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ImmunoHorizons 2021, 5 (7) 543-556
doi: https://doi.org/10.4049/immunohorizons.2100032
http://www.immunohorizons.org/content/5/7/543

This information is current as of August 6, 2021.

Supplementary Material
http://www.immunohorizons.org/content/suppl/2021/07/15/immunohorizons.2100032.DCSupplemental

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Visualization of Resident Memory CD8 T Cells in the Lungs of Young and Aged Influenza Memory Mice and after Heterosubtypic Challenge

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ABSTRACT

Memory T cells that are resident in the tissues (T resident memory [Trm]) serve as frontline responders to prevent reinfection by pathogens. Trm in the lung protect against respiratory viruses. Although these cells have been well characterized, little is known about the impact of immune aging on the establishment, maintenance, function and recall of lung-resident Trm in the context of an influenza virus infection. Aging is associated with a progressive decline in immune function and a generalized inflammatory syndrome, referred to as inflamming. In this study, we analyzed inflamming in the lung and assessed numbers and function of lung Trm after primary influenza infection and heterosubtypic challenge of young and aged mice. Our analysis showed that aged mice had more severe and sustained lung inflammation than young mice. Analysis of Trm numbers by flow cytometry and direct imaging showed comparable or higher numbers of Trm in aged compared with young mice, with a similar rate of decline over time in both groups of mice. Furthermore, influenza virus-specific Trm from young and aged memory mice were both functional in vitro, and the mice were protected from heterosubtypic challenge. Finally, there were enhanced numbers of T cells resident in the lungs of aged compared with young mice after heterosubtypic viral challenge. The data suggest that the generation, maintenance, and function of Trm in aged mice are not severely impaired and the increased numbers in aged compared with young mice after heterosubtypic challenge may be associated with enhanced lung inflammation in the aged mice. ImmunoHorizons, 2021, 5: 543–556.

INTRODUCTION

Immunity declines with aging, resulting in impaired responses to infection and reduced vaccination efficacy, termed immunosenescence (1–3). Age-associated immune defects include impaired numbers, repertoire, and function of B cells (4–6), impaired CD4 T cell function (7–10), reduced numbers and repertoire diversity of CD8 T cells (11–18), and increased numbers of regulatory T cells (19). In addition, age-associated structural changes in the airways and lungs render the elderly especially susceptible to respiratory infections (20). Aging is also associated with generalized inflammation that is not linked to any particular disease, termed inflamming (21–25).

Influenza virus elicits humoral and cellular immunity. Abs protect against reinfection with serologically similar viruses, whereas T cells can provide cross-protection to serologically distinct influenza virus strains that share internal components recognized by T cells. Understanding T cell immunity is also
important because it has been shown that T cell responses are strong correlates of vaccine-induced protection in the elderly (26). Following recovery from respiratory viral infections, such as influenza virus, Ag-specific memory CD4 and CD8 T cells are established. These memory T cells can be divided into several distinct subpopulations, including effector memory T cells (Tem), central memory T cells (Tcm), and tissue-resident memory T cells (Trm) (27, 28). Tem and Tcm circulate between the secondary lymphoid organs. In contrast, Trm reside within nonlymphoid tissues and do not circulate. Influenza-specific T cells resident in the lung (lung Trm) reside in both the lung interstitium (bronchioles and alveolar walls) and airways (29, 30).

Lung Trm play an important role in controlling respiratory virus infections because they are situated at the site of reinfection, allowing them to respond rapidly to secondary exposure (31). They have been shown to be essential for effective protection against infection with heterosubtypic influenza viruses (32, 33). In addition to mediating direct antiviral action in the lung, Trm can also elicit inflammatory responses, thereby recruiting circulating T cells (effector T cells and Tem) to the site of infection (34). Another mechanism resulting in increased numbers of Trm after viral challenge is that Trm can proliferate after reinfection (32, 35–37). Importantly, several studies have shown that lung Trm, in contrast to Trm at other sites, such as the skin, are not maintained long-term, resulting in waning heterosubtypic immunity with time (32, 38, 39).

