A Distinct Lung-Interstitium-Resident Memory CD8+ T Cell Subset Confers Enhanced Protection to Lower Respiratory Tract Infection

Highlights
- Intranasal T cell epitope-targeted vaccination confers enhanced protection
- Intranasal vaccination preferentially induces CXCR3LO lung-resident memory CD8+ T cells
- Protective memory T cells localize to vulnerable sites of the lung interstitium
- Interstitial memory CD8+ T cells rapidly protect the lung against infection

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In Brief
Mucosal surveillance ensures rapid protection when a pathogen breaches the barrier. Gilchuk et al. report that local vaccination elicits a distinct resident memory CD8+ T cell subset that localizes to the lung interstitium. These T cells protect against respiratory infections by positioning at vulnerable sites and acting quickly against infections.
A Distinct Lung-Interstitium-Resident Memory CD8+ T Cell Subset Confers Enhanced Protection to Lower Respiratory Tract Infection

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SUMMARY

The nature and anatomic location of the protective memory CD8+ T cell subset induced by intranasal vaccination remain poorly understood. We developed a vaccination model to assess the anatomic location of protective memory CD8+ T cells and their role in lower airway infections. Memory CD8+ T cells elicited by local intranasal, but not systemic, vaccination with an engineered non-replicative CD8+ T cell-targeted antigen confer enhanced protection to a lethal respiratory viral challenge. This protection depends on a distinct CXCR3LO resident memory CD8+ T (TRM) cell population that preferentially localizes to the pulmonary interstitium. Because they are positioned close to the mucosa, where infection occurs, interstitial TRM cells act before inflammation can recruit circulating memory CD8+ T cells into the lung tissue. This results in a local protective immune response as early as 1 day post-infection. Hence, vaccine strategies that induce lung interstitial TRM cells may confer better protection against respiratory pathogens.

INTRODUCTION

Memory of past encounters with pathogens is a key feature of the adaptive immune system and the basis for successful vaccination. Antigen-specific memory CD8+ T cells play key roles in protection against infectious diseases. A delay in the generation of effector CD8+ T cells and/or the recall of circulating memory CD8+ T cells to the site of infection allows pathogens to replicate and cause disease. Non-circulating tissue-resident memory CD8+ T (TRM) cells, by virtue of their optimal positioning at barriers (e.g., skin, intestine, female reproductive tract, and respiratory tract), keep infections in check at sites of pathogen entry to confer protective immunity (Ariotti et al., 2012; Bergsbaken and Bevan, 2015; Bivas-Benita et al., 2013; Hickman et al., 2015; Jiang et al., 2012; Li et al., 2013; Mackay et al., 2013; Salek-Ardakani et al., 2011; Schenkel et al., 2014; Sheridan et al., 2014; Shin and Iwasaki, 2012; Slutter et al., 2013; Takamura et al., 2010). Hence, insights into the generation and function of TRM cells are essential for devising ways to induce protective immunity by vaccination (Clark, 2015; Schenkel and Masopust, 2014).

The respiratory mucosa is a major site for pathogen invasion and, hence, a site for constant immune surveillance. Safeguarding the lungs (especially the terminal respiratory tree that consists of alveoli and associated capillary beds) from injury is critical to preserve lung function. Thus, severe morbidity and mortality in respiratory infectious diseases are associated with viral dissemination and inflammation, which can damage the lung parenchyma and alveoli (Gilchuk et al., 2013; Manicassamy et al., 2010). To protect from disease, memory CD8+ T cells are strategically distributed within distinct anatomical compartments of the lungs (Hasenberg et al., 2013; Lelkes et al., 2014; Rangel-Moreno et al., 2011). The anatomical niche that memory CD8+ T cells occupy in the lungs depends on the route of infection (Anderson et al., 2012). Nonetheless, the precise location and protective capacity of different CD8+ TRM cell subsets in the lungs that are elicited by vaccination are poorly understood.

Most vaccines in clinical use, including those protecting against respiratory infections, are administrated to elicit systemic rather than local immune responses. Live and attenuated microbes are dangerous for use as pulmonary vaccines, and inactivated microbes poorly elicit cellular immunity. Consequently, there is a need for physiologically relevant experimental models that closely recapitulate protective CD8+ TRM cell responses to vaccination in the lungs. Here, we describe a model in which lower airway vaccination with a non-replicative pathogen-derived protein antigen elicits high frequency of epitope-specific...
CD8+ TRM cells within the lung tissue that conferred rapid protection to mice against lethal vaccinia virus (VACV) infection of the lower respiratory tract. A comparative analysis of the outcomes of distinct vaccination routes revealed that the protective CD8+ TRM cell subset localized to a spatially distinct niche within the lung parenchyma and exhibited unique phenotypic features.

RESULTS

Intranasal Vaccination with Antigenic Protein Plus Adjuvant Confers Enhanced Protection against Respiratory Virus Infection

We previously reported two HLA-B7.2-restricted VACV-derived CD8+ T cell epitopes: B8R70–79 (B8R), which elicited a protective polyclonal CD8+ T cell response upon peptide vaccination; and L4R37–45, which elicited a robust CD8+ T cell response but was non-protective for reasons previously reported (Gilchuk et al., 2013). This finding was exploited to dissect the protective capabilities of endogenous (non-T cell receptor [TCR] transgenic), epitope-specific CD8+ T cells. We engineered two recombinant VACV-derived proteins (L4R and L4R-b8r). To create L4R-b8r, the native non-protective L4R37–45 peptide in L4R was substituted with the protective B8R epitope. Thus, L4R and L4R-b8r are made of the same protein scaffold but differ only in the epitope they contain (Figure 1A).

