza virus-specific CD8 T cells in the lung (196) whereas *in-situ* T cell proliferation, but not peripheral T cell supply, plays a significant role

in the maintenance of Ag-specific CD8 T cells in our model of RM viral vectored vaccination (197).

As shown in study two (Chapter 3), RM delivery of a specific Ag, Ag85 protein, but not a nonspecific inflammatory agonist, CpG, to parenterally vaccinated hosts, induces Ag-specific CD8 $T_{RM}$ in the lung characterized by high-level expression of $T_{RM}$ associated-molecules CD69, CD103, and VLA-1. Intriguingly, such RM modulation during memory, but not effector phase of T cell responses, results in robust induction of Ag-specific CD8 $T_{RM}$ in the lung. Our results are in alignment with a previous study demonstrating induction of Ag-specific CD8 $T_{RM}$ upon RM delivery of CpG plus specific-Ag peptide in parenterally viral pathogen-infected hosts (180). However, the latter study showed comparably robust induction of Ag-specific CD8 $T_{RM}$ in the lung upon RM modulation during effector or memory phase of T cell responses. This discrepancy is likely attributed to distinct quality of systemically activated recruited T cells with effector versus effector memory phenotype induced by different viral species (198), replication-efficient vs replication-deficient viruses (195), and distinct innate immune activation (199), as well as differences in the lung inflammatory signals and antigenic persistence induced upon RM modulation (114).

Moreover, we have provided evidence that RM delivery of specific-Ag in parenterally vaccinated hosts also induces Ag-specific CD8 T cells of $T_{RM}$ phenotype in dMLNs, which is consistent with generation of such cells in dLNs of the intestine upon parenteral viral pathogen infection (200). As such, while Ag-specific CD8 $T_{RM}$ from our work express both CD69 and CD103, the Ag-specific CD8 $T_{RM}$ from the latter study

express only CD69. On the other hand, our finding contrasts with the lack of such CD8 $T_{RM}$ in dMLNs upon RM viral infection/vaccination (153, 201). Together, such discrepancies are likely attributed to differences in Ag presentation to CD4 T cells, which in turn affects the acquisition of $T_{RM}$-phenotype by Ag-specific CD8 T cells in dMLNs. In support of this immunologic scenario, we think upon Ag presentation (202, 203), such activated CD4 T cells provide help to CD8 T cells to express CD103 (149). Still, it remains to be determined how such Ag-specific CD8 T cells can be developed in dMLNs, whether they share similar development pathway with their counterparts in NLTs, whether they are authentic $T_{RM}$, and what their importance would be in protection against mucosal intracellular pathogens.

We demonstrate that VLA-1 is expressed by Ag-specific CD8 T cells in the lung upon RM modulation in parenterally replication-deficient viral vector-based vaccinated hosts in an Ag-independent manner. This finding is consistent with Ag-independent inflammatory models of rheumatoid arthritis, DTH (204) and cancer (205), where VLA-1 expression is non-specifically upregulated on T cells during inflammation. However, this observation is at odds with a previous study demonstrating Ag-dependent expression of VLA-1 by lung Ag-specific CD8 $T_{RM}$ upon RM modulation in parenterally viral pathogen infected hosts (180). Such discrepancies suggest that VLA-1 expression by T cells differs based on the model and tissue microenvironment. Moreover, consistent with the model of RM viral-vectored vaccination, here in parenteral vaccination model we show that VLA-1 expression is acquired on Ag-specific CD8 T cells during their priming in systemic SLOs

such as dMLNs and further upregulated after arrival into the lung inflammatory microenvironment.

Altogether, we show that both local inflammatory cues and specific-Ag are critical for $T_{RM}$ generation in the lung. Our finding disagrees with the mechanism for $T_{RM}$ formation in other NLTs such as skin or female genital tract which does not require specific Ags (206, 207). Based on differential transcriptional profiling among $T_{RM}$ cells from distinct tissues, these findings together suggest a major role for tissue-derived signals in $T_{RM}$ formation in specific peripheral sites (208, 209).

### 4.3 Mechanisms underlying the regulation of lung $T_{RM}$ cells

Despite current understanding that memory T cells including $T_{EM}$ and $T_{CM}$ are established soon after T cell priming (21), it remains unclear whether generation of noncirculating $T_{RM}$ occurs early after initial activation in the lymph node, or after tissue-specific localization based on specific signals within the tissue microenvironment, or a combination of these two scenarios. It is believed that specific migratory cues are required for tissue-specific entry of T cells, which consequently leads to acquisition of $T_{RM}$ characteristics within those peripheral tissues. This suggests that some early signals during T cell priming help differentiate cells towards a $T_{RM}$ fate (20, 32).