It has been established that influenza virus infection of aged mice results in the generation of fewer circulating memory CD8 Tem and Tcm than in young mice (15, 40–44). However, little is known about the impact of age on the generation of Trm. There is recent evidence that influenza virus infection elicits higher numbers of Trm in aged compared with young mice. However, these Trm were reported to be functionally defective and failed to protect against heterosubtypic challenge (48). Our studies address the impact of age on both the development and maintenance of Trm, using the well-developed influenza mouse model. In light of reports that isolation of CD8 Tem and Tcm is highly inefficient and misrepresents the makeup of different T cell subsets (30), we have focused our analysis not only on flow cytometry but also using direct in situ visualization of Trm in lung sections. A complete understanding of the impact of age on the development of Trm is necessary to develop vaccination strategies that protect the growing elderly population.

### MATERIALS AND METHODS

#### **Mice, viruses, and infections**

C57BL/6J and B6.SJL-pta6Pepc<sup>−/−</sup>/BoyJ (CD45.1) mice were obtained from the Trudeau Institute animal facility or purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen–free conditions. Mice were housed in a biosafety level 2 environment for the duration of these studies. All animal procedures were approved by the Institutional Care and Use Committee at Trudeau Institute. Influenza viruses A/PR/8/34 (PR8, H1N1) were grown, stored, and titrated as previously described (46). Young (8–10 wk old) and aged (>18 mo old) mice were anesthetized with 2,2,2,tribromoethanol or with isoflurane and infected intranasally (i.n.) with 3,000 50% egg ID<sub>50</sub> x31. For heterologous protection studies, mice previously infected with x31 were challenged with 1,000 EID<sub>50</sub> PR8 i.n. at 30, 60, or 90 d after x31 infection. Viral load was determined as previously described (47).

### Intravital labeling, cell isolation, and flow cytometry

Intravital staining was performed immediately before mouse euthanasia and tissue harvest. Briefly, to identify T cells resident in various tissues, 1.5 μg of fluorophore-conjugated CD3e (145-2C11; BD Biosciences) Ab in 1× PBS was injected i.v.; 5 min postinjection, mice were euthanized and exsanguinated prior to harvest of bronchoalveolar lavage (BAL), a negative control for intravital staining, and lungs. BAL cells were collected by lavage of the lungs five times with 1 ml of HBSS. Lung tissue was prepared by coarsely chopping the tissue followed by incubation in a 0.5 mg/ml solution of collagenase D (Roche) and DNase (Sigma-Aldrich) for 30 min at 37°C. Lymphocytes were enriched from digested lung tissue by differential centrifugation using a gradient of 40/80% Percoll (GE Healthcare). After RBC lysis with ammonium-buffered chloride, live cell numbers were determined by counting and trypan blue exclusion. Single-cell suspensions were incubated with Fc block (anti-CD16/32) for 15 min on ice followed by staining with Abs to CD4 (RM4-5), CD19 (1D3) (both from BD Biosciences), CD8a (53-6.7), CD44 (IM7), CD69 (H1.2F3), and CD103 (2E7) (BioLegend). Samples were acquired on a FACSCanto II or LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software.

### Intravital labeling, immunofluorescence, and microscopy

Intravital staining was performed immediately before mouse euthanasia and tissue harvest. Briefly, to identify T cells resident in lung tissue, 1.5 μg of fluorophore-conjugated CD8b.2 (5.3-5.5) (BioLegend) Ab in 1× PBS was injected i.v.; 5 min postinjection, mice were euthanized and exsanguinated prior to harvest of lung tissue. Lung sections were cut 200-μm thick on a vibrating microtome and were subsequently incubated with Fc block (anti-CD16/32) for 45 min on ice with Abs to CD8α (53-6.7; BD Bioscience) and EpCAM CD326 (G8.8; eBioscience) to distinguish airways. Slides were imaged using a Leica SP5 confocal microscope with 405, 488, 543, and 633 laser lines. Emission spectra were collected using the appropriate bandwidth settings for each fluorophore. A total of 30 optical sections covering 40 μm of tissue were taken for each image. The data were collected as Leica image files using LAS AF version 2.6.0.7266 software (Leica) and converted into TIF files using Fiji software (ImageJ; National Institutes of Health).
Whole lung sections (Supplemental Fig. 1) were prepared and stained as described above. Images were stitched using the Tile Scan feature in Leica LAS AF software (version 2.7.3.9723). Each tile was imaged using a 10× objective and the appropriate excitation and collection bandpass filters for each fluorochrome.

