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CD8 airway resident memory T (T_{RM}) cells are a distinctive T_{RM} population with a high turnover rate and a unique phenotype influenced by their localization within the airways. Their role in mediating protective immunity to respiratory pathogens, although suggested by many studies, has not been directly proven. This study provides definitive evidence that airway CD8 T_{RM} cells are sufficient to mediate protection against respiratory virus challenge. Despite being poorly cytolytic in vivo and failing to expand after encountering Ag, airway CD8 T_{RM} cells rapidly express effector cytokines, with IFN-γ being produced most robustly. Notably, established airway CD8 T_{RM} cells possess the ability to produce IFN-γ faster than systemic effector memory CD8 T cells. Furthermore, naive mice receiving intratracheal transfer of airway CD8 T_{RM} cells lacking the ability to produce IFN-γ were less effective at controlling pathogen load upon heterologous challenge. This direct evidence of airway CD8 T_{RM} cell–mediated protection demonstrates the importance of these cells as a first line of defense for optimal immunity against respiratory pathogens and suggests they should be considered in the development of future cell-mediated vaccines. ***The Journal of Immunology*****, 2015, 195: 203–209.**

Clearance of a primary respiratory virus infection results in the establishment of virus-specific central memory T cells that reside in secondary lymphoid organs, effector memory T (T_{EM}) cells that recirculate through tissues, and resident memory T (T_{RM}) cells that remain in the lung parenchyma and lung airways (1). At the population level, both airway and parenchymal T_{RM} cells display similar kinetics where the number of Ag-specific memory CD8 T cells is highest in these sites at 1 mo postinfection and gradually declines before stabilizing at a relatively low number of cells 6–8 mo postinfection (2). However, the homeostasis of these populations at the level of individual cells is quite different. Whereas lung parenchymal T_{RM} cells are long-lived in the tissue, airway T_{RM} cells have a relatively short half-life of ∼14 d and must be continually replenished to maintain the population (3). Thus, even though these resident memory populations occupy the same tissue, the differences between them at the level of individual cells make it unclear whether they equally contribute to cellular immunity in the lung.

Memory CD8 T cells canonically aid in controlling and clearing a pathogen through targeted lysis of infected cells (4) and modulation of the innate immune response at the site of infection through the local production of cytokines (5). There is ample evidence from animal models that memory CD8 T cells confer protective immunity to respiratory viruses by significantly decreasing viral loads, leading to faster clearance and decreased immunopathology (6–8). Recent studies in humans showed that increased numbers of circulating cross-reactive memory CD8 T cells correlated with significant decreases in viral loads and lower disease burden following heterosubtypic influenza challenge (9). Notably, studies in animal models that allow sampling of peripheral tissues have shown the number of memory CD8 T cells in the lung correlates with the efficacy of cellular immunity to respiratory virus challenge, and a similar phenomenon has been observed in models of *Mycobacterium tuberculosis* immunity (10, 11). Furthermore, the protective efficacy of cellular immunity to influenza virus slowly declines over several months after infection with kinetics identical to the decline in the number of airway CD8 T_{RM} cells (12). Previous studies have shown that airway CD4 T_{RM} cells could mediate protection in mice lacking CD8 T cells (13), but despite the potential correlation between airway CD8 T_{RM} cells and protective cellular immunity in the lung, there is currently no direct evidence that demonstrates the protective efficacy or protective mechanism of these cells.

T_{RM} cells are generated in response to regional infections and have been documented in the lungs, skin, gut, and reproductive tract where they would have the ability to provide an initial line of defense against invading pathogens (14–19). T_{RM} populations consist of noncirculating cells characterized by permanent residence in peripheral tissues; expression of the tissue retention molecules CD69 and CD103; downregulated expression of CD62L, CCR7, and sphingosine-1-phosphate receptor 1; and a transcription program distinct from their circulating T_{EM} cell counterparts (20, 21). Despite sharing these hallmarks with T_{RM} populations in other tissues, lung airway T_{RM} cells have a distinct phenotype and are short-lived, likely due to the harsh airway microenvironment. Key features of this distinct phenotype are the downregulation of the integrin CD11a and poor in vivo cytolytic capacity, which call

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Abbreviations used in this article: BAL, bronchoalveolar lavage; EID_{50}, 50% egg infectious dose; i.n., intranasal(ly); i.t., intratracheal(ly); NP, nuclear protein; T_{EM}, effector memory T; T_{RM}, resident memory T; WT, wild-type.

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into question the ability of these cells to participate in protective immunity (22, 23). Nevertheless, airway CD8 T_{RM} cells are in prime position to respond to a challenge from pathogens that infect the respiratory epithelium (24). Therefore, it is important to know whether these cells are sufficient to protect against secondary challenge and, if so, how they mediate said protection.