To test the immunogenicity of L4R and L4R-b8r, HLA-B7.2 transgenic (B7.2 tg) mice were primed and boosted intraperitoneally (IP) (Figure 1B) with the protein antigen formulated with α-galactosylceramide (αGC) as the adjuvant (Gilchuk et al., 2013; Semmling et al., 2010). Staining with the corresponding B7.2-peptide tetramer (Tet) showed that both protein antigens induced a robust systemic CD8+ T cell response (Figure 1C). The response was specific to the epitope used for immunization, because the elicited CD8+ T cells reacted only with the cognate tetramer (Figure S1). Moreover, under conditions promoting severe lower airway infection, intraperitoneal vaccination with L4R-b8r was sufficient to protect mice from a lethal respiratory VACV challenge, while L4R-vaccinated mice succumbed to disease (Figure 1D). Thus, B8R-specific CD8+ T cells were the sole mediators of protection, with undetectable contribution by other adaptive immune cells to the antigen or the adjuvant. We next compared the protective potential of memory B8R-specific CD8+ T cells generated by intraperitoneal and intranasal (IN) vaccinations. Strikingly, intranasally vaccinated mice lethally challenged with VACV were more resistant to disease and showed lower VACV titers in the lung as early as day 1 post-infection (p.i.) (Figures 1E and 1F). We concluded that IN vaccination, when compared to intraperitoneal vaccination, with CD8+ T cell-targeted antigen more efficiently protected mice from lethal respiratory VACV infection.
Epitope-Specific CD8+ T Cells Elicited by Protein Vaccination Mediate Long-Term Protection against Lethal Virus Infection

IN αGC administration rapidly induces local inflammatory responses and interferon-γ (IFN-γ) production (Courtney et al., 2011), potentially explaining the enhanced protection observed when αGC was used as an adjuvant. To investigate antigen specificity of the observed protective immune responses, we primed and boosted mice intranasally with αGC alone or with αGC plus L4R-b8R and then challenged them with VACV on day 14 or on day 24 after boost. We chose day 24 after boost because αGC-induced IFN-γ responses in the lungs wane substantially by then (Courtney et al., 2011). We found that IN αGC treatment 14 days before challenge played very little role in conferring protective immunity that waned after 3 weeks. (Figures 2A, 2B, S2A, and S2B). Thus, B8R-specific CD8+ T cells in the lungs participate in enhanced protection to respiratory infection.

To directly demonstrate that CD8+ T cells elicited upon IN vaccination participated in protection, we locally depleted them by IN αCD8 antibody (Ab) delivery (Slutter et al., 2013). Lung TRM cells were quantified after intravascular (IV) staining with αCD45 Ab in vivo to identify circulating blood leucocytes (Anderson et al., 2014). Local CD8+ T cell depletion significantly decreased the number of CD8+ TRM cells in the lungs (Tet+ αCD45 Ab-) but maintained intravascular CD8+ T cells (Tet+ αCD45 Ab+; Figures 2C and S2C–S2E). This depletion rendered otherwise resistant intranasally vaccinated mice more susceptible to respiratory VACV challenge (Figures 2C and S2C–S2E), showing that lung TRM cells participate in protection.

Finally, we investigated the duration of protection by memory CD8+ T cells elicited after intraperitoneal or IN vaccination. Notably, only intranasally vaccinated and not IP-vaccinated mice were protected when challenged intranasally with VACV at day 50 after boost, suggesting a key role for IN vaccination in establishing long-lasting protective immunity (Figure 2D). These data together established a non-TCR transgenic mouse model to study the basis of immune protection rendered against lethal respiratory infection by endogenous lung CD8+ TRM cells.

Intranasal Vaccination Elicits a Distinct CXCR3LO CD8+ TRM Cell Subset in the Lung Interstitium

We next investigated the immunologic properties of B8R-specific CD8+ T cells elicited by intraperitoneal or IN vaccination. We found that both immunization routes elicited robust systemic and local pulmonary memory CD8+ T cell responses of comparable frequency (Figure 3A). B8R-specific CD8+ T cells elicited by both vaccination routes responded to antigen by producing IFN-γ and mobilizing CD107a to the cell surface (Figures S3A and S3B). Thus, B8R-specific CD8+ T cells in the lungs participate in enhanced protection to respiratory infection.

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suggesting that local versus systemic vaccination results in differential localization of memory CD8⁺ T cells within the lungs. By staining with αCD45 Ab in vivo and with αCD8 Ab and Tet ex vivo, the distribution of airway (AW)-resident CD8⁺ T cells in bronchoalveolar lavage (BAL) and parenchymal CD8⁺ T cells in two anatomically distinct compartments of the lungs (the vasculature [“marginated” vascular (MV)] and interstitium [interstitial (IST)]) were assessed in response to the two immunization routes. We found that the Tet⁺ αCD45 Ab⁻ CD8⁺ T cells in the BAL and lung parenchyma were attributed to AW and IST TrM cells, respectively (Figure 3B), as previously suggested by others (Anderson et al., 2014; Barletta et al., 2012).