**Intracellular cytokine staining**

For measurement of cytokine production, single-cell suspensions from lung or spleen were incubated in the presence of 10 μg/ml influenza nucleoprotein (NP)366-374 or γ-herpesvirus-68 ORF6487-495 for 1.5 h at 37°C; brefeldin A (50 μg/ml) was added, and the incubation was continued for an additional 3.5 h. Surface staining for CD4, CD8a, CD19, and CD44 was performed, the cells were fixed, permeabilized (Cytofix/Cytoperm Kit; BD Biosciences), and stained for intracellular cytokines with Abs to TNF-α (MP6-XT22; BioLegend). Samples were acquired on FACSVerse II or LSR II flow cytometers (BD Biosciences), and data were analyzed with FlowJo software.

**In vitro CTL assay**

Following intravitral labeling with CD3ε, lymphocytes were isolated from the lungs of young and aged x31-infected mice (70–85 dpi postinfection [dpi]), as described above. Lymphocytes were pooled for each group and stained with Abs for CD8a and CD44. The cells were flow cytometrically sorted into two subsets, CD44highCD3ε+ (systemic) and CD8a+CD44highCD3ε− (resident). Influenza NP366-374D1 tetramer quantification was used to adjust the concentration of effector cells to equalize the number of NP+ cells per mL. Congenic target cells (CD45.1+) were prepared by pulsing splenocytes with 1 μg/ml influenza NP366-374 or γ-herpesvirus-68 ORF6487-495 peptides for 3 h at 37°C. NP-pulsed targets were then labeled with 2.5 μM CFSE, the NP- and ORF6-pulsed targets were mixed at a 1:1 ratio, and sorted memory effector cells were added at an E:T ratio of 4:1, 2:1, or 1:1. Target cells incubated alone served as negative controls. The plate was gently centrifuged (300 × g for 1 min) to bring effector and target cells into contact and incubated for 18 h at 37°C. The ratio of live specific targets (NP366-374) to live nonspecific targets (ORF6487-495) was assessed by flow cytometry by gating on propidium iodide–negative, CD45.1+, CFSE+/− cells. Specific lysis was calculated with the equation [1 - (ratio of targets only/ratio of targets + effectors)] × 100.

**Histology and scoring**

Lungs were inflated with 10% neutral buffered formalin, embedded in paraffin, and whole lung sections were prepared at 4–5 microns and stained with H&E. The lung lesions were scored in the following categories: whole lung score (involvement of total lung section area), bronchial/bronchiolar necrosis, bronchiolar/bronchiolar hyperplasia, bronchiolar lymphocyte cuffing, perivascular lymphocyte cuffing, alveolar inflammation, alveolar epithelialization, squamous metaplasia, alveolar macrophages, and neutrophils. Scores included the following: 0, normal or none; 1, minimal; 2, mild; 3, moderate; 4, severe. Whole lung scores included the following: 0, no lesions; 1, 0–25% of the lung involved; 2, 26–50%; 3, 51–75%; 4, 76–100%.

**Statistical analysis**

Statistical analysis was performed using Prism 8 (GraphPad Software), as indicated in the legends to individual figures. The p values < 0.05 were considered significant.

**RESULTS**

**Aged memory mice are protected against sublethal heterosubtypic influenza virus challenge**

The mouse influenza virus model provides an important experimental system for studying T cell immunity in aging (48). The initial infection generates neutralizing Abs specific for the external coat proteins of the virus, including hemagglutinin (HA) and neuraminidase (NA), and T cells specific for internal viral proteins, including NP. To allow us to characterize cross-reactive T cell responses while avoiding neutralizing Abs, we used two different strains of influenza virus, A/HK-x31 (x31, H3N2) and A/PR8/34 (PR8, H1N1). Cohorts of young and aged memory mice were monitored for weight, survival, and viral clearance after heterosubtypic challenge on day 45 postinfection with a dose of the highly pathogenic PR8 strain of virus that has been determined to be sublethal for young memory mice. Aged mice lost more weight than young mice after challenge (Fig. 1A) and, importantly, continued losing weight for several days after the young mice started regaining weight. However, aged mice were largely protected, exhibiting only modest (18%) morbidity (Fig. 1B). Analysis of viral clearance demonstrated that, although aged mice had higher titers of virus than young mice on day 5, viral clearance at day 7 was comparable in both groups. Virus was completely cleared from both young and aged mice by day 10 postinfection (Fig. 1C). Whereas clear differences have been reported in the viral clearance and survival of aged mice after initial infection with influenza virus (49–53), these data show that both young and aged memory mice have similar levels of survival and clear virus relatively comparably following heterosubtypic challenge.