In this study, we use an intratracheal (i.t.) transfer approach to show that airway CD8 T_{RM} cells are sufficient to convey protection against respiratory virus challenge in an Ag-specific manner and quickly produce IFN-γ upon Ag exposure to limit early viral replication in the lung. We used murine models of influenza and Sendai virus infection to demonstrate that airway CD8 T_{RM} cells are equally sensitive to Ag as spleen-derived T_{EM} cells; however, airway CD8 T_{RM} cells respond more quickly, with the predominant responsive population being long-term airway resident cells rather than cells having recently migrated from the lung parenchyma or vasculature. Finally, we show that transfer of airway CD8 T_{RM} cells lacking IFN-γ have a significant defect in their protective efficacy. Our findings on the protective capacity of airway CD8 T_{RM} cells demonstrate their utility in providing protective immunity against respiratory pathogens, lending insight into a protective cellular population that could be elicited through future targeted cellular-based vaccines or immunotherapies.

Materials and Methods

Mice and infections

C57BL/6J (wild-type [WT]), B6.PL-*Thy1*^{a}/CyJ (CD90.1), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1), and B6.129S7-*Ifng*^{tm1Ts}/J (IFN-γ knockout) mice from The Jackson Laboratory were housed under specific Animal Biosafety Level 2 conditions at Emory University and Trudeau Institute. Intranasal (i.n.) infection with influenza A/HKx31 (H3N2) at 30,000 EID_{50} (50% egg infectious doses) and Sendai virus at 282 EID_{50} established virus-specific T cells in mice as previously described (25). Influenza A/PR8 (H1N1) at 6000 EID_{50} was used for challenge of transfer recipient mice. All experiments were completed in accordance with the Institutional Animal Care and Use Committee guidelines of Emory University and Trudeau Institute.

Cellular isolation, i.t. transfer, intravital labeling, and flow cytometry

Memory CD8 T cells, harvested from mice 35–45 d postinfection, were negatively selected from bronchoalveolar lavage (BAL) using a Miltenyi Biotec CD8α T cell isolation kit II. Influenza nuclear protein $(NP)_{366–374}$/D^{b+} tetramer quantification allowed for equal numbers of Ag-specific cells to be i.t. transferred from donor mice to naive recipient mice. No more than 1.5×10^5 Ag-specific airway CD8 T_{RM} cells were transferred per recipient to approximate physiological numbers of airway T_{RM} cells. Abs used for flow cytometry and cell sorting were CD62L (MEL-14), CD8α (53-6.7), CXCR3 (CXCR-173) (BioLegend), CD11a (M17/4), CD44 (IM7) (eBioscience), CD3ε (145-2C11), CD45.2 (104), CD90.2 (53-2.1), and IFN-γ (XMG1.2) (BD Biosciences). Intravital staining was performed immediately before mouse euthanasia and tissue harvest as previously described (15). Briefly, to identify T cells resident in various tissues, including the lung parenchyma, 1.5 μg fluorophore-conjugated anti-CD3ε Ab in 200 μl 1× PBS was i.v. injected into the tail vein of mice; 5 min postinjection, mice were euthanized with Avertin (2,2,2-tribromoethanol; Sigma-Aldrich) and exsanguinated prior to harvest of BAL and other tissues. Staining for intracellular cytokines was performed as previously described following stimulation in the presence of brefeldin A for the indicated periods of time (25). To study cell proliferation, mice were given an i.p. bolus of BrdU (0.8 mg) at the time of infection and maintained on BrdU drinking water (0.8 mg/ml) until harvest. BrdU incorporation was measured using a BrdU flow kit (BD Biosciences) following tetramer and Ab staining. Samples were run on a BD Biosciences FACSCanto II or LSR II flow cytometer and analyzed with FlowJo software. Sorting was performed on an Influx or FACSAria II cell sorter (BD Biosciences).

CTL assay

Donor airway CD8 T_{RM} cells were harvested from the airways of PBS control or PR8-challenged mice and sorted based on CD90.2 expression. Congenic ($CD45.1^+$) targets were pulsed with $FluNP_{366–374}$ peptide (specific targets) or a nonspecific peptide (γHV $p79_{524–531}$ or $SendaiNP_{324–332}$) for 2–4 h at 37°C with nonspecific targets being labeled with 2.5 μM CFSE; nonspecific and FluNP targets were then mixed at a 1:1 ratio. Sorted airway CD8 T_{RM}, lung parenchymal CD8 T_{RM}, or splenic CD8 T_{EM} cells were incubated with targets at an E:T ratio of 4:1 or 1:1 for 6, 14, or 24 h. The ratio of live specific targets to live nonspecific targets was determined by gating on propidium $iodide^-$/$CD45.1^+$/$CFSE^{+/-}$ cells following flow cytometry. Specific lysis was calculated by the formula: [1 − (Ratio of targets only/Ratio of targets following incubation with effector cells)] × 100.

Peptide stimulation and Luminex assay

Mice who received PBS, Sendai-specific airway CD8 T_{RM} cells, or influenza-specific airway CD8 T_{RM} cells followed by i.n. influenza virus challenge the following day had BAL isolated 3 d postchallenge. The supernatant from the single BAL pull was separated from cells via centrifugation prior to cytokine and chemokine analysis by Luminex. Alternatively, BAL and spleens were harvested from Sendai memory mice, sorted to isolate $CD44^{hi}$/$CD62L^-$/$CD8^+$ cells, and stimulated 6 h using irradiated congenic APCs pulsed with 1 μg/ml $SendaiNP_{324–332}$ (FAPGNYPAL) or 1 μg/ml $FluNP_{366–374}$ (ASNENMETM) prior to cytokine and chemokine analysis by Luminex.