Most B8R-specific CD8⁺ T cells (>95%), after intraperitoneal immunization but prior to challenge were Tet⁺ αCD45 Ab⁺ representing the MV memory pool confined to the lung vasculature (Figures 3B and 3C). In contrast, after IN immunization, B8R-spe-

were confined to lung parenchyma and were not blood or LN contaminants. Tet⁺ αCD45 Ab⁻ TrM cells recovered from the BAL uniformly expressed CXCR3HI, a marker for AW TrM cells (Kohlmeier et al., 2009; Slütter et al., 2013). In contrast, Tet⁺ αCD45 Ab⁻ TrM cells retained in the lung parenchyma after perfusion were CXCR3LO, suggesting no cross-contamination between AW and IST TrM cells (Figure S4C).

Resting IST TrM cells were composed of CD103HI and CD103LO/NEG subsets that expressed higher levels of CD69 and CD27 than MV CD8⁺ T cells and were KLRG1LO, CD62LLO, CCRL2LO, CD122LO, and CD127HI, a phenotype consistent with the current definition of a TrM cell (Figure 3D). Unlike CXCR3HI AW TrM cells, IST TrM cells were CXCR3LO, thereby discriminating between the two TrM cell subsets in the lungs (Figures 3D and 3C). Resting and activated IST TrM cells were responsive to re-stimulation with antigenic peptide and stained for GzmB ex vivo (Figure 3D; data not shown). Thus, IN
immunization with a non-replicative immunogen and αGC elicited functionally competent and CXCR3LO memory CD8+ T cells that occupied a distinct anatomical niche in immune lungs and acquired a tissue resident phenotype.

**IST TRM Cells Persist in the Lungs and Respond before Systemic Memory CD8+ T Cells Are Recruited to the Site of Infection**

We next determined the prevalence of B8R-specific CD8+ T cells within the lung parenchyma and BAL after intraperitoneal or IN vaccination and during infection (Figure 4A). Regardless of the vaccination route, similar numbers of MV Tet+ CD8+ T cells were detected in the lungs at day 14 post-boost and until day 4 after lethal IN VACV challenge. Strikingly, at the same time points there were ~15- to 40-fold higher numbers of IST TRM cells in intranasally vaccinated lungs as compared to IP-immunized lungs (Figure 4A). Moreover, IN vaccination established ~3 × 10^6 IST TRM cells per lung, which accounted for ~98% of total lung CD8+ TRM cells during early infection. This IST TRM cell number is likely an underestimate, owing to dramatic cell losses (>98%) incurred during tissue processing (Steinert et al., 2015). IN immunization also induced ~500 AW CD8+ TRM cells per lung, which were undetectable in IP-immunized mouse lungs (Figure 4A). This small population of AW TRM cells was sustained in the airways without substantial change in number until day 6 p.i. The dramatically higher number of pre-existing IST TRM cells present during early infection was associated with the rapid reduction of viral load in intranasally vaccinated mice. Conversely, the delayed accumulation of IP-induced memory CD8+ T cells in the lung tissue was consistent with the increased VACV burden in these mice (Figure 1F).

Efficient CD8+ T cell-mediated immune surveillance in infected tissues requires a high density of responders (Halle et al., 2016), with an estimate of ~3,000 TRM cells per million nucleated cells in the lungs (Steinert et al., 2015). Hence, we hypothesized that the highly abundant IST TRM cells, in comparison to ~500 AW TRM cells, participated in the rapid control of the infection established with ~10^5 PFU VACV that spreads through ~2 × 10^6 alveoli. To test this hypothesis, we attempted adoptive transfer or selective local depletion of AW or IST TRM cells before VACV challenge. We could not repopulate the lungs with a physiological density of endogenous epitope-specific IST TRM cells because of poor yields (Steinert et al., 2015) and poor homing efficiency (data not shown). IN application of αCD8 Ab depleted AW TRM cells as expected (Slüter et al., 2013), but significantly reduced IST TRM as well (Figures S2C–S2E). As an alternative approach, we repopulated the airways of IP-vaccinated mice with ~770 BBR-specific AW TRM cells per lung, which approximates two times their physiological number, harvested from intranasally vaccinated and VACV-challenged mice. The transferred AW TRM cells neither egressed into the lung parenchyma nor provided resistance to VACV infection (Figure S5). Together these data suggest that IST TRM cells elicited by IN, but not intraperitoneal, vaccination conferred early virus control and enhanced protection.

To determine why the pre-existing systemic memory CD8+ T cells accumulated slowly in the infected lungs, we assessed their proliferation kinetics. For this, IP-vaccinated mice were intranasally challenged with VACV and then pulsed with EdU (5-ethyl-2’-deoxyuridine) for 1 day, BBR-specific AW and IST TRM cells showed modest EdU incorporation by day 4 p.i. and profound incorporation by day 6 p.i. By contrast, proliferation of BBR-specific blood, splenic, and lung MV CD8+ T cells was obvious only by day 6 p.i. (Figure 4B). Although it is likely that memory CD8+ T cells were proliferating in the draining LN at this time point (Moyron-Quiroz et al., 2006), proliferating cells were not detected in the blood and spleen until day 6 p.i. This result suggested that systemic CD8+ T cells had not yet left the LN to participate in viral clearance. The increased total number of BBR-specific IST TRM cells in the lungs observed on day 4 p.i. was inversely related to ~3- to 8-fold lower BBR-specific CD8+ T cells in blood, spleen, and lung vasculature (Figure 4C). Thus, the delayed recruitment of systemic resting memory CD8+ T cells to the lungs accounted for late accumulation of IST and AW TRM cells during infection, which then extensively proliferated in situ.