**Aged mice exhibit enhanced chronic lung inflammation compared with young mice with influenza virus postinfection**

The prolonged weight loss in influenza virus–infected aged mice may have resulted from enhanced inflammation and pathology. To assess the impact of influenza infection on lung inflammation, young and aged mice were infected with x31 (H3N2) influenza virus. Lung tissues were collected 45 dpi, and the pathology of H&E-stained tissues from young and aged memory mice was compared. The data on the left side of graphs for Fig. 2 show overall histopathology (Fig. 2A), the presence of bronchiolo lymphocyte cuffs (Fig. 2B), alveolar macrophages (Fig. 2C), and alveolar epithelialization (Fig. 2D).
in memory mice. Representative images show that the lungs of young memory mice (Fig. 3A, 3B) had less extensive and less severe lung lesions than did aged memory mice (Fig. 3C–F). Interstitial lesions in young mice (Fig. 3B) were much less extensive than in aged mice (Fig. 3C–F). The lesions included alveolar bronchiolization (Fig. 3D, 3E), macrophages with crystals (Fig. 3E), and lymphocyte foci, also known as induced BALT (iBALT) (Fig. 3F). Taken together, the aged mice showed enhanced pathology scores compared with young mice after influenza infection.

**Influenza virus infection elicits higher numbers of Trm in the lungs of aged compared with young mice**

Trm in the lung play a key role in heterosubtypic protection against influenza virus challenge (32, 33). We therefore set out to examine whether the difference in pathology and weight loss observed in aged animals reflected differences in the generation and/or maintenance of lung Trm cells. To accomplish this, we compared the numbers of CD8 Trm in influenza NP-specific CD8 Trm by using a tetramer specific for NP (NP366-374/Db) in young and aged influenza memory mice. Lung Trm were identified using an i.v. Ab-labeling approach based on the failure of resident cells to be labeled with i.v.-injected anti-CD3 Abs (Fig. 4A) (54). Lung cells that were not protected from staining, likely because they were circulating through the capillaries during the time of the short in vivo staining, are referred to as systemic T cells. We analyzed the numbers of total CD8 Trm (Fig. 4B), NP-specific Trm (Fig. 4C), and CD8 and NP-specific systemic T cells (Fig. 4D, 4E) in young and aged memory mice 1 mo postinfection. The data show that the numbers of CD8 Trm and NP+ Trm are increased in the lungs of aged mice (Fig. 4B, 4C). Consistent with these findings, a

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**FIGURE 1. Aged influenza memory mice are protected from heterosubtypic influenza virus challenge.**

Young and aged female mice were infected i.n. with 3,000 EID_{50} x31 (H3N2) and challenged at 45 dpi with 1000 EID_{50} of A/PR8 (H1N1) influenza virus. (A) Mice were monitored daily for weight loss. Data points are presented as mean ± SEM. (B) Survival was monitored for 21 d postchallenge. Moribund mice were humanely sacrificed. Data points are presented as mean. (C) Viral titers were determined at 5, 7, and 10 d after PR8 challenge. Data points are presented as median with range. Dotted line indicates limit of detection. Data in (A) and (B) are pooled from three independent experiments. n = 40 young mice and 54 aged mice. For data in (C), n = 8–10 mice per group for each time point. **p < 0.01, Wilcoxon signed-rank test.

**FIGURE 2. Lungs from aged mice have enhanced numbers of inflammatory cells and histological features associated with inflammation after influenza infection and heterosubtypic challenge.**

The lungs of young and aged mice were analyzed for histological changes and inflammatory cells after primary x31 (H3N2) influenza infection and 14 and 21 d after heterosubtypic A/PR8 (H1N1) challenge. (A) Whole lung histopathology score. (B) Bronchioles lymphocyte cuffs. (C) Alveolar macrophages. (D) Alveolar epithelialization. Data are presented as individual data points, with bars representing mean ± SD. n = 5 mice per group.
recent report also showed increased numbers of Trm in aged mice after influenza infection that were correlated with enhanced levels of TGF-β associated with aging (45).