Measurement of viral load

Sendai and influenza virus PFU titers were completed as previously described (7) following day 3 postchallenge with Sendai or x31 influenza virus, respectively. Quantitative PCR on influenza virus polymerase gene (PA) was completed as described (26) using high-capacity cDNA reverse transcription kit (Life Technologies)–generated cDNA from 2 μg RNA isolated from lung homogenates by TRIzol and a RiboPure RNA purification kit (Ambion).

Results

Airway CD8 T_{RM} cells are sufficient to convey protection in an Ag-specific manner

Given their proximity to the respiratory epithelium, airway T_{RM} cells are ideally located to rapidly recognize and respond to respiratory viral infections. However, prior ex vivo studies have shown airway CD8 T_{RM} cells have a unique phenotype and effector function when compared with their systemic counterparts. Because of these differences, it is unclear if, and in what capacity, these cells contribute to protective immunity. To specifically test the protective capacity of airway CD8 T_{RM} cells in the absence of parenchymal T_{RM} and circulating T_{EM} cells, we i.t. transferred Sendai or influenza virus–specific airway CD8 T_{RM} cells from the airways of immune mice directly into the airways of naive recipient mice (Fig. 1A). The transferred airway CD8 T_{RM} population expressed high levels of CXCR3 (Supplemental Fig. 1), which has been shown to be upregulated on CD8 T cells in the airways during an acute infection and continues to be expressed into immunological memory (27). These cells also remain in the airways following i.t. transfer and do not egress from the airways to the lung parenchyma or mediastinal lymph node (Supplemental Fig. 2). Recipient mice were challenged with influenza or Sendai virus 1 d after transfer, and viral titers were measured 3 d postinfection. As shown in Fig. 1B and 1C, mice receiving airway CD8 T_{RM} cells specific to the challenge virus had a significant decrease in viral titers. In contrast, airway T_{RM} cells specific for a different virus showed no difference in titers compared with PBS controls. Thus, airway CD8 T_{RM} cells are sufficient to limit early viral replication through a mechanism that requires cognate Ag recognition.

Ag-specific airway CD8 T_{RM} cells result in decreased expression of proinflammatory cytokines and chemokines within the lung airways upon challenge with cognate Ag

Three days after i.n. challenge, the BAL supernatant was harvested from naive mice who received an i.t. transfer of PBS, Sendai-

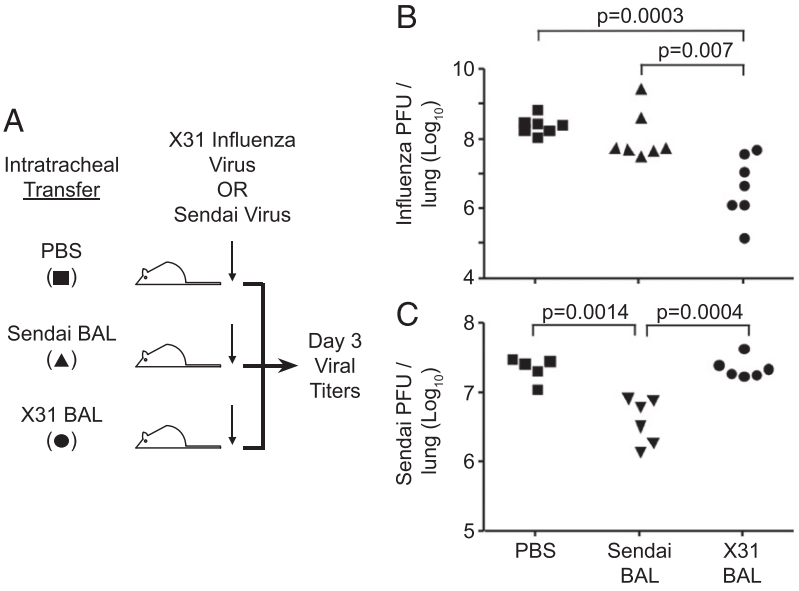

FIGURE 1. Airway CD8 T_{RM} cells significantly decrease viral replication in an Ag-specific manner. (**A**) Intratracheal transfer experimental model where PBS or airway CD8 T_{RM} cells from Sendai virus or influenza virus (x31) memory mice were harvested from BAL and i.t. transferred into naive WT mice, which were i.n. challenged the following day and viral titers were measured 3 d later. (**B**) Influenza titers from each of the three groups receiving PBS, Sendai-specific airway CD8 T_{RM} cells, or x31-specific airway CD8 T_{RM} cells as noted in (A). n = 7 mice/group; unpaired two-tailed t tests; compiled from two and representative of four independent experiments. (**C**) Sendai titers from each of the three mouse groups receiving PBS, Sendai-specific airway CD8 T_{RM} cells, or x31-specific airway CD8 T_{RM} cells as noted in (A). n = 5–6 mice/group; unpaired two-tailed t tests; compiled from two and representative of four independent experiments.

specific airway CD8 T_{RM} cells, or influenza-specific airway CD8 T_{RM} cells 1 d before challenge (Fig. 2A). Despite having the greatest reduction in viral titers upon challenge, mice receiving influenza-specific airway CD8 T_{RM} cells i.t. produced significantly lower levels of CXCL1, CCL2, IL-6, and TNF-α when compared with mice receiving PBS or Sendai-specific airway CD8 T_{RM} cells (Fig. 2B). In contrast, the airways of naive mice receiving Sendai-specific airway CD8 T_{RM} cells i.t. had higher levels of all four inflammatory cytokines than even the PBS controls as a result of the non–Ag-specific influenza virus challenge. Therefore, upon exposure to cognate Ag, the ability of airway CD8 T_{RM} cells to rapidly decrease viral loads aids in restraining the local proinflammatory immune response and limiting unnecessary damage to the lungs.

