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Tissue-Resident Memory T Cells in the Lungs Protect against Acute Respiratory Syncytial Virus Infection

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ABSTRACT
Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in young children. The T cell response plays a critical role in facilitating clearance of an acute RSV infection, and memory T cell responses are vital for protection against secondary RSV exposures. Tissue-resident memory (TRM) T cells have been identified as a subset of memory T cells that reside in nonlymphoid tissues and are critical for providing long-term immunity. There is currently limited information regarding the establishment and longevity of TRM T cell responses elicited following an acute RSV infection as well as their role in protection against repeated RSV infections. In this study, we examined the magnitude, phenotype, and protective capacity of TRM CD4 and CD8 T cells in the lungs of BALB/c mice following an acute RSV infection. TRM CD4 and CD8 T cells were established within the lungs and waned by 149 d following RSV infection. To determine the protective capacity of TRMs, FTY720 administration was used to prevent trafficking of peripheral memory T cells into the lungs prior to challenge of RSV-immune mice, with a recombinant influenza virus expressing either an RSV-derived CD4 or CD8 T cell epitope. We observed enhanced viral clearance in RSV-immune mice, suggesting that TRM CD8 T cells can contribute to protection against a secondary RSV infection. Given the protective capacity of TRMs, future RSV vaccine candidates should focus on the generation of these cell populations within the lung to induce effective immunity against RSV infection. ImmunoHorizons, 2021, 5: 59–69.

INTRODUCTION
Respiratory syncytial virus (RSV) is a common cause of lower respiratory tract infections in children worldwide, with an estimated 33.1 million cases per year leading to 3.2 million hospital admissions and 59,600 deaths (1). By 2 y of age, nearly all children have been infected with RSV at least once (2). Despite the high morbidity and mortality associated with RSV infection, there is currently no licensed RSV vaccine. In the 1960s, a formalin-inactivated RSV vaccine (FI-RSV) was produced, but a majority of patients vaccinated still experienced a natural RSV infection. Of those infected, 80% required hospitalization, and two patients died secondary to their pulmonary disease, with increased pulmonary eosinophils noted on autopsy (3). Further investigation has demonstrated that the FI-RSV failed to generate neutralizing Abs as well as RSV-specific memory CD8 T cells, the combination...
of which allowed for the development of a robust mixed Th1 and Th2 CD4 T cell memory response in the lung that resulted in mucus overproduction and airway hypersensitivity leading to vaccine-enhanced disease (4–7). The lack of CD8 T cell production is particularly noteworthy, as they have been shown to ameliorate the Th2 response and vaccine-enhanced disease (7, 8).

The T cell response to RSV infection plays a critical role in mediating both viral clearance and disease severity. After Ab depletion of either CD4 or CD8 T cells, mice infected with RSV demonstrated prolonged detectable viral titers and decreased manifestations of disease (9). A Th2-skewed CD4 T cell response has been implicated as particularly critical in mediating RSV-induced disease following acute infection of infants. Nasal washes from infants with RSV-confirmed lower respiratory tract infections exhibited increased IL-4/IFN-γ ratios compared with infants with upper respiratory tract infections (10). The role of CD8 T cells in protection and immunopathology has been demonstrated in irradiated mice, who were able to clear RSV following transfer of CD8 T cells; however, they also experienced increased weight loss and pulmonary disease (11). The importance of T cells for viral clearance has also been shown in humans, as children with immunodeficiencies that result in impaired T cell function exhibit prolonged viral shedding (12).