By virtue of their location, IST TRM cells can quickly engage in contact-dependent killing of infected lung tissue to confer enhanced immune protection, whereas MV CD8+ T cells are spatially isolated and cannot do so. To assess early activation of CD8+ T cell subsets in the lungs, intranasally immunized mice were challenged with VACV, and BBR epitope-specific MV CD8+ T cells (mostly CD69loCD44lo) and IST TRM (mostly CD69hi) were assessed for CD69 induction within 36 hr p.i. Remarkably, only IST TRM cells and not MV CD8+ T cells upregulated CD69 expression (Figure 4D), suggesting that IST TRM cells are key initial responders to infection.

**Interstitial TRM Localize to Sites of the Lungs that Are Vulnerable to Infection**

We previously showed that mortality in naive VACV-challenged mice was due to severe damage of alveoli characterized by widespread necrosis and fibrin-filled and edematous alveolar spaces (Gichuk et al., 2013). Here, we determined the sites of VACV infection following IN inoculation with GFP reporter virus (Norbury et al., 2002) in conjunction with fluorescent labeling of type I alveolar epithelial cells with anti-rodoplanin Ab (Vanderbilt et al., 2008) on day 4 p.i. Confocal microscopy revealed that VACV localized to two spatially distinct niches surrounding the bronchioles and type I epithelial cells that form the alveolar walls (Figure 5A). Accordingly, imaging of αCD45 Ab−CD8+ T cells in acutely infected lungs of vaccinated mice showed localization to the sites that are vulnerable to VACV infection, including the bronchioles and alveolar walls (Figure 5B).

Imaging of endogenous antigen-specific CD8+ T cells in the lungs was reported previously (Khanna et al., 2008; Moyron-Quiroz et al., 2006), but whether CD8+ TRM cells occupy niches in the lung parenchyma where fatal infection occurs remains unknown. The pulmonary interstitium is the support tissue within the lungs; it includes the alveolar epithelium, capillary bed endothelium, basement membrane, and the perivascular and perilymphatic tissues. Collectively, they constitute the walls of the bronchioles and alveoli. To determine the location of VACV-specific IST TRM cells, we stained thick fresh lung sections with fluorescent BBR tetramer (red) after IV labeling of vascular leucocytes with αCD45 Ab (blue) and vascular endothelium of alveolar capillary beds with fluorescent tomato lectin (green). Using selective
Figure 4. IST TRM Cells Persist in the Lungs and Respond before Systemic Memory CD8+ T Cells Accumulate to the Site of Infection

(A) Counts of B8R-specific MV, IST, and AW CD8+ T cells that were determined after intraperitoneal or IN vaccination (day 0) and at the indicated time points after IN challenge with VACV (n = 3–12 mice/group, mean ± SEM).

(B) Proliferation kinetics of splenic, blood, and lung B8R-specific CD8+ T cells of IP-vaccinated mice was assessed by EdU incorporation at indicated time points p.i. Representative of three or four mice per group per time point.

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plane illumination microscopy (SPIM), we achieved volumetric imaging that allowed visualization of VACV-specific CD8+ T cells in the alveolar interstitium (IST T RM, red) and alveolar capillary beds (MV CD8+ T cells, magenta from the merge of red and blue) (see Figure 5C). We found that IN vaccination induced a substantial number of IST T RM cells that localized within the alveolar walls, outside of alveolar capillaries (Figure 5C). Such a location of IST T RM cells was even more prominent in acutely infected lungs of intranasally vaccinated mice, showing that infected alveoli are common sites of immune surveillance by IST T RM cells that are located at the most vulnerable sites within the lung parenchyma.

DISCUSSION

At the internal barriers, the mucosal immune response confers the first line of defense against invading pathogens. Here, we report a distinct protective CD8+ T RM cell that is elicited by IN protein antigen plus adjuvant vaccination and localized to the lungs interstitium, an anatomical site intimately connected to the respiratory mucosa. Sublethal respiratory infections induce bronchus-associated lymphoid tissue that serves as a site for naïve CD8+ T cell priming and a niche for T RM cell maintenance (Halle et al., 2009). Accordingly, IN infection with live influenza virus elicited a robust T RM cell response that localized mostly around the large airways and blood vessels of the lungs (Wu et al., 2014). Other studies have suggested a protective role for CXCR3+ memory CD8+ T cells also induced by viral infection but located within the airway lumen (McMaster et al., 2015; Slütter et al., 2013). Our findings extend the types of protective T RM cells that patrol the lungs. Thus, in our IN protein antigen plus αGC adjuvant vaccination model, we identified a major CXCR3+ IST T RM cell subset that localizes to the most vulnerable sites of infection—the alveolar and bronchiolar walls. Efficient CD8+ T cell-mediated tissue surveillance depends on high responder density (Halle et al., 2016; Steinert et al., 2015). Accordingly, upon IN vaccination, IST T RM cells were elicited at physiologically relevant numbers and accounted for most of the lung T RM cells, suggesting these T RM cells are key initial responders to infection. Nonetheless, at the late stages of infection, we do not discount the possibility that IST T RM cells may replenish AW T RM cells, which together with systemic effector memory CD8+ T cells may substantially contribute to the resolution of infection.