Airway CD8 T_{RM} cells fail to gain rapid cytolytic function in vivo, even in the presence of cognate Ag

To understand the mechanism by which airway CD8 T_{RM} cells mediate protection, we examined the capacity of these cells to induce target cell death in vitro and their ability to proliferate upon secondary infection. In Fig. 3A, we isolated and sorted airway CD8 T_{RM} and splenic CD8 T_{EM} cells from x31 influenza memory mice to compare their respective cytolytic capabilities. The specific lysis of airway CD8 T_{RM} cells was relatively negligible, remaining at ∼10%, for E:T ratios ranging from 1:1 to 4:1, whereas the specific lysis of splenic CD8 T_{EM} cells from the same mice increase as the E:T ratio increases (Fig. 3A). For all three E:T ratios, the specific lysis of the splenic CD8 T_{EM} cells was significantly higher than that of the airway CD8 T_{RM} cells. To directly compare the CTL activity of the airway (BAL) and lung parenchymal CD8 T_{RM} cells 35 d after x31 influenza virus infection, we sorted cells from the airways and lung tissue that were protected from an intravital staining Ab, providing a $CD44^{hi}$/$CD62L^{lo}$ CD8 T_{RM} population from each resident compartment (Fig. 3B). Fig. 3C shows that, at a 1:1 E:T ratio, the lung parenchymal CD8 T_{RM} cells have significantly higher CTL activity than the airway CD8 T_{RM} cells, even after incubating with targets for 14 h. Finally, to understand whether the airway CD8 T_{RM} population gains CTL function by encountering cognate Ag, we transferred airway CD8 T_{RM} cells from x31 influenza-primed mice i.t. into congenic naive mice and challenged those mice with PBS (control) or PR8 influenza. On day 3 postchallenge, the transferred airway CD8 T_{RM} cells were isolated by cell sorting and assessed for cytolytic function. Even in the presence of cognate Ag stimulation in vivo, airway CD8 T_{RM} cells remained poorly cytolytic in a short-term CTL assay irrespective of the E:T ratio and did not display robust cytolytic function until 24 h of target incubation (Fig. 3D). Therefore, the airway CD8 T_{RM} population, once established, is poorly cytolytic and remains poorly cytolytic even during a secondary infection, whereas the lung parenchymal CD8 T_{RM} population retains its cytolytic capacity.

Airway CD8 T_{RM} cells fail to proliferate in vivo, even in the presence of cognate Ag

To investigate whether the rapid proliferation and expansion of airway CD8 T_{RM} cells may be important for their protective function, we transferred airway CD8 T_{RM} cells ($CD90.2^{+}$) from

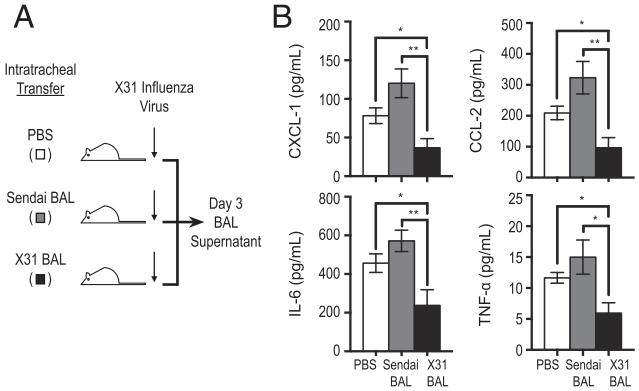

FIGURE 2. Ag-specific airway CD8 T_{RM} cells result in lower production of inflammatory cytokines within the lung airways upon challenge with cognate Ag. (**A**) PBS or airway CD8 T_{RM} cells from Sendai virus or influenza virus (x31) memory mice were harvested from BAL and i.t. transferred into naive WT mice, which were i.n. challenged the following day and BAL supernatant was harvested 3 d later. (**B**) BAL supernatant from each of the three groups receiving PBS, Sendai-specific airway CD8 T_{RM} cells, or x31-specific airway CD8 T_{RM} cells i.t. as noted in (A) was isolated and Luminex assay was performed to quantify the amount of CXCL1 (KC), CCL2 (MCP-1), IL-6, and TNF-α produced in the airways of the recipient mice. n = 6–7 mice/group; unpaired two-tailed t tests; representative of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