In our first study, we show VLA-1 to be expressed on Ag-specific CD8 T cells upon their activation in the dMLNs following RM replication-deficient viral vector-based TB vaccination. However, using a well-established VLA-1 function blocking mAb, we show that VLA-1 is not required for the homing of vaccine-activated circulating CD8 T cells to the lung mucosa during the effector phase of T cell responses. This finding is in line with

the previous report in a model of influenza viral infection (192). However, these findings contradict the observations from the Ag-independent inflammatory models of rheumatoid arthritis, DTH (204) and cancer (205), where VLA-1 is observed to play a role in T cell localization in the inflammatory sites. These data suggest that relative contribution of VLA-1 to T cell homing varies based on the model and tissue microenvironment. It remains to be determined which specific migratory molecules are required for Ag-specific CD8 T cells to traffick into the lung following RM viral vector-based TB vaccination.

After the arrival of T cells to the lung, one of the main requirements for development of $T_{RM}$ cells is the ability of T cells to survive during T cell contraction phase and become memory T cells. Intriguingly, for the first time we find VLA-1 to negatively regulate RM TB vaccine-induced CD8 T cells during the contraction phase in the lung**.** In this regard, VLA-1 markedly accelerates the rate of CD8 T cell contraction, leading to reduced numbers and activation of CD8 T cells. While it remains to be determined how VLA-1 negatively governs the contraction of CD8 T cells destined to be $T_{RM}$, VLA-1 may play a role in controlling the survival (210), resources competition (211, 212), and differentiation (213) of RM TB vaccine-induced CD8 T cells.

After establishment of $T_{RM}$ cells in the lung, the maintenance of such cells is critical for protection against subsequent reinfection. In this regard, in the first study we demonstrated that while the lung CD8 T cells after the contraction phase keep on expressing VLA-1, this $T_{RM}$-associated molecule does not have a pivotal role in maintaining CD8 $T_{RM}$ during the memory phase. This observation is at odds with the finding from the model of respiratory influenza viral infection where VLA-1 plays a critical role in the maintenance

of Ag-specific memory CD8 T cells in the lung (192). It is probable that in our RM viral vector-based TB vaccination model, VLA-1 deficiency may well be compensated for by other $T_{RM}$-associated molecules including CD103, which is stably expressed at high levels on the RM TB vaccine-induced CD8 $T_{RM}$. CD103 may augment the retention of CD8 $T_{RM}$ in the lung via its binding to E-cadherin on epithelial cells (214).

Finding biological correlates of immune protection is another urgent need for development of effective prophylactic and therapeutic vaccination strategies for mucosal infectious diseases, in particular TB (215, 216). In this respect, generation of lung $T_{RM}$ may prospectively be considered as a biological correlate for protective immunity upon TB vaccination (217). However, it may be challenging to directly evaluate $T_{RM}$ in human lungs. One possible approach is to identify biological correlates of immune protection of lung in the circulation. Our first study demonstrates that VLA-1, in contrast to CD103, is expressed by most of the circulating RM TB vaccine-induced Ag-specific CD8 T cells which may be considered as a surrogate marker to anticipate $T_{RM}$ induction in the restricted entry sites of the lung. It has been well established that mucosal migratory molecules would be prospective biological correlates of protective immunity as they are associated with the T cell subsets that tend to reside in mucosal sites (218) (219). Based on our current findings, we suggest that not only the mucosal migratory molecules but also the molecules that are expressed on activated T cells during priming in dLNs may potentially be further explored for their value as biological correlates of immune protection.

Our second study shows that Ag-specific CD8 T cells induced by parenteral viral vector-based TB vaccination fails to be recruited to the lung restricted-entry sites, but they

traffick into such tissue sites upon RM immune modulation using Ag-specific or non-specific inflammatory agonists. Moreover, that Ag-specific CD8 T cells can be recruited to the lung restricted-entry sites independent of CD4 T cell help after RM delivery of specific-Ag in parenterally immunized hosts is at odds with a recent study documenting the importance of CD4 T cell help for acquisition of homing molecules by effector CD8 T cells for homing and extravasation (220). In the current study, while we could not rule out a similar role for CD4 T cells, CD8 T cell recruitment to the lung in the absence of CD4 T cell help might be due to the lung inflammatory conditions induced by RM delivery of inflammatory agonists, where upregulation of inflammatory chemokines and ligands for adhesion molecules might compensate for the requirements for tissue-specific homing of T cells into inflamed peripheral tissues (119).