The mechanism by which CXCR3+ IST T RM cells are recruited and preferentially retained in the lung interstitium upon vaccination remains unclear. In this regard, our preliminary transcriptome analysis of naïve lung CD8+ T cells and purified IST T RM cells revealed many differentially expressed genes that could be associated with trafficking and/or residence in non-lymphoid tissues, including CCR7, Itgae, Cxcr6, Cxcr3, Klrk1, Klfl2, and S1pr1 (P.G. and S.J., unpublished data). The role of these genes in IST T RM cell recruitment, retention, and function awaits further study.

Devising vaccination strategies to elicit T RM cells in barrier tissues by immunization through an accessible tissue, such as skin, is an important goal for human vaccine design (Park and Kupper, 2015). In this regard, it is noteworthy that vaccination with VACV or replication-deficient modified vaccinia virus Ankara by skin scarification elicited memory CD8+ T cells that protected mice from lethal respiratory infection, although inactivated viruses failed to do so (Liu et al., 2010). Local immunization with CD8+ T cell-targeted epitope plus adjuvant could be a promising alternative to distal vaccination approach that uses virus vectors. Future studies will determine if vaccination by skin scarification with subunit vaccine formulation have the potential to elicit protective T RM cells that also localize to distal barrier tissues. We hope our findings will have implications for the design of safe and effective CD8+ T cell-targeted vaccines against respiratory infections.

EXPERIMENTAL PROCEDURES

Mice, Viruses, and Antigens

B6-K1D2;B10.07,02tg (B7th) transgenic mice have been described elsewhere (Alexander et al., 2003). Mouse breeding, maintenance, and experimentation complied with institutional animal care and use committee (IACUC) regulations.

The Western Reserve strain of VACV and VACV-GFP (a gift from J.W. Yewdell) were grown in and titrated with BSC-40 cells. The design of recombinant L4R-derived antigen has been described elsewhere (Gilchuk et al., 2013). L4R-b8r DNA was generated by substituting the sequence that encodes FPRSMLSIF (L4R37–45) in L4R33–249 DNA with a sequence that encodes FPNKDFVS(F8R70–76). Recombinant antigens were purified to high purity as described previously (Gilchuk et al., 2013).

Vaccination, Infections, and Burden

Ketamine-xylazine-anesthetized, 6- to 8-week-old mice were inoculated intranasally or intraperitoneally twice with 50 μg antigenic protein formulated with 1 μg αGC (Funakoshi) in 100 μl PBS. To challenge, anesthetized mice were inoculated with 105 PFU VACV in 50 μl PBS. Mice were monitored daily for morbidity, and those losing over 30% of initial body weight were euthanized per IACUC regulations.

Lungs from individual mice were washed with PBS, homogenized, and subjected to one freeze/thaw cycle, and serial 10-fold dilutions were plated on confluent BSC-40 cells. Plaques were visualized by crystal violet staining.

IV Staining and Flow Cytometry

IV staining was performed with fluorescently labeled αCD45 Ab as described previously (Anderson et al., 2014) but modified to precisely discriminate MV, AW, and IST CD8+ T cells. After IV staining, lungs were perfused, which removes cells from the vasculature and airway lumen while retaining MV and IST CD8+ T cells within the lung parenchyma. To account for BAL CD8+ T cells, the backflow from the airways, typically generated upon perfusion, was quantitatively recovered as detailed in the Supplemental Experimental Procedures.

(C) Number of Tet+ CD8+ T cells in spleen, blood, and lungs of mice vaccinated and challenged as in (B) was assessed at the indicated times (n = 3-4 mice/group, mean ± SEM).

(D) Intranasally vaccinated mice were challenged with VACV or mock-inoculated with PBS (n = 4–5 mice/group). 36 hr p.i., Tet+ MV CD8+ T cells and IST T RM cells were assessed for CD69 induction from pooled lungs in each group. See also Figure S3.
A. Bronchiolar infection

I. Bronchiolar infection

II. Alveolar infection

VACV-GFP Type I epithelium

B. Bronchiolar CD8 T cells

Alveolar CD8 T cells

Vascular endothelium CD45 (IV) CD8 Colocalization

C. IN vaccination (uninfected lung)

IN vaccination + VACV (d7 p.i.)

Vascular endothelium CD45 (IV) B8R-tetramer Colocalization

(legend on next page)
In Vivo CD8\(^+\) T Cell Proliferation

Mice were injected with 1 mg EdU (Life Technologies) IP 24 hr before analysis. After IV staining, lung cells were labeled with viability dye to discriminate dead cells, followed by staining with tetramer and Abs. Cells were stained with Click-IT Plus EdU Alexa488 (Life Technologies) according to the manufacturer’s protocol.