Memory CD4 T cells have been implicated in immunopathology following RSV infection. Previous work in our laboratory has demonstrated that the memory T cell effector response that develops following RSV challenge of FI-RSV–immunized mice consists of both Th1 and Th2 CD4 T cells. Th1 memory CD4 T cells mediate the induction of airway obstruction and weight loss, and Th2 memory CD4 T cells induce increased mucus production and airway hyperresponsiveness (5). Furthermore, when CD4 T cells were depleted in mice previously immunized with FI-RSV, there was a significant decrease in airway obstruction, weight loss, and airway resistance following RSV challenge (5). Memory CD4 T cells are also crucial in mediating viral clearance. In mice infected with RSV, adoptive transfer of in vitro–stimulated, RSV-specific memory CD4 T cells led to reduced RSV titers. At the same time, transfer of these cells also led to enhanced weight loss and pulmonary disease. The effects of transferred CD4 T cells were specific to RSV-infected mice and were not seen in mice infected with influenza or naive mice (13). Memory CD8 T cells have been demonstrated to provide protection against RSV. Mice infected with murine CMV expressing the RSV matrix protein developed effector memory CD8 T cells that mediated enhanced viral clearance following RSV challenge (14). Additionally, work in our laboratory demonstrated that eliciting a high magnitude of RSV-specific memory CD8 T cells via a dendritic cell–Listeria monocytogenes prime-boost immunization regimen caused enhanced viral clearance at the expense of severe and fatal CD8 T cell–mediated immunopathology (15).

Tissue-resident memory (TRM) T cells represent a recently identified subset of memory T cells that are retained in non-lymphoid tissues and contribute to protective immunity (16). TRMs are typically defined by the cell markers CD69 and CD103, with TRM CD4 T cells defined as CD69+CD103− and TRM CD8 T cells defined as CD69+CD103+ (17). CD69 is an early marker of T cell activation and antagonizes sphingosine-1-phosphate receptor function to facilitate lymphocyte retention within tissues (18), and CD103 binds E-cadherin on epithelial cells (19). TRM CD4 and CD8 T cells have been identified in a variety of tissue types, including the murine female genital tract and intestine (20–23). Additionally, TRM CD8 T cells have been demonstrated in the brain and skin of mice (24, 25). Both TRM CD4 and CD8 T cells have been identified in murine lungs following infection with influenza (17). TRM CD4 and CD8 T cells are also generated following influenza A virus (IAV) infection in mice and mediate protection against nonvaccine viral strains (26). In the setting of RSV infection, TRM CD8 T cells are established in the airways of African green monkeys and healthy human adults (27, 28). Adults infected with RSV also demonstrated decreased viral load and disease severity that was associated with increased TRM CD8 T cell populations (28). Similarly, TRM CD4 T cells were demonstrated in the airway following RSV infection in healthy adults without any correlations with disease severity or viral clearance (29). These studies showcase the presence of TRM CD4 and CD8 T cells following RSV infection in various models. However, there is a lack of exhaustive studies examining the kinetics and phenotypes of TRMs in the lungs following acute RSV infection. Furthermore, although there was a correlation with protection by TRM CD8 T cells in adults with RSV, direct experimental evidence of protection against RSV by TRMs is currently lacking.

We sought to establish the population kinetics of TRM CD4 and CD8 T cells following acute RSV infection to a late memory timepoint using a murine model. We demonstrated that both TRM CD4 and CD8 T cells are established in the lungs following acute RSV infection and wane with time by 5 mo following infection. We also delineated the protective capacity of TRM CD4 and CD8 T cells in the lungs using FTY720 administration prior to infection of RSV-immune mice with a recombinant influenza virus expressing RSV-derived CD4 and CD8 T cell epitopes. Our data demonstrate that TRM CD8 T cells in the lung contribute to protection and that protection is not mediated by circulating memory or effector cells. This has important implications in the development of an RSV vaccine. Vaccines capable of generating an RSV-specific memory T cell response, particularly a TRM CD8 T cell response in the lung, may offer a great opportunity for generating effective immunity to RSV.