In the second study, we also find that upon recruitment of parenteral TB vaccine-induced memory CD8 T cells to the lung by RM delivery of specific-Ag, such cells underwent contraction to a lesser extent compared to their effector counterparts. This observation is attributed to the quality of effector versus memory T cells, as well as T cell survival and proliferation. As a case in point, in this study we report that reduced rates of apoptosis and higher *in-situ* T cell proliferation leads to increased survival and proliferation of such recruited memory T cells in the lung, respectively. As such, our finding that CD4 helped Ag-specific CD8 T cells in the lung restricted-entry sites showed improved survival agrees with the previous study demonstrating increased apoptosis in non-helped CD8 T cells upon secondary stimulation (221). Furthermore, our finding that compared to the un-helped cells, CD4 helped Ag-specific CD8 T cells in the lung underwent enhanced

proliferation agrees with the previous studies showing high capacity of CD4 helped Ag-specific CD8 T cells for autonomous secondary proliferation upon re-encounter with Ag (222-225). These findings together suggest that reduced contraction of Ag-specific memory CD8 T cells occurs in an Ag-dependent manner following RM modulation of parenteral TB vaccination. It still remains to be determined whether VLA-1 also plays an immunoregulatory role in T cell contraction following RM modulation of parenteral TB vaccination.

After T cell recruitment to peripheral tissue sites and establishment of long-term memory T cells, $T_{RM}$ precursors from such memory T cell population differentiate into $T_{RM}$ cells, which is a critical stage for the development of $T_{RM}$ cells. In this regard, the presence of Ag and lung microenvironment cues such as $T_{RM}$-inducing cytokines including TGF-β, IL-33, and tumour necrosis factor-α (TNF-α) play a critical role in establishment of lung $T_{RM}$ cells (149, 183, 184, 209). Our second study shows that RM modulation by using specific-Ag during memory, but not effector, phase in parenteral viral vector-based TB vaccinated hosts elicits robust generation of Ag-specific CD4 and CD8 T cells in the lung restricted entry sites. In this regard, CD4 T cell help is required for induction of such lung CD8 T cells exhibiting $T_{RM}$ phenotype, through augmenting the numbers of such cells, as well as the expression of $T_{RM}$-associated molecule on a per cell basis. This finding is consistent with a previous study demonstrating a critical role for CD4 T cells in formation of airway luminal CD8 $T_{RM}$ cells during primary respiratory influenza virus infection (149). However, our finding contradicts this study which shows unaltered magnitude of Ag-specific CD8 T cells in the lung parenchyma in the absence of CD4 T cells (149). This

difference is likely because of the difference in models (RM replication-efficient viral infection versus RM modulation upon parenteral replication-deficient immunization), the formula of RM delivered specific-Ag (viral infection versus protein Ag85 complex), and lung microenvironment. Furthermore, our finding is in line with the current dogma of CD4 T cell-dependent CD8 T cell priming, expansion, and generation of long-lasting memory during effector phase of T cell responses (226), but our study further adds a role of CD4 T cells in the activities of systemically induced memory CD8 T cells mobilized into the mucosal tissue. Specifically, we show that CD4 T cells help the conversion of circulating CD8 $T_{EM}$ to CD8 $T_{RM}$ cells in the lung. Of note, we have previously shown that CD4 T cells are dispensable in protective CD8 T cell responses induced by RM viral vector-based vaccination (106). These findings together suggest that relative roles of CD4 T cells in CD8 T cell responses vary depending on the route of immunization and lung microenvironment.

We have used an intravascular staining approach to differentiate the intravascular T cells from those residing in lung tissues. By this approach, fluorochrome-labeled mAbs are administered directly into blood via the tail vein immediately before sacrificing mice to label circulating leukocytes. This approach has been utilized to identify bona fide $T_{RM}$ cells (227, 228). In fact, this strategy has allowed us to precisely distinguish resident immune cells located in the restricted entry sites of the lung (airway lumen and parenchyma) from circulating cells positioned in the vasculature compartment of the lung in parenteral and mucosal vaccination models (201, 229). Future studies may also use microscopic analysis of the lung to determine precisely the localization of $T_{RM}$ cells to obtain better understanding about the cellular associations and tissue microarchitecture for supporting

$T_{RM}$ development and/or maintenance. Moreover, conjoining the vasculature of two mice through parabiotic surgery will allow us to more precisely determine the relationship of circulating T cells to the development of $T_{RM}$ in the lung. Using this approach, it has been previously shown that while circulating $T_{EM}$ and $T_{CM}$ equilibrate between the two parabionts, non-circulating $T_{RM}$ do not undergo such equilibration and persistently remain in the lung mucosal sites where they originally developed (148).