Two characteristic phenotypic markers of Trm are CD103 and CD69 (30, 55). CD103 is the αε subunit of the αεβ7 integrin heterodimer that binds E-cadherin, and CD69 is a c-type lectin that binds to and sequesters S1PR1, required for tissue egress (56–58). Although CD103 is a marker for Trm, CD103 alone is not sufficient to identify Trm cells. A more consistent marker is CD69, which is an important indicator of Trm differentiation and plays a role in retaining Trm in peripheral tissues (59, 60). We found no statistical difference in expression of CD69 or coexpression of CD103 and CD69 in total CD8 Trm (Fig. 4F) or NP-specific Trm (Fig. 4G) in aged mice. These data show that the generation of Trm was not impaired in aged mice and, at least phenotypically, they appear to be similar to Trm cells found in young mice.

**Trm numbers are maintained comparably over time in young and aged mice**

In contrast to Trm found in other sites in the body, such as skin, the numbers of Trm in the lungs of young influenza-infected mice have been shown to decline rapidly during the first 6 mo postinfection (32, 38, 39). To compare Trm maintenance over time in young and aged mice, we enumerated CD8 Trm and NP⁺ Trm out to 261 dpi, again using CD8α and CD3ε intravital staining to distinguish resident and systemic CD8 T cells (54). The numbers of Trm (Fig. 5A) and NP⁺ Trm (Fig. 5B) declined steadily over the first 105 d before stabilizing at a minimal level in both young and aged mice. Notably, the rate of decline of Trm in both young and aged mice was similar, as shown by the slopes of decline over the first 105 d (determined by linear regression analysis). Despite the relatively comparable rate of decline, there were statistically more CD8 Trm in aged compared with young mice at 30 dpi but not at 70–105 dpi.

**FIGURE 3. Lungs from aged compared with young memory mice exhibit enhanced inflammation.**

Young and aged female mice were infected i.n. with 3,000 EID₅₀ ×31. Lungs were examined for histological characteristics and inflammatory cells at 45 dpi with H&E staining. Representative images from young and aged mice. (A) Young mouse showing two small foci of inflammation (upper middle) (original magnification ×40). (B) Young mouse showing chronic interstitial inflammation with alveolar bronchiolization (original magnification ×200). (C) Aged mouse with extensive inflammation in two lung lobes (original magnification ×40). (D) Aged mouse with extensive alveolar bronchiolization with lymphocyte foci (original magnification ×200). (E) Aged mouse with alveolar bronchiolization and a large multinucleated macrophage with intracytoplasmic eosinophilic crystals (original magnification ×200). (F) Aged mouse showing two large lymphocyte foci (iBALT) and alveolar bronchiolization (original magnification ×200).
Taken together with the previous finding, these results show that the generation and maintenance of Trm in the lungs of mice after influenza infection in an aged environment are not impaired.

**The function of Trm in young and aged mice is comparable on a per cell basis**

To directly compare the function of CD8 Trm in young and aged memory mice (~70–85 dpi), we analyzed in vitro function in terms of CTL activity for Trm and systemic T cells (Fig. 6A, 6B). Resident and systemic CD8 lung T cells were flow cytometrically sorted from young and aged mice. Cell numbers were adjusted to achieve comparable numbers of NP-specific cells in the young and aged populations, based on the percentage of NP+ cells determined by NP366-374 tetramer staining. Cells from young and aged memory mice as well as cells from acutely infected young mice, which served as a positive control, were tested for Ag-specific cytotoxicity. Comparable levels of activity were observed for Trm (Fig. 6A) and systemic T cells (Fig. 6B) in both young and aged mice, indicating Trm from aged memory mice were not impaired in CTL function.