**Materials and Methods**

**Mice**

Female BALB/c mice between 6 and 8 wk old were purchased from the National Cancer Institute (Frederick, MD). All experimental procedures were approved by the University of Iowa Animal Care and Use Committee under Animal Protocol no. 4101196 and no. 7041999. The experiments were performed under strict accordance to the Office of Laboratory Animal Welfare guidelines and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.
Viruses and infection

The A2 strain of RSV was a gift from Dr. B. Graham (National Institutes of Health, Bethesda, MD). Mice were infected intranasally (i.n.) with SV, wild-type IAV-A/PR8/34 (IAV-PR8) or recombinant IAV strains following sedation with isoflurane. Recombinant IAV expressing CD8 epitope M2s2–90 (IAV-M2s2) and IAV expressing CD4 epitope F51–67 (IAV-F51) were provided by Dr. R. Langlois (University of Minnesota, Minneapolis, MN). The viruses were created using standard reverse genetics as previously described (30). They were rescued and grown in 10-d-old embryonated chicken eggs (Charles River Laboratories). M2s2 and F51 epitopes were inserted into the mRNA at nucleotide position 186 encoding the neuraminidase stalk region, which has been previously demonstrated to be tolerant of such insertions (31).

FTY720 treatment

In the TRM protection studies, mice were treated with 1 mg/kg of FTY720 (Cayman Chemical, Ann Arbor, MI) i.p. once daily. A stock solution was prepared in DMSO and subsequently diluted to the desired concentration in endotoxin-free water. Treatment lasted for 7 d, beginning 3 d prior to recombinant influenza challenge and ending on day 4 postinfection (p.i.).

Flow cytometry analysis and tissue collection

Mice were injected i.v. with 1 µg CD45-APC (clone 30-F11) or CD45-FITC (clone 30-F11) Ab 3 min prior to euthanasia. Cells from the lungs were processed as previously described (32). Lungs were harvested and made into single-cell suspensions as previously described (33, 34). Cells from the lung were stained for extracellular surface molecules with Abs specific to CD90.2 (clone 53–2.1), CD4 (clone GK1.5), CD8 (clone 53–6.7), CD49d (clone R1-2), CD11a (clone M17/4), CD103 (clone 2E7), CD69 (clone H1.2F3), CD62L (MEL-14), CD122 (SH4), and CXCR3 (clone CXCR3-173) for 30 min at 4°C and fixed with fix/lyse solution (eBioscience) for 10 min at room temperature. RSV-specific CD8 T cells were identified using an M2s2 tetramer, which is made in our laboratory. Stained cells were run on an LSRFortessa (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Plaque assays for IAV

Whole lungs were harvested from mice, weighed, and mechanically homogenized, and supernatants were stored at −80°C. For IAV plaque assays, MDCK (American Type Culture Collection) cells in six-well plates were washed three times with PBS. One milliliter of sterile DMEM was added afterward. Plates were infected with 100 µl of serially diluted recombinant IAV–infected lung samples (10-fold dilutions) for 1 h at 37°C and subsequently washed twice with PBS. Wells were overlaid with 2 ml of 1:1 mixture of 2× Eagle’s MEM and 1.6% agarose containing 1 mg/ml TPCK–trypsin. After incubating at 37°C and 5% CO2 for 3 d, the agarose plugs were carefully removed. Monolayers were fixed with 2 ml 70% ethanol for 20 min at room temperature and subsequently stained with 1 ml of 1% crystal violet in methanol for 10 min at room temperature. Plates were washed with water and allowed to dry overnight, and plaques were counted the next morning.

Statistical analysis

Statistical analyses are detailed in each figure legend and were performed using Prism software (GraphPad Software, San Diego, CA). Data were evaluated using unpaired, two-tailed Student t test between two groups with Holm–Sidak posttest analysis to determine if there was a statistical significance of at least α = 0.05.