Confocal Microscopy and SPIM

For imaging of VACV-GFP, 10 µm fresh-frozen lung sections were stained with sCD8R-Alexa Fluor 488-conjugated Ab and mouse rodoplin Ab followed by secondary Alexa Fluor 647-conjugated Ab. For imaging of CD8\(^+\) T cells, lungs were harvested after IV staining with sCD45-BV421 Ab and fixed with 4% paraformaldehyde (PFA). Frozen 10 µm sections were stained with sCD8\(^+\)-PE Ab and AF488-labeled tomato lectin (Vector Laboratories). Confocal microscopy was performed using an AxioObserverZ.1 inverted microscope (Zeiss) equipped with a 20 x 1.0 numerical aperture (NA) objective; 405-nm, 488-nm, and 561-nm laser lines; and dual sCMOS cameras. Image analysis was performed with Imaris software (Bitplane Scientific). For imaging of BRR-specific CD8\(^+\) T cells in the lungs, mice were intravenously (i.v.) injected with AF488-labeled tomato lectin and sCD45-BV421 Ab, which visualizes vasculature and vascular leukocytes, respectively. Fresh, 1-mm-thick sections were cut with tissue slicer followed by overnight incubation with BRR tetramer-PE. Washed tissues were mounted in 1% agarose and analyzed using the LightsheetZ.1 platform (Zeiss) equipped with a CU-22 spinning disc (Yokogawa), 473-nm and 660-nm laser lines, a 20 – 0.75 NA Plan-Apo objective, and an Evolve EMCCD camera (Photometrics). Images were analyzed using Slidebook software (Intelligent Imaging Innovations).

Statistics

Descriptive statistics (mean ± SEM or mean ± SD) are provided for continuous variables as noted. The Wilcoxon rank-sum test or two-sample t test was applied to two-group comparisons or the post hoc group comparisons in ANOVA; all tests were two tailed and unpaired (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant). Statistical analyses were performed using the open source R software (version 2.15).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.037.

AUTHOR CONTRIBUTIONS


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bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. J. Exp. Med. 206, 2593–2601.
Supplemental Information

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Figure S1 – Related to Figure 1
Figure S2 – Related to Figure 2

**A**

Boost with antigen+αGC, IP or IN

IN αGC to IP group

Challenge with VACV, IN

- L4R-b8r/αGC (IN) + VACV
- L4R-b8r/αGC (IP) + αGC (IN) + VACV

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**B**

d6 p.i

![Graph showing VACV pfu per lungs](image)

**C**

Pulmonary-vaccinated with antigen

αCD8 Abs

IV staining, assess lung CD8+ T cells

- d-24
- d-1
- d0

**D**

PBS
αCD8a Ab

- Lung parenchyma
- BAL

**E**

MV
IST
AW

- IN L4R-b8r/αGC+PBS
- IN L4R-b8r/αGC+αCD8a Ab

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Figure S3 – Related to Figure 3

A
Prime with antigen/αGC, IP or IN

Boost with antigen/αGC, IP or IN

Assess splenic and lungs CD8⁺ T cells

Challenge with VACV, IN

Assess splenic and lungs CD8⁺ T cell response

d-28  d-14  d-8 or d0  d0  d7

B
Post vaccination (d0)

Tetramer staining

Spleen CD8⁺ T cells

Lung CD8⁺ T cells

ICCS

Responding (CD107α⁺IFN-γ⁺) of Tet⁺ CD8⁺ T cells

CD8

CD107α

Tetramer

IFN-γ

% of responding Tet⁺ CD8⁺ T cells

IN vaccinated

IP vaccinated

Spleen

Lung

C
After VACV challenge (d7 p.i.)

Spleen

Lung

% BBR-Tet⁺ CD8⁺ T cells

Vaccination route

IP

IN

D
After VACV challenge (d7 p.i.)

Spleen

Lung

% CD107α⁺IFN-γ⁺ of BBR-Tet⁺ CD8⁺ T cells

Vaccination route

IP

IN
Figure S4 – Related to Figure 3

A

CD8⁺ T cells

B

% BBR-Tet⁺ CD8⁺ T cells

C

Lung CD8⁺ T cells

B8R-tetramer

Lung parenchyma

CXCR3

Cells, mode
Figure S5 – Related to Figure 4

A

B

C

D

E
Figure S1. Adjuvant plus protein antigen prime and boost elicited memory CD8+ T cells are reactive only to the homologous epitope of protein that was used for immunization –Related to Figure 1
Mice were primed and boosted with L4R or L4R-bbr protein and adjuvant by intranasal route. Lungs were harvested on day 6 after the boost. Epitope-specific CD8+ T cells for each immunized group were assessed in same staining reaction with two, homologous or heterologous, tetramers which are labeled with PE or APC as indicated. Data represent one of two or three mice for each group. Plots are gated on live CD8+ T cells.

Figure S2. CD8+ T cells induced by IN vaccination participate in protection –Related to Figure 2
(A—B). IN αGC treatment of IP vaccinated mice did not protect mice from lethal VACV challenge on d24 after the treatment. Mice were immunized IN or IP with L4r-bbr plus αGC. On d50 after boost, IP vaccinated mice were administered αGC IN. On d24 after IN αGC administration, i.e., d74 after boost, both groups were challenged with VACV and monitored for protection by assessing weight loss kinetics (A) and virus titers in the lungs on d6 after challenge (B). One of two independent experiments are shown, n=3—5 mice per group; mean±SEM. Number indicates % survival based on endpoint criteria.
(C—E) IN vaccinated mice are more susceptible to VACV after local depletion of CD8+ T cells.
(D) Representative flow plots show gates for CD8+ T cells of mice that were treated with PBS or αCD8 Ab as in (C).
(E) Quantification of CD8+ T cell subsets from mice treated as in (C). (n=3 per group); mean±SEM. AW: airway, MV: marginated vascular, IST: interstitial resident CD8+ T cells.