In addition, we assessed intracellular cytokine production of IFN-γ and TNF-α by Trm following in vitro stimulation with recombinant NP peptide in young and aged memory mice (Fig. 6C, 6D). The percentages of cells among CD8 T cells producing intracellular IFN-γ (Fig. 6C) and coproducing IFN-γ and TNF-α (Fig. 6D) showed comparable activity in young and aged mice in two independent experiments, based on the proportion of NP+ cells in young and aged mice at each timepoint. These findings differ from a recent analysis of influenza memory mice, demonstrating that CD8 Trm from aged mice were impaired in production of IFN-γ and TNF-α relative to Trm from young mice following stimulation with recombinant NP (45).
We next visualized CD8 Trm in the lungs of young and aged memory mice at 1 and 3 mo postinfection. Although flow cytometry is commonly used to allow quantitation of large numbers of phenotypically different cells isolated from tissues, it was previously demonstrated by quantitative immunofluorescence microscopy that the isolation of CD8 T cells from non-lymphoid tissues is highly inefficient and misrepresents the relative presence of different T cell subsets (30). For example, Trm in the lung were preferentially underrepresented compared with Tem by flow cytometry, whereas quantitative immunofluorescence microscopy showed that a high percentage of lung memory T cells were Trm. Therefore, to obtain a more accurate assessment of the presence of Trm in the lung, we stained unfixed thick (200 μm) lung tissue sections for direct microscopic examination. This allowed us to directly visualize cells and determine their distribution without isolating individual cells. Mice were injected i.v. with anti-CD8b.2 Abs 5 min prior to euthanization, exsanguination, and preparation of lung tissue sections. Tissue sections were then stained with Abs to CD8a to identify total CD8 T cells present in the lung. EpCAM staining was added to identify airways. Resident memory CD8 T cells in the lung section stain exclusively with the CD8a Ab because they are protected from i.v. staining. In contrast, circulating CD8 T cells in the lung sections costain with both CD8a and CD8b.2 Abs and represent cells present in small capillaries in the lung stained during the brief in vivo labeling period. The data in Fig. 7 show representative sections from stained lungs of memory mice at 1 mo postinfection for young (Fig. 7A) and aged (Fig. 7B) mice and at 3 mo postinfection for young (Fig. 7C) and aged (Fig. 7D) mice. The numbers of Trm in 1- and 3-mo young and aged memory mice enumerated by direct counting show that there were not statistically different numbers of Trm or systemic T cells in aged compared with young memory mice (Fig. 8A–D, left side). Taken together with flow cytometry data (Figs. 4, 5), these data clearly demonstrate that there are no defects in the generation or maintenance of Trm populations in an aged environment.

**Aged, influenza memory mice exhibit enhanced inflammation 14 and 21 d after sublethal heterosubtypic influenza virus challenge**

We challenged the young and aged memory mice with a sublethal dose of a heterosubtypic influenza virus (PR8, H1N1) 45 d after initial x31 infection and examined inflammation in the lung by H&E at 14 and 21 d after challenge (Figs. 2, 9). Pathology was generally more severe in aged compared with young mice after challenge (see data for day 14 and day 21 postchallenge on right side of graphs), in terms of overall histopathology score (Fig. 2A), bronchioles lymphocyte cuffs (Fig. 2B), alveolar macrophages (Fig. 2C), and alveolar epithelialization...
Examination of the H&E analysis revealed that the young challenged mice showed minimal evidence of inflammation (Fig. 9A) and lymphocyte foci (Fig. 9C). In contrast, the lesions in aged mice after challenge were much more severe. The aged lungs showed extensive inflammatory lesions (Fig. 9B), alveolar bronchiolization (Fig. 9D), foci of

**FIGURE 7. Visualization of CD8 Trm in young and aged mice following influenza x31 infection.**

Young and aged female mice were infected i.n. with 3,000 EID$_{50}$ x31 (H3N2). Lungs were harvested at 1 and 3 mo postinfection following intravital labeling with anti-CD8b and processed into 200-um thick unfixed sections. A total of 30 optical sections covering 40 μM tissue were taken for each image. Sections were stained with anti-CD8a and anti-CD326 (EpCAM). Representative images show resident CD8 (CD8a$^+$CD8b$^-$), systemic CD8 (CD8a$^+$CD8b$^+$) and EpCAM for visualizing airways. (A) Young, 1 mo postinfection. (B) Aged, 1 mo postinfection. (C) Young, 3 mo postinfection. (D) Aged, 3 mo postinfection. Images are representative from $n = 2–3$ mice per group, with 5–7 images per lung.