RESULTS

Kinetics and persistence of RSV-specific CD4 and CD8 T cells following an acute RSV infection

Following an acute RSV infection, most virus-specific CD4 and CD8 effector T cells remain in the lung parenchyma for up to 30 d (35). However, it is not clear how long RSV-specific memory T cells remain in the lungs. We sought to assess the duration of virus-specific memory CD4 and CD8 T cells in the lungs following acute RSV infection. BALB/c mice were infected i.n. with RSV, and lungs were harvested on days 8, 10, 15, 30, 79, and 149 p.i. Importantly, mice were injected with an anti-CD45 Ab i.v. prior to euthanasia to distinguish cells in the lung parenchyma versus the pulmonary vasculature (32, 35, 36). RSV-specific CD4 T cells were identified using the surrogate activation marker approach CD49d+CD11ahi (37, 38) that identifies Ag-experienced CD4 T cells, and RSV-specific CD8 T cells were identified using an M2s2 tetramer. Following acute RSV infection, all CD4 T cell populations peaked within the lung parenchyma at day 8 p.i. and decreased in frequency up to 5 mo following infection (Fig. 1A). However, virus-specific CD49d+CD11a8i CD4 T cells were seen at higher frequencies than bulk CD4 T cells or CD49d−CD11a8o nonactivated CD4 T cells in the lung parenchyma, including at late memory timepoints (Fig. 1A). Additionally, RSV-specific CD4 T cells were significantly increased in frequency and total number in the lung parenchyma compared with the vasculature (Fig. 1B, 1C). Although the total number of RSV-specific CD49d+CD11a8i CD4 T cells declined, the frequency was maintained up to 149 d p.i. (Fig. 1B, 1C).

RSV-specific CD8 T cells were established in the lungs early following acute RSV infection, and their frequency declined steadily over time (Fig. 1D). M2s2 tetramer–positive CD8 T cells were observed in the highest frequencies in the lung parenchyma compared with Ag-experienced CD11a8i, nonactivated CD11a8o, or bulk CD8 T cells (Fig. 1D). The frequency of Ag-experienced CD11a8i CD8 T cells in the lungs was maintained at a higher level into memory as compared with the frequency maintained as identified by the peripheral blood intravascular stain (IV%), similar to what was observed with Ag-experienced CD49d+CD11a8i CD4 T cells (Fig. 1E). Additionally, the number of Ag-experienced CD11a8i CD8 T cells in the lungs was significantly increased compared with the number in the periphery at early timepoints (p < 0.001) but decreased and showed no significant difference by day 30 p.i. (Fig. 1F). M2s2–specific CD8 T cells were found almost exclusively in the lung tissue compared with the vasculature; however, there was a notable decrease in frequency at 149 d p.i. (Fig. 1G, 1H). Thus, we demonstrate that RSV-specific CD4 and
CD8 T cells are primarily found in the lung parenchyma following acute RSV infection, but their numbers gradually wane over time, which may contribute to the continued risk of reinfection with RSV observed in human adults (2, 39, 40).

TRM CD4 and CD8 T cell numbers decline over time
We next sought to evaluate the kinetics of TRM CD4 and CD8 T cells following acute RSV infection. TRM CD4 T cells were classified as CD69⁺CD103⁻, and CD49d⁻CD11a⁺ CD4 T cells in the lung parenchyma after RSV infection. (A) Frequency of CD4, CD49d⁻CD11a⁻, and CD49d⁺CD11a⁺ CD4 T cells in the lung parenchyma (IV⁻) and vasculature (IV⁺). (B) Frequency and (C) number of virus-specific CD49d⁺CD11a⁻ CD4 T cells in the lung parenchyma (IV⁻) and vasculature (IV⁺). (D) Frequency of CD8, CD11a⁻, CD11a⁺, and M282 tetramer- positive CD8 T cells in the lung parenchyma after RSV infection. (E) Frequency and (F) number of IV⁻ and IV⁺ CD11a⁻ CD8 T cells in the lung. (G) Frequency and (H) number of IV⁻ and IV⁺ M282 tetramer⁺ CD8 T cells in the lung. Data are presented as mean ± SEM from two independent experiments that have been combined (n = 3–4 mice per experiment). *p < 0.05, **p < 0.01, ***p < 0.001, performed with Student t test. IV⁻, lung tissue intravascular stain.