Figure S3. Frequency and functional competence of epitope-specific CD8+ T cells that generated via IN and IP vaccinations – Related to Figure 3
(A) Schema of immunization and CD8+ T cell analysis.
(B) Responsiveness of B8R-specific CD8+ T cells that generated by IN or IP vaccination to peptide re-stimulation in vitro. Spleens or lungs were harvested on d6 or d14 after boost with L4r-bbr and αGC and split into two separate reactions, which included tetramer staining, or re-stimulation with B8R peptide followed by intracellular IFN-γ and CD107a staining (ICCS). Representative flow cytometry plots are shown for spleen and lungs of IN vaccinated mice (left panel) and percent of responding antigen-specific CD8+ T cells CD107a+IFN-γ+ cells (right panel) that calculated as fraction of Tet+ CD8+ T cells. One of two independent experiments with pooled spleens or lungs (n=3). Mean ±SD of assay triplicates.
(C). Frequency of B8R-specific CD8+ T cells identified by tetramer staining in lungs and spleen of IP or IN vaccinated mice on d7 after IN VACV challenge.
(F) Frequency of IFN-γ CD107αγ B8R epitope-specific CD8+ T cells from (C) that calculated as in (B). Each symbol indicates an individual mouse, n=6—10 mice/group; mean±SEM.

Figure S4. Feasibility of the approach to discriminate different CD8+ T cell subsets in the lungs –Related to Figure 3
Mice (n=4—5) were immunized IN with L4r-bbr plus αGC. Lungs parenchyma, BAL, blood, and mediastinal lymph nodes were harvested for the analysis on d8-14 after the boost. IV staining with CD45 Ab followed by tetramer staining were used to discriminate TRM. Representative flow cytometry plots (A) and frequency (B) showing feasibility of the approach to identify different immune CD8+ T cells in the lungs. Each connecting line on (B) represents individual mouse.
(C) Representative flow cytometry plots showing discrimination of IST and AW TRM by CXCR3 staining.

Figure S5. Low number of AW TRM could not account for the rapid virus control in infected lungs –Related to Figure 4
(A) Experimental design. Fourteen mice were primed and boosted IN with L4R-bbr protein and αGC and then inoculated IN with VACV. On day 45 p.i. BAL CD8+ T cells were purified by magnetic sorting, counted and adoptively transferred by intra-tracheal route to non-immune recipient mice (n=9) in 100 µl of sterile PBS. Each mouse received ~17,000 of alive B8R-specific AW TRM. Other group (n=6) received ~30,000 of purified naïve splenic CD8+ T cells. Third group (n=9) that were primed and boosted IN with L4R-bbr protein and αGC served as positive control for protection. Twenty-four hours after transfer mice were challenged IN with VACV.
(B) Virus titers assessed 24 hours p.i in the lungs of individual mice from (A).
(C) Aliquot of magnetically purified recipients BAL CD8+ T cells that were used for adoptive transfer. Gating strategy to identify count viable B8R-spetope specific AW TRM is shown.
(D) Repopulation efficiency of the airways after adoptive intra-tracheal transfer of airway resident memory CD8+ T cells. BAL and lungs parenchyma of three donor mice were assessed for B8R-specific AW TRM 24 hours after adoptive transfer. The efficiency of the
airway repopulation/recovery of adoptively transferred AW Trm is ~5% (mean 770 cells per mouse lungs) which is approximately two-fold excess of their physiological number upon protein antigen and adjuvant vaccination (mean 495 cells per mouse lungs).
Supplemental Experimental Procedures

IV staining and flow cytometry
Mice were injected i.v. with 2μg fluorescently labeled αCD45 Ab and after 5 min, lungs were slowly perfused with 10ml PBS to ensure blood removal before lungs inflation. AW CD8⁺ T cells was quantitatively collected with the backflow (3—5ml) from the airways typically generated after lung inflation. Of note, recent study suggested avoiding lung perfusion to preserve airway resident populations (Anderson et al., 2014) although for our study lung perfusion was required to minimize contamination of IST TrM with αCD45- AW TrM for phenotyping analysis. Collagenase-digested lung was used without further purification for CD8⁺ T cells assays and counting. For direct ex vivo intracellular staining, lungs were processed in presence of 10μg/ml brefeldin A (Sigma). For in vitro re-stimulation assays, cells were incubated with 10μg/ml of antigenic peptide and αCD107a Ab as described (Gilchuk et al., 2013). CD8⁺ T cells counting was performed with counting beads as described (Gilchuk et al., 2013). Preparation of fluorescent tetramers and CD8⁺ T cells staining were described previously (Gilchuk et al., 2013). Dead cells were discriminated using viability dye Flow cytometric data were collected using an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

CD8 depletion
Local depletion of CD8⁺ T cells in lung was performed as described previously (Slutter et al., 2013). Briefly, 5 μg of anti-CD8α antibody (clone 2.43) was administrated in 50 μl of sterile PBS by intranasal route to anesthetized mice, and after 24h CD8⁺ T cells were assessed in different lung compartments with tetramer. Mock (PBS)-treated mice served as a baseline control for CD8⁺ T cells counting, and for VACV challenge experiments.