**FIGURE 8. Numbers of Trm and systemic T cells in young and aged memory mice before and after heterosubtypic challenge.**

Cell counts were determined from representative lung images as shown in Fig. 7 for 1- and 3-mo young and aged memory mice and Fig. 10 for 1- and 3-mo young and aged memory mice 6 d after heterosubtypic challenge. (A) Numbers of Trm cells in young and aged 1-mo memory mice before and after challenge. (B) Numbers of systemic T cells in young and aged 1-mo memory mice before and after challenge. (C) Numbers of Trm cells in young and aged 3-mo memory mice before and after challenge. (D) Numbers of systemic T cells in young and aged 3-mo memory mice before and after challenge. Data are pooled from two to three mice per group with five to seven images per lung. *$p < 0.05$, **$p < 0.01$, **** $p < 0.0001$, Mann–Whitney.
Intracellular and extracellular eosinophilic crystals within the alveolar macrophage foci of the aged mice (Fig. 9E), alveolar bronchiolization (Fig. 9F) and bronchiolar mucus metaplasia. Overall, the aged memory mice showed enhanced lung inflammation after challenge.

Visualization of T cells in the lungs of young and aged memory mice following heterosubtypic influenza virus challenge

To visualize T cells in the lungs of young and aged influenza memory mice 6 d following sublethal heterosubtypic challenge at 1 and 3 mo after the initial infection, we again analyzed unfixed thick Z-stacked lung sections harvested 5 min after in vivo administration of CD8b.2 Ab (Fig. 10). Numbers of cells resident in the lung, which likely include bona fide Trm as well as Tem and T effector cells that have entered the lung in the 6 d following challenge and are thus protected from staining with in vivo administered Ab, are plotted on the right side of the graphs in Fig. 8A, 8C. It can be seen that, not unexpectedly, there was a dramatic increase in the numbers of T cells in the lungs of both young and aged memory mice after challenge at 1 and 3 mo after initial infection. There was also an increase in systemic T cells (Fig. 8B, 8D).

The data show a high degree of heterogeneity among sections within the lungs of an individual mouse and illustrate the necessity of visualizing and counting multiple areas of the lung within each lung section. The images in Fig. 10 are representative sections from young and aged mice with intermediate numbers of cells. To clearly illustrate the heterogeneity within individual lungs, we show stitched panels of the entire lungs of representative mice (Supplemental Fig. 1). Three individual sections are enlarged from a young mouse (Supplemental Fig. 1A) and an aged mouse (Supplemental Fig. 1B), showing various numbers of cells from separate sections of the same lung.

Despite the heterogeneity, the mean numbers of lung T cells in young and aged mice 6 d after challenge at 1 mo after initial infection were statistically different (Fig. 8A, right side). This difference at 6 d postchallenge was enhanced when mice were challenged at 3 mo after initial infection (Fig. 8C, right side). The total numbers of systemic CD8 T cells showed no difference between young and aged mice challenged at either
timepoint after initial infection (Fig. 8B, 8D, right side). Taken together, these data show that the numbers of T cells in the lung, likely consisting of Trm, Tem, and effector T cells, increase dramatically after heterosubtypic challenge of young and aged memory mice. Importantly, there were a statistically significant higher number of CD8 T cells in the lungs of aged compared with young mice, with the enhancement in aged mice being more dramatic when mice were challenged at 3 mo after initial infection.

**DISCUSSION**

**Review of key results**

Lung Trm are the frontline responders to pulmonary infections, and they also elicit inflammatory responses that can recruit circulating T cells (effector T cells and Tem) to the site of infection (34). In this study, we have monitored the number, longevity, and function of Trm, assessed inflammation, and visualized numbers of Trm generated after influenza infection of young and aged mice. Because immune function declines with age, we anticipated that the numbers and function of lung Trm following influenza virus infection might be compromised in aged compared with young mice. However, our data show that aged mice have more severe lung pathology and generate comparable (determined by direct counting of cells in lung tissue) or greater (determined by flow cytometry) numbers of Trm than young mice after initial infection. We also show that there is a gradual and comparable decline in lung Trm numbers over time in both young and aged mice such that numbers in both groups of mice are relatively comparable 3 mo postinfection. Furthermore, Trm from young and aged memory mice are comparably functional on a per cell basis. Finally, aged memory mice have enhanced lung inflammation and more T cells in the lungs compared with young memory mice after challenge with a heterosubtypic viral infection.

**Inflammation and Trm in memory mice**

Aging is associated with generalized heightened inflammation (inflammaging), and systemic inflammation has been shown to increase CD8 T cell responses, resulting in increased numbers of memory CD8 T cells (61). Thus, it was perhaps not surprising to observe that aged mice had more severe lung pathology and generated comparable or more Trm after initial infection. It has been shown that the majority of Trm in the lung are

![FIGURE 10. Visualization of CD8 Trm in young and aged memory mice following heterosubtypic challenge.](image-url)
maintained within repair-associated memory depots (RAMD) that are temporarily created at the site of tissue injury (62). RAMDs consist of peribronchiolar foci surrounding Krt pods, which are known to be created after regeneration of damaged tissue and are distinct from conventional iBALT structures. Thus, more inflammation in aged memory mice may generate more RAMD-associated reservoirs for Trm.