FIGURE 1. RSV-specific CD4 and CD8 T cells localize to the lungs.
BALB/c mice were infected with RSV i.n., and lungs were harvested at days 8, 10, 15, 30, 79, and 149 p.i. (A) Frequency of CD4, CD49d⁻CD11a⁻, and CD49d⁺CD11a⁺ CD4 T cells in the lung parenchyma after RSV infection. (B) Frequency and (C) number of virus-specific CD49d⁺CD11a⁻ CD4 T cells in the lung parenchyma (IV⁻) and vasculature (IV⁺). (D) Frequency of CD8, CD11a⁻, CD11a⁺, and M282 tetramer- positive CD8 T cells in the lung parenchyma after RSV infection. (E) Frequency and (F) number of IV⁻ and IV⁺ CD11a⁻ CD8 T cells in the lung. (G) Frequency and (H) number of IV⁻ and IV⁺ M282 tetramer⁺ CD8 T cells in the lung. Data are presented as mean ± SEM from two independent experiments that have been combined (n = 3–4 mice per experiment). *p < 0.05, **p < 0.01, ***p < 0.001, performed with Student t test. IV⁻, lung tissue intravascular stain.

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TRM CD4 and CD8 T cell numbers decline over time
We next sought to evaluate the kinetics of TRM CD4 and CD8 T cells following acute RSV infection. TRM CD4 T cells were classified as CD69⁺CD103⁻, and TRM CD8 T cells were defined as CD69⁺CD103⁻ (Fig. 2A, 2B, respectively). The full gating strategy for TRM CD4 and CD8 T cells is outlined in Supplemental Fig. 1. Following RSV infection, the frequency and total number of TRM CD4 T cells was significantly increased in the pulmonary tissue compared with the vasculature (Fig. 2C, 2D). The frequency of TRM CD4 T cells remained relatively stable through 149 d p.i. (Fig. 2C). However, the total number of TRM CD4 T cells declined over time (Fig. 2D). In comparison with the number of total RSV-specific CD49d⁺CD11a⁻ CD4 T cells in the lung parenchyma, the number of TRM CD4 T cells in the lung tissue was decreased, although the difference was not statistically significant (Fig. 2E). Both cell populations declined with time at a similar rate (Fig. 2E).

As with TRM CD4 T cells, RSV-specific TRM CD8 T cells were significantly increased in both frequency and total number in the lung tissue compared with the vasculature at early timepoints following RSV infection (Fig. 2F, 2G). Unlike TRM CD4 T cells, the frequency and total number of TRM CD8 T cells declined with time and showed no significant difference compared with that in the pulmonary vasculature at late time points (Fig. 2F, 2G). M282⁺ specific TRM CD8 T cells also exhibited significantly lower numbers in the lung tissue compared with all M282 tetramer⁺ CD8
FIGURE 2. CD4 and CD8 T cell numbers wane with time after RSV infection.

BALB/c mice were infected with RSV i.n., and lungs were harvested at days 8, 10, 15, 30, 79, and 149 p.i. Cells were analyzed by flow cytometry and gated on RSV-specific (A) TRM CD4 T cells and (B) TRM CD8 T cells in the lung parenchyma as shown. Representative staining panels are from day 30 p.i. (C) Frequency and (D) number of CD69<sup>-</sup>CD103<sup>-</sup>CD49d<sup>-</sup>CD11ahi CD4 T cells in the pulmonary parenchyma (IV<sup>-</sup>) and vasculature (IV<sup>+</sup>). (E) Number of IV<sup>-</sup>CD69<sup>-</sup>CD103<sup>-</sup>CD49d<sup>-</sup>CD11ahi TRM and total CD49d<sup>-</sup>CD11ahi CD4 T cells in the lung. (F) Frequency and (G) number of IV<sup>-</sup> and IV<sup>+</sup>CD69<sup>-</sup>CD103<sup>-</sup> M282 tetramer<sup>+</sup> CD8 T cells in the lung. (H) Number of CD69<sup>-</sup>CD103<sup>-</sup> M282 tetramer<sup>+</sup> TRM and total M282 tetramer<sup>+</sup> CD8 T cells in the lung. Data are presented as mean ± SEM from two independent experiments that have been combined (n = 3–4 mice per experiment). *p < 0.05, **p < 0.01, ***p < 0.001, performed with Student t test. IV<sup>-</sup>, lung tissue intravascular stain.
FIGURE 3. Expression of phenotypic markers by TRM CD4 T cells.