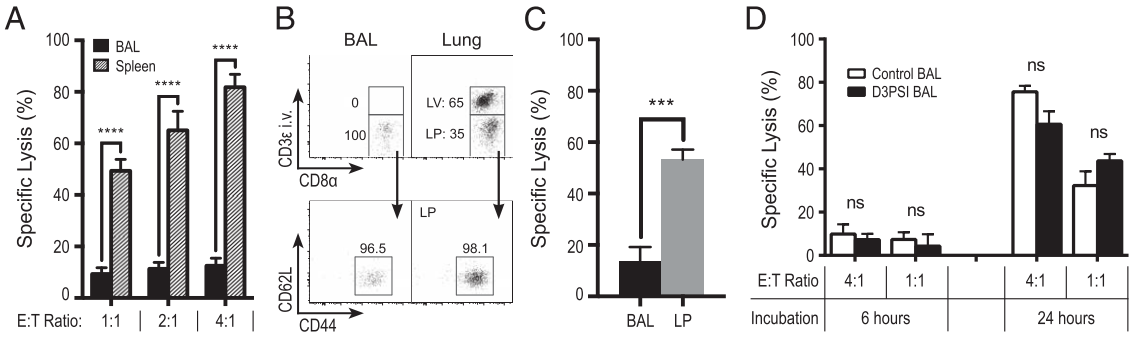

FIGURE 3. Airway CD8 T_{RM} cells are slow to gain cytolytic function following secondary infection. (**A**) Sorted $CD44^{hi}/CD62L^{lo}$ CD8 T cells from the airways and spleen of x31 memory mice and incubated with targets at an E:T ratio of 1:1, 2:1, or 4:1 for 6 h. $n = 5$ replicates per E:T ratio; representative of four independent experiments; mean ± SEM; two-way ANOVA with Sidak test corrected multiple comparisons. $^{****}p < 0.0001$. (**B**) $CD44^{hi}/CD62L^{lo}$ CD8 T cells were sorted from the airways and lung parenchyma (LP) of x31 memory mice. Cells resident in the lung parenchyma were identified by intravital labeling using a fluorophore-conjugated anti-CD3ε Ab administered immediately prior to mouse euthanasia; cells within the LP are protected from being labeled by the i.v. administered Ab, whereas those within the lung vasculature (LV) are labeled. (**C**) Cells sorted in (B) were incubated with targets at an E:T ratio of 1:1 for 14 h to determine cytolytic capacity. $n = 4$–5 replicates; representative of two independent experiments; mean ± SEM; unpaired two-tailed t test. $^{***}p < 0.001$. (**D**) Sorted $CD11a^{lo}$ airway CD8 T_{RM} cells from x31 memory mice were transferred i.t. into naive congenic recipients challenged with PBS (Control) or PR8 for 3 d (D3PSI). Donor cells were isolated from the airways on day 3 and incubated with targets at an E:T ratio of 4:1 or 1:1 for 6 or 24 h. $n = 4$ replicates/time point/E:T ratio; representative of three independent experiments; mean ± SEM; two-way ANOVA with Sidak test corrected multiple comparisons.

x31 influenza–primed mice i.t. into congenic ($CD90.1^+$) x31 influenza–primed mice, challenged them with PR8 the following day, and maintained the mice on BrdU water for 7 d (Fig. 4A). The i.t. transferred population maintained their $CD11a^{lo}$ status, did not incorporate BrdU, and failed to expand throughout the secondary response (Fig. 4B, 4C). Notably, the only flu-specific CD8 T cells in the airways to incorporate BrdU were host cells that recently migrated to the airways, as noted by their $CD11a^{hi}$ status; these host cells eventually dominate the secondary response. Taken together, these data infer that the airway CD8 T_{RM} cells do not need to proliferate within the airways or gain rapid cytolytic function to mediate protection to a secondary challenge.

Airway CD8 T_{RM} cells are capable of rapidly producing antiviral cytokines

Given their suboptimal cytolytic activity, we hypothesized that airway CD8 T_{RM} cells may provide protection by rapidly detecting cognate Ag and secreting antiviral cytokines in response to secondary challenge. To test this, we examined the cytokine profile of airway CD8 T_{RM} cells and splenic-derived CD8 T_{EM} cells from Sendai-immune mice in response to their cognate Ag (SendNP) or an unrelated peptide (FluNP). As shown in Fig. 5A, after 6 h of stimulation with cognate Ag, airway CD8 T_{RM} cells produced significant amounts of IFN-γ, TNF-α, and IL-10; splenic CD8 T_{EM} cells also produced significant amounts of these cytokines plus IL-2. Notably, out of the cytokines produced by airway CD8 T_{RM} cells, IFN-γ was most impressive with respect to magnitude and merited further investigation.