We have also used blocking antibodies as a choice of loss-of-function approaches to investigate the function of a select molecule or cell type. For instance, we have used a well-established VLA-1 function-blocking mAb to examine the role of VLA-1 in the context of CD8 T cell responses. Studies using VLA-1-deficient mice furthermore support that the mode of action for this mAb is to diminish the function of VLA-1 (230). As such, there is consistency in findings obtained by using both this mAb and VLA-1-deficient mice in a model of influenza viral infection (153). To fully evaluate the role of VLA-1 in the development and maintenance of CD8 $T_{RM}$ cells, it would be required to develop an inducible knockout specific to CD8 T cells, so that the adhesion molecule could be eliminated at specific time points post vaccination. We have also used a CD4 T cell depletion approach to address its role in the prime-pull induced CD8 T cell responses. Compared to using MHC class II-deficient mice with 100% deficiency in CD4 T cells, in our study CD4 T cell-depleted mice still had some levels of CD4 T cells before and after RM modulation. We think that this may not necessarily diminish the significance of our results since most of immunocompromised individuals demonstrate only partial CD4 T cell deficiency (106).

Overall, both RM-viral vector-based vaccination and RM modulation upon parenteral viral vector-based vaccination result in induction of lung $T_{RM}$ in the presence of local inflammatory cues and specific-Ag. However, such $T_{RM}$ generation occurs by distinct underlying mechanisms. On this point, lung $T_{RM}$ formation by the former vaccination strategy is CD4 T cell-independent, but by the latter it is CD4 T cell-dependent to a certain extent. Moreover, compared to RM route of vaccination, RM modulation following parenteral viral vector-based vaccination seems to induce more balanced anti-TB CD4 and CD8 T cells in the lung.

## 4.4 Conclusions and future directions

$T_{RM}$ located in the lung mucosal sites play a pivotal role in defense against intracellular respiratory infections. To this date, it remains to be investigated whether viral vectored RM vaccination or immunologically modulated parenteral vaccination may induce $T_{RM}$ in the lung, and if so, what are the potential underlying mechanisms regulating such cells. The research presented in this thesis not only helps fill up the current gap of knowledge, but it also holds implications in the development of effective vaccination strategies to fight against mucosal intracellular infectious diseases such as TB.

First, we found that RM vaccination induces generation of lung CD8 $T_{RM}$ identified by the expression of $T_{RM}$-associated molecules including CD69, CD103, and VLA-1. In this regard, VLA-1 is acquired during T cell activation in the dMLNs whereas CD103 is acquired after T cells entered the lung. After arrival to the lung, Ag-specific CD8 T cells stably express VLA-1 at high levels through the effector/expansion, contraction, and memory phases of T cell responses. We find that VLA-1 is not required for trafficking of

these cells to the lung, but it negatively regulates them in the contraction phase. Further, VLA-1 has a negligible role in the maintenance of such cells in the lung. Such TB vaccination strategy may be applied for immune competent individuals and those with CD4 T cell deficiency, as it leads to CD4 T cell-independent formation of long-lasting and protective CD8 T cell responses in the lung (106). Further studies may address (i) whether Ag-specific CD8 T cell recruitment to the lung requires tissue-specific migratory molecules or occurs non-specifically due to the lung inflammatory conditions induced by RM vaccination; (ii) if tissue-specific migratory molecules are required, where and how such molecules are acquired; and (iii) how VLA-1 negatively regulates the contraction of T cells destined to become $T_{RM}$ cells.

Secondly, we show that although parenteral vaccination alone does not elicit lung CD8 $T_{RM}$, subsequent RM delivery of a specific Ag preparation, but not a non-specific inflammatory agonist, leads to the generation of CD8 $T_{RM}$ in the lung. Of note, Ag-specific CD8 $T_{RM}$ are present not only in the lung, but also in dMLNs. Generation of lung CD8 $T_{RM}$ requires CD4 T cell help through enhancing cell survival and proliferation within the lung. While these results support the potential of such parenteral prime-local mucosal pull vaccination strategy with some of TB vaccine candidates, it may not be suitable for individuals with CD4 T cell deficiency. Further investigations are required to address (i) how CD4 T cell help enhances CD8 T cell survival and proliferation; (ii) the connection between $T_{RM}$-inducing cytokines in the lung and CD4 T cell-mediated CD8 $T_{RM}$ generation; (iii) the role of CD4 and/or CD8 T cells induced by such vaccination strategy in immune protection against pulmonary TB, and (iv) the impact of parenteral BCG prime-local

modulation pull on CD8 $T_{RM}$ generation in the lung, and consequently immune protection against pulmonary TB.

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