(A) Expression of CD62L, CXCR3, and CD122 by CD69+CD103+CD49d+CD11ahi CD4 T cells was compared with circulating (IV+) CD49d+CD11ahi CD4 T cells. Representative histograms from day 30 p.i. are shown. (B) Frequency and (C) number of CD62L+ cells in the lung. (D) Frequency and (E) number of CXCR3+ cells in the lung. (F) Frequency and (G) number of CD122+ cells in the lung. Data are represented as mean ± SEM from two independent experiments that have been combined (n = 3–4 mice per experiment). *p < 0.05, **p < 0.01, ***p < 0.001, performed with Student t test.

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FIGURE 4. Expression of phenotypic markers by CD8 T cells.

(A) Expression of CD62L, CXCR3, and CD122 by CD69\(^+\)CD103\(^+\) M282 tetramer\(^+\) CD8 T cells was compared with circulating (IV\(^+\)) M282 tetramer\(^+\) CD8 T cells. Representative histograms from day 30 p.i. are shown. (B) Frequency and (C) number of CD62L\(^+\) cells in the lung. (D) Frequency and (E) number of CXCR3\(^+\) cells in the lung. (F) Frequency and (G) number of CD122\(^+\) cells in the lung. Data are presented as mean ± SEM from two independent experiments that have been combined (n = 3–4 mice per experiment). *p < 0.05, **p < 0.01, ***p < 0.001, performed with Student t test.

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T cells and showed a more rapid decline (Fig. 2H). Therefore, acute RSV infection leads to the generation of RSV-specific TRM CD4 and CD8 T cells in the lung parenchyma. However, these populations appear to be transient and exhibit a more rapid decline as compared with the total number of virus-specific memory T cells identified using surrogate activation markers or tetramers.

We further examined the phenotypes of TRMs generated following RSV infection. CD62L, typically expressed at high levels on central memory cells, was expressed at low levels by both TRM CD4 (Fig. 3A–C) and TRM CD8 (Fig. 4A–C) T cells, which is consistent with previous descriptions of TRMs (16, 41). The frequency of TRM CD4 T cells that expressed either CXCR3 (Fig. 3A, 3D) or CD122 (Fig. 3A, 3F) was low throughout all memory timepoints examined after day 30. However, the number of TRM CD4 T cells expressing either marker was significantly increased compared with circulating (IV+)CD49d+CD11ahi CD4 T cells at early timepoints (Fig. 3E, 3G). CXCR3 expression was significantly increased on TRM CD8 T cells compared with circulating (IV+) M282 tetramer+ CD8 T cells through day 30 (Fig. 4A). However, both the frequency and number of CXCR3+ TRM CD8 T cells decreased with time, correlating with the decline of TRM CD8 T cells in the lung (Fig. 4D, 4E). The frequency of CD122-expressing TRM CD8 T cells and RSV-specific circulating (IV+) CD8 T cells was high on days 8–10 and then declined, remaining stable through day 149 (Fig. 4A, 4F). The total number of TRM CD8 T cells expressing CD122 was significantly higher than circulating CD8 T cells at early timepoints (Fig. 4G). This may represent the necessity of CD122 for TRM CD8 establishment and survival, which has been previously demonstrated in the skin of mice (42). Our data demonstrate that TRM CD4 and CD8 T cells generated after RSV infection have low expression levels of CD62L, which is consistent with general descriptions of TRMs. Expression of CD122 and CXCR3, however, is generally increased at acute timepoints and subsequently decreases over time.