We suspected that the rate at which the airway CD8 T_{RM} population senses its cognate Ag could be another difference between the two populations, as rapid cytokine production is a hallmark of T_{RM}-mediated protection in other peripheral sites (18). This would corroborate the idea that the airway CD8 T_{RM} population acts as an early warning sensor to mediate protection in an Ag-specific manner. Thus, when we compared the airway CD8 T_{RM} and splenic T_{EM} cell IFN-γ production at early times after cognate peptide stimulation (Fig. 5B), we observed that the airway CD8 T_{RM} population reacted faster (within 2 h) than the splenic CD8 T_{EM} population. Furthermore, it was the $CD11a^{lo}$ airway CD8 T_{RM} population, which has resided in the airway the longest, that had the fastest rate of IFN-γ production. One explanation for the quicker IFN-γ response by the airway CD8 T_{RM} cells is that they are more sensitive to Ag than are splenic CD8 T_{EM} cells. However, there was no difference in peptide affinity between

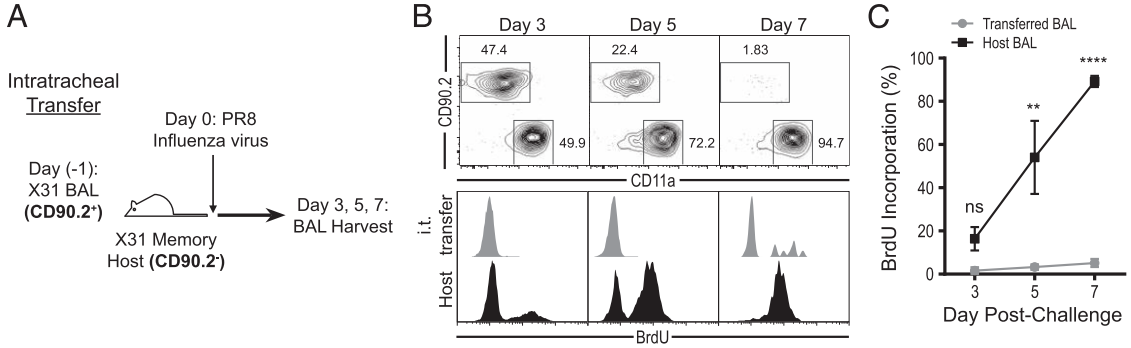

FIGURE 4. Airway CD8 T_{RM} cells do not proliferate in vivo following secondary infection. (**A**) Schematic of the experimental design used in (**B**) and (**C**). (B) $CD90.2^+$ airway CD8 T_{RM} cells were i.t. transferred into x31 memory $CD90.1^+/CD90.2^-$ mice 1 d prior to PR8 i.n. challenge; host mice were maintained on BrdU following cell transfer through day 7 of viral challenge. BAL was isolated at days 3, 5, and 7 following challenge to evaluate donor airway CD8 T_{RM} expansion, CD11a expression (*top row*) and BrdU incorporation (*bottom row*). Plots shown are gated of FluNP-specific cells. $n = 3$ mice/time point/group; representative of three independent experiments. (C) BrdU incorporation for donor and recipient airway CD8 T_{RM} cells from the plots in (B) is graphed as the mean ± SEM; two-way ANOVA with Sidak test corrected multiple comparisons. $^{**}p < 0.01$, $^{****}p < 0.0001$.