Intra-tracheal transfer of AW TrM
Isolation and adoptive transfer of AW TrM were performed as described previously (McMaster et al., 2015). Briefly, mice were primed and boosted IN with L4R-b8r protein and αGC and then inoculated IN with VACV. On day 45 p.i. BAL CD8⁺ T cells were purified using a Miltenyi Biotec CD8α T cell isolation kit II, counted and adoptively transferred by intra-tracheal route to non-immune recipient mice in 100 μl of sterile PBS.
Flow cytometry and microscopy reagents

Tetramers
B8R<sub>70-79</sub> (FPKNDFVSF)/HLA-B7.2 PE
B8R<sub>70-79</sub> (FPKNDFVSF)/HLA-B7.2 APC
B8R<sub>70-79</sub> (FPKNDFVSF)/HLA-B7.2 BV421
NP<sub>366-374</sub> (ASKENMETM)/H2D<sup>β</sup> PE
L4R<sub>17-14</sub> (FPRSMSLIF)/B7.2 PE

Antibodies
Anti-CD8α-APC-Cy7 (clone 53-6.7; BD Biosciences, San Jose, CA)
Anti-IL-2-PE (clone JES6-5H4; BD Biosciences)
Anti-CD44-APC (clone IM7; BD Biosciences)
Anti-CD45-BV421 (clone 30-F11; BD Biosciences)
Anti-CD8α-PerCP-Cy5.5 (clone 53-6.7; BD Biosciences)
Anti-CD3ε-PE (clone 145-2C11; BD Biosciences)
Anti-CD25-PE (clone BC96; BD Biosciences)
Anti-CD4-APC (clone RM4-4; BD Biosciences)
Anti-CD62L-APC (clone MEL-14; BD Biosciences)
Anti-CD4-APC-Cy7 (clone MEL-14; BD Biosciences)
Anti-CD8α-APC (clone 53-6.7; BD Biosciences)
Anti-CD279-PE (clone J43; BD Biosciences)
Anti-CD69-PerCP-Cy5.5 (clone H1.2F3; BD Biosciences)
Anti-CD103-PE (clone 2E7; BioLegend)
Anti-CD122-PE (clone TM-β1; BioLegend, San Diego, CA)
Anti-CD103-PE (clone 2E7; BioLegend)
Anti-CD49b-PE (clone DX5; BioLegend)
Anti-CD43-PE (clone 1B11; BioLegend)
Anti-CD49b-PE (clone DX5; BioLegend)
Anti-CD27-PE (clone LG.3A10; BioLegend)
Anti-CD47-PE (clone Miap301; BioLegend)
Anti-CD184 (CXCR4)-PE (clone L276F12; BioLegend)
Anti-CD107a-APC (clone 1D4B; BD Biosciences)
Anti-CD11c-FITC (clone N418; Tonbo Biosciences, San Diego, CA)
Anti-CD107a-FITC (clone 1D4B; BD Biosciences)
Anti-CD107a-FITC (clone 1D4B; BD Biosciences)
Anti-CD11a-FITC (clone 2D7; BD Biosciences)
Anti-KLRG1-APC (clone 2F1; eBioscience, San Diego, CA)
Anti-CD183 (CXCR3)-APC (clone CXCR3-173; BD Biosciences)
Anti-CD279 (PD-1)-PE (clone J43; BD Biosciences)
Anti-CD69-PerCP-Cy5.5 (clone H1.2F3; BD Biosciences)
Anti-CD45.2-PE (clone 104; BD Biosciences)
Anti-IFN-γ-APC (clone XMG1.2; BD Biosciences)
Anti-IFN-γ-PE (clone XMG1.2; BD Biosciences)
Anti-IFN-γ-APC (clone XMG1.2; BD Biosciences)
Anti-IFN-γ-PE (clone XMG1.2; BD Biosciences)
Anti-CD112-PE (clone TM-β1; BioLegend, San Diego, CA)
Anti-CD103-PE (clone 2E7; BioLegend)
Anti-CD49b-PE (clone DX5; BioLegend)
Anti-CD43-PE (clone 1B11; BioLegend)
Anti-CD49b-PE (clone DX5; BioLegend)
Anti-CD27-PE (clone LG.3A10; BioLegend)
Anti-CD47-PE (clone Miap301; BioLegend)
Anti-CD184 (CXCR4)-PE (clone L276F12; BioLegend)
Anti-CD49a-PE (clone Ha31/8; BioLegend)
Anti-CTLA-4-PE (clone UC10-4F10-11; Tonbo Biosciences)
Anti-TNF-PE (clone MP6-XT22; BD Biosciences)
Anti-CD4-FITC (clone RM4-4; BD Biosciences)
Anti-Granzyme B-PE (clone GB11; Life Technologies, Grand Island, NY)
Anti-Podoplanin unconjugated (clone 8.1.1; BioLegend)
Anti-GFP-Alexa Fluor 488 (rabbit polyclonal; Life Technologies)
Anti-Syrian Hamster IgG Alexa Fluor 647-conjugated (goat polyclonal: Jackson ImmunoResearch, West Grove, PA)

Viability dyes
Propidium iodide (BD Biosciences)
eFluor450 amino-reactive viability dye (eBioscience)
Other reagents
Brilliant Violet 421 streptavidin conjugate (BioLegend)
R-phycoerythrin (PE) streptavidin conjugate (Life Technologies)
Allophycocyanin (APC) streptavidin conjugate (Life Technologies)
DyLight 488 lycopersicon Esculentum (tomato) lectin (Vector Laboratories, Burlingame, CA)
**Supplemental References**