Establishing the protective capacity of RSV-specific TRM CD4 and CD8 T cells

Although previous studies have shown correlations with protection against RSV by TRMs, direct experimental evidence of the protective capacity of RSV-specific TRMs is currently lacking (43). To directly assess the protective capacity of RSV-specific TRMs, BALB/c mice were infected with RSV, and 1 mo later, mice were challenged with IAV-PR8, IAV-F51, or IAV-M282 (Fig. 5A). RSV naive mice were used as negative controls. Beginning 3 d prior to influenza challenge, mice were treated i.p. once daily with FTY720, a sphingosine-1-phosphate receptor 1 agonist, to prevent...
trafficking of lymphocytes from secondary lymph organs into the lungs (14, 44). The effect of FTY720 has been demonstrated in several studies. In mice infected with Bordetella pertussis, treatment with oral FTY720 prevented egress of lymphocytes from lymphoid tissue to the lungs, with only TRMs found in the lungs (41). Similarly, in mice immunized with a recombinant influenza A vaccine expressing Mycobacterium tuberculosis peptides, treatment with FTY720 i.p. led to decreased circulating T cells, but stable Mycobacterium tuberculosis–specific TRM CD4 T cells, in the lungs (45). FTY720 treatment was continued throughout infection with recombinant influenza. Four days after influenza challenge, lungs were harvested, and plaque assays were performed to measure viral titers in the lungs. RSV-immune mice challenged with IAV-PR8 unexpectedly exhibited a significant (p < 0.05) 1-log decrease in influenza viral titers in the lung compared with naive mice, suggesting that RSV-immune mice may exhibit enhanced protection against a subsequent influenza virus infection. Infection with either IAV-F51 or IAV-M282 exhibited significantly (p < 0.05 and p < 0.001, respectively) decreased influenza viral titers in the lung compared with naive mice not exposed to RSV (Fig. 5B–D), with IAV-M282 demonstrating a greater decrease than was observed with the IAV-PR8 control. Thus, these data demonstrate that RSV-specific TRM CD8 T cells are directly protective when stimulated by their respective RSV-derived Ags.

DISCUSSION

T cells play a crucial role in the pathophysiology of acute RSV infection, contributing to both viral clearance and disease burden. TRMs have been demonstrated in multiple tissues, including the lungs following respiratory infections (46). Our data demonstrate that RSV-specific CD4 and CD8 T cells localize to the lungs and remain present at increased frequencies and numbers for at least 149 d p.i. This builds upon previous work in our laboratory demonstrating that RSV-specific T cells are found within the lung parenchyma up to 30 d p.i. (35). Furthermore, our data demonstrate that TRM CD4 and CD8 T cells are established in the lungs following acute RSV infection but that the number of TRMs wanes with time. The development of TRMs following acute RSV infection has been demonstrated previously in African green monkeys. However, these cells were identified in the bronchial alveolar fluid at acute or short-term memory time points (27). Recently, both TRM CD4 and CD8 T cells were identified in the airways of adult humans experimentally infected with RSV (28, 29). Our study is able to expand upon this work to define the presence of TRMs within the pulmonary tissue and delineate the kinetics of TRMs after RSV infection.