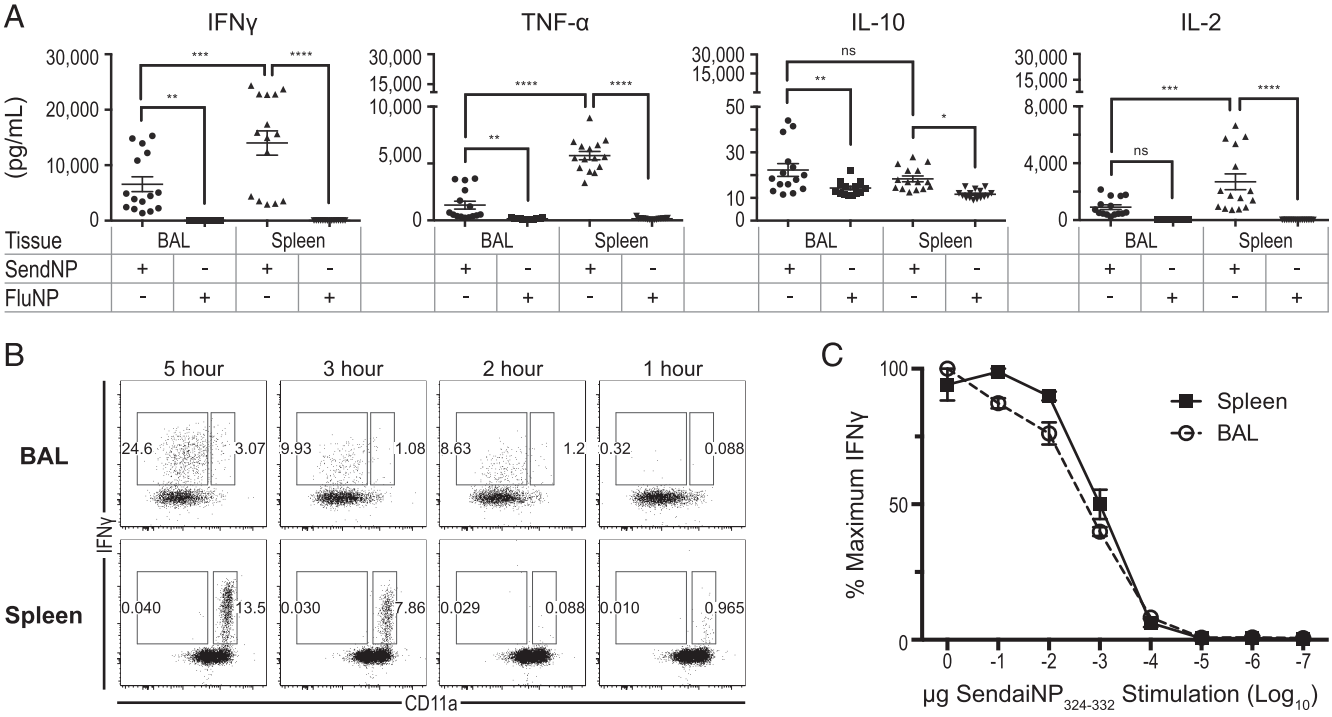

FIGURE 5. Airway CD8 T_{RM} cells are capable of quickly producing cytokines, especially IFN-γ, upon recognition of cognate Ag. (**A**) Mice i.n. infected with Sendai virus and rested to immunological memory were sacrificed on day 45. Sorted $CD62L^{-}/CD8^{+}$ cells from BAL and spleen were stimulated with $SendaiNP_{324–332}$ or $FluNP_{366–374}$ peptide for six hours before Luminex assay. $n = 15$ mice/group; compiled from three independent experiments; mean ± SEM; one-way ANOVA with Tukey test corrected multiple comparisons. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. (**B**) Airway CD8 T_{RM} cells and splenic-derived CD8 T_{EM} cells were stimulated with 1 μg/ml SendNP for 1–5 h prior to intracellular staining for IFN-γ production. $n = 4$ replicates/time point; representative of three independent experiments. (**C**) Percentage maximum of IFN-γ production of airway CD8 T_{RM} cells (BAL) or splenic-derived CD8 T_{EM} cells (Spleen) following stimulation with serial dilutions of SendNP for 5 h before ICS for IFN-γ production. $n = 2$ replicates/group/time point; representative of four independent experiments; mean ± SEM. (B) and (C) results were gated on $CD8^{+}/CD45.2^{+}$ cells.

airway CD8 T_{RM} and splenic CD8 T_{EM} cells (Fig. 5C). This lack of difference is especially true at lower concentrations where a divergence would be expected if the airway CD8 T_{RM} cells had greater functional avidity to their cognate Ag than did the splenic CD8 T_{EM} cells. Taken together, these data demonstrate that airway CD8 T_{RM} cells are able to rapidly produce antiviral cytokines upon Ag recognition and suggest that airway CD8 T_{RM} cell–derived IFN-γ may be a crucial mediator of protection against respiratory virus challenge.

IFN-γ–deficient airway CD8 T_{RM} cells show a significant defect in protective immunity

To test whether IFN-γ was important for airway CD8 T_{RM} cell–mediated protection during an influenza virus infection, we i.t. transferred equal numbers of FluNP-specific airway CD8 T_{RM} cells from either WT or IFN-γ–deficient mice into naive recipients, followed by PR8 influenza challenge 1 d later (Fig. 6A). We found that mice receiving influenza-specific WT airway CD8 T_{RM} cells have significantly lower viral copies than do those mice receiving airway CD8 T_{RM} cells from IFN-γ–deficient mice following PR8 challenge (Fig. 6B). Moreover, mice receiving IFN-γ–deficient airway CD8 T_{RM} cells still showed a significant decrease in virus copies compared with PBS control mice, suggesting that other antiviral mechanisms are likely involved, such as production of TNF-α by airway CD8 T_{RM} cells observed in Fig. 5A. Nevertheless, although it has been shown that IFN-γ is not necessary to survive a lethal primary influenza virus infection (28), these data show that IFN-γ produced by airway CD8 T_{RM} cells plays an important role in limiting viral loads following secondary challenge, which can be important in limiting immunopathology during an infection (29).

Discussion

Tissue-resident memory T cells established at thresholds of pathogen entry play a crucial role in protective immunity. To our knowledge, these findings provide the first direct evidence that airway CD8 T_{RM} cells, a unique population of T_{RM} cells based on their limited lifespan and microenvironment-constrained phenotype, serve as a first line of defense in the lung against pathogen challenge and are sufficient to limit early viral replication. Their fast response upon Ag exposure to produce IFN-γ and other effector cytokines makes them ideal for limiting early viral replication. Furthermore, these cells fail to proliferate within the airways and remain

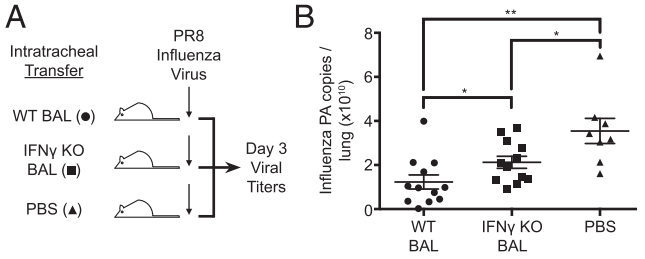

FIGURE 6. IFN-γ–deficient airway CD8 T_{RM} cells are less effective at conveying heterologous protection. (**A**) Experimental i.t. transfer model where PBS or influenza-specific airway CD8 T_{RM} cells from WT or IFN-γ knockout mice were i.t. transferred into naive WT mice, which were i.n. challenged with PR8 influenza virus 1 d after transfer, and viral titers were measured 3 d later. (**B**) Influenza titers from each of the three groups receiving PBS, IFN-γ knockout airway CD8 T_{RM} cells, or WT airway CD8 T_{RM} cells as noted in (A). $n = 8–12$ mice/group; compiled from two independent experiments; mean ± SEM; unpaired two-tailed t tests. $*p < 0.05$, $**p < 0.01$.

poorly cytolytic even in the presence of their cognate Ag, suggesting that the ability to rapidly produce cytokines is critical for their protective efficacy. In support of this, airway CD8 T_{RM} cells lacking IFN-γ had a significant defect in protective immunity compared with WT controls. Taken together, these data demonstrate that the airway CD8 T_{RM} population plays an important role in secondary cellular immunity against respiratory viruses by providing a rapid, local source of cytokines to promote an early antiviral state.

Many studies have observed a correlation between the steady decline in numbers of airway CD8 T_{RM} cells in the months after primary infection and the steady decline in heterosubtypic immunity against influenza virus challenge. However, demonstrating that the decline in protective immunity is a direct consequence of a decline in airway CD8 T_{RM} population has been difficult because delineating the individual contributions of airway T_{RM}, lung parenchymal T_{RM}, and circulating T_{EM} populations are not possible through traditional Ab depletion approaches. The importance of analyzing the role of airway CD8 T_{RM} cells independently of these other subsets was further highlighted in a recent study that observed that lung parenchymal T_{RM} cell numbers also decline in the months postinfection, and the decline in protection may have been solely attributable to this phenomenon (10). Our data do not preclude a role for parenchymal T_{RM} cells in heterologous immunity, but rather suggest that these populations may act in concert to limit early viral replication. Unlike the airway T_{RM} population, T_{RM} populations within other tissues display strong cytolytic activity, and the lung parenchymal T_{RM} population maintains expression of CD11a, enabling their cytolytic activity (Fig. 3C). It has been shown that infected lung epithelial cells can present Ag to T cells on the apical surface lining the airways in addition to the basolateral surface; thus, it is possible that an infected epithelial cell would be presenting Ag to both the airway and lung parenchymal T_{RM} subsets (30). Therefore, there may be a division of labor between these populations where airway T_{RM} cells serve more of a sentinel function through the rapid production of cytokines to condition the local microenvironment and lung parenchymal T_{RM} cells mediate direct killing of infected cells.

In addition to their cytolytic defect, it is intriguing that airway CD8 T_{RM} cells fail to proliferate even when triggered by their cognate Ag. It was previously shown that airway CD8 T_{RM} cells transferred i.v. into naive hosts were capable of generating a complete secondary effector and memory response upon challenge, demonstrating that these are not terminally differentiated and are able to undergo clonal expansion (31). In contrast, our study examined proliferation in situ within the airways, where the local microenvironment does not provide abundant nutrient and growth factors to support an expanding T cell population. Clonal expansion of CD8 T cells following Ag stimulation is accompanied by a metabolic switch to glycolysis (32), and the concentration of glucose in airway fluid is 10- to 15-fold lower than blood plasma (33, 34). Therefore, the inability of these cells to proliferate in the airways may simply be a consequence of insufficient nutrients within the local airway microenvironment.

Although IFN-γ–deficient mice show no defect in antiviral immunity following a primary influenza infection (28), its impact on protective cellular immunity to heterologous influenza challenge is less clear, with several conflicting reports regarding the protective role of IFN-γ during secondary challenge (35–38). Our data show that the inability of airway CD8 T_{RM} cells to produce IFN-γ resulted in a significant increase in viral titers compared with WT airway CD8 T_{RM} cells; it may be that the impact, positive or negative, of IFN-γ on protective immunity during influenza challenge depends on the timing of IFN-γ production. For example, it has been shown that IFN-γ production at the later stages of the acute response can lead to enhanced pathology (39), whereas our data suggest that early production of IFN-γ by airway CD8 T_{RM} cells results in decreased levels of proinflammatory cytokines, likely due to decreased viral replication. Note also that airway CD8 T_{RM} cells also produced TNF-α and IL-10 and that these cytokines may account for the limited protective effect observed when IFN-γ–deficient airway CD8 T_{RM} cells were transferred into the airways of naive mice compared with PBS controls. In particular, the low levels of IL-10 produced may also limit early proinflammatory cytokine production and decrease pathology (40).

In summary, we show that airway CD8 T_{RM} cells are sufficient to limit early viral replication following secondary influenza virus challenge, resulting in an attenuated duration of proinflammatory cytokine expression that can promote immunopathology. Furthermore, the protective efficacy was dependent on IFN-γ production by airway CD8 T_{RM} cells and did not require local proliferation or enhanced cytolytic activity. We think these data support the idea that Ag-specific airway CD8 T_{RM} cells act as sentinels capable of rapidly responding to invading pathogens and alerting the immune system. Identifying approaches to generate or boost this airway CD8 T_{RM} population through targeted vaccines and immunotherapies may afford greater protection against respiratory pathogens.

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Disclosures

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