We have also shown that RSV-specific TRM CD8 T cells can provide protective immunity. Previous studies have indicated that RSV-specific TRM CD8 T cells are correlated with decreased severity of RSV disease and decreased viral load in healthy adults undergoing an experimental RSV human challenge (28). Kinnear et al. (47) transferred CD8 T cells from the airways of mice exposed to RSV to naive mice, which led to decreased RSV disease severity and viral load. However, only 25% of the transferred CD8 T cells were TRMs. Thus, although TRMs may have contributed to protection, their protective effect could not be separated from that of the transferred effector CD8 T cells or T cells from secondary lymphoid organs. Using FTY720, we were able to limit the immune response to T cells already present in the lung at the time of infection, the majority of which were TRMs. In addition, by using recombinant influenza viruses engineered to express either a CD4 or a CD8 T cell–specific RSV epitope, we were able to independently assess the protective capacity of TRM CD4 and CD8 T cells and demonstrate that TRM CD8 T cells can provide protection when stimulated with their respective RSV-derived Ags.

Alternative mechanisms may also have contributed to the protection observed following infection with the recombinant influenza strains, including nonspecific protection by TRM T cell present in the lung that have the capacity to make antiviral cytokines in response to cytokine stimulation, such as with IL-12 and IL-18 (48–50). In addition, nonspecific protection could be conferred by the innate immune response. Mice infected with the influenza strain X-31 or 4 d prior to RSV challenge demonstrated significantly decreased RSV titers compared with unvaccinated mice; however, this effect was lost if mice were infected with X-31 14 or 28 d before RSV challenge. The protection observed was Ab independent and conferred nonspecifically by TLR3/TLR7-mediated innate immune responses (51). Similarly, immune cells and club cells that survived an initial IAV infection nonspecifically mediated protection against an influenza B virus challenge through alterations in the composition of cellular infiltration and inflammatory cytokine profiles; it is possible cells in the lung following the initial RSV infection in this study were similarly primed to protect against the secondary influenza virus challenge (52). Consistent with results in mice, infection with influenza virus or adenovirus has been documented to provide protection against subsequent febrile respiratory illnesses in humans (53). Thus, it is possible that memory T cells and innate immune responses may have contributed nonspecifically to the TRM-mediated protection against IAV infection observed in this study.

We surprisingly observed that RSV-immune mice exhibited enhanced protection against a wild-type influenza virus infection, resulting in significantly reduced viral titers in the lung. This result made it difficult to determine if RSV-specific TRM CD4 T cells were capable of exhibiting protective immunity because the protection observed was similar between the wild-type influenza virus infection and the recombinant influenza strain expressing an RSV-derives CD4 T cell epitope. Despite this complication, we did observe a nearly 2-log reduction in viral titers in the lungs of RSV-immune mice challenged with a recombinant influenza strain expressing an RSV-derives CD8 T cell epitope, suggesting that RSV-specific TRM CD8 T cells can exhibit protective functions.

Immunity to RSV has been demonstrated to wane with time, with nearly half of children experiencing a second infection in the first 24 mo of life (2). Recent studies have demonstrated that influenza virus–specific TRM CD8 T cells wane over time, which also correlated with a loss of heterosubtypic protection (54). Our
protection experiments were performed at day 30 p.i., a time when we could readily detect RSV-specific TRM CD4 and CD8 T cells in the lung. Given that RSV-specific TRMs also waned by 149 d p.i., it is possible that the loss of long-term RSV immunity observed in humans is associated with declining TRMs in the lungs.

As TRMs have been demonstrated to provide protection against acute infection, future RSV vaccine designs should include the induction of this critical cell population. One challenge will be against acute infection, future RSV vaccine designs should include TRMs within the lungs is vital to developing protective immunity. The generation of TRMs within the lungs is vital to developing protective immunity against RSV and should be a focus of ongoing RSV vaccine development.

In conclusion, our data demonstrate that RSV-specific TRM CD4 and CD8 T cells are established in the lungs and diminish with time following initial infection. Furthermore, TRM CD8 T cells exhibit the capacity to provide protective immunity. The generation of TRMs within the lungs is vital to developing protective immunity against RSV and should be a focus of ongoing RSV vaccine development.

DISCLOSURES

The authors have no financial conflicts of interest.

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