Another potential reason to expect fewer and functionally impaired Trm in aged mice is because of the well-characterized decline in function of CD4 T cells with aging (7–10, 41, 63–66). CD4 T cells have been shown to be required for normal CD8 T cell and CD8 Trm function (67–71) as well as the optimal development of CD8 T cell memory (67, 68, 70–73). Specifically, it has been shown that CD4 T cells are required for the generation of CD103–CD8 Trm after influenza virus infection. Infection in the absence of CD4 T cells resulted in mislocalization of CD8 Trm in the lung airways (69) that the authors speculated was due to lower levels of CD103 expression on CD8 T cells from CD4-depleted mice, resulting in poorer binding to E-cadherin (69, 74). These authors also showed that CD8 Trm established in the absence of CD4 T cells were functionally impaired in that they were unable to recruit CD8 T cells to the lung airways after heterosubtypic influenza virus challenge (69). Thus, it was possible that aged mice in our studies would manifest the Trm characteristics of CD4-deficient mice; they are mislocalized and nonfunctional. However, our analysis failed to confirm these predictions. First, although we did not use the same software for analysis of localization as the previous study (69), our direct visualization of cells in thick lung sections failed to show gross mislocalization of Trm in the lung airways in aged compared with young, mice, as would have been predicted as a consequence of CD4 defects associated with aging. Second, our in vitro analysis of Trm function in young and aged mice failed to show that aged mice were functionally impaired in terms of CTL activity or intracellular cytokine production, as had been previously reported for CD4-deficient young (69) and aged (45) mice.

Longevity of Trm

It has been shown that Trm in the lung are short-lived (32, 75), contrasting with Trm in other sites such as the skin (76). The decline of lung Trm correlates with the relatively rapid loss of heterosubtypic immunity to influenza virus, described early on by Gerhardt et al. (32, 77). The numbers of lung Trm have been shown to be greatly diminished by 7 mo postinfection (32). Other studies have defined more precise kinetics of decay. For example, Slutter et al. (38) showed an initial steep drop within the first 50 days, followed by a slower decline. Zarnitsyna et al. (39) determined a steep rate of decay of both NP- and polymerase acidic protein (PA)-specific cells by linear regression during the first 45 days after the establishment of memory. It has been shown that interstitial lung Trm are localized within niches in RAMDs, which decline as tissue repair progresses (29). The decline of RAMDs may account for the shorter half-life of lung CD8 Trm compared with those in other mucosal tissues (62). Our data confirm the short half-life of lung CD8 Trm in young mice and, importantly, confirm that the rate of decay is similar in aged mice.

Response after challenge

Both young and aged mice showed a dramatic increase in numbers of lung CD8 T cells, likely consisting of Trm, Tem, and effector T cells after heterosubtypic challenge, with the number in aged memory mice being significantly higher compared with young memory mice, especially at 3 mo after initial infection. There are several possible explanations. It is possible that the higher numbers of lung T cells after heterosubtypic challenge may be a consequence of greater influx of peripheral T cells (Tem and effector T cells), as has been shown for lung airways (78). In support of this possibility, the decline of numbers or function of Trm over time has been shown to lead to a greater influx of peripheral CD8 T cells following heterosubtypic infection (32). However, our studies showed that young and aged Trm were comparable in numbers and function at 70–85 days after initial infection. Alternatively, it is possible that aged mice exhibit enhanced recruitment of cells to the lung after heterosubtypic infection because of the sustained inflammatory state of the aged lung, as it has been shown that inflammation enhances secondary T cell responses (61). Finally, although lung Trm were originally not thought to proliferate (74, 78–80), recent data show that Trm can proliferate after reinfection (35–37). Therefore, enhanced numbers of lung T cells in memory mice after heterosubtypic influenza challenge may be a consequence of recruitment of Ag-specific cells from the periphery, influx of non-Ag-specific T cells due to inflammation, and/or low levels of proliferation in situ (32, 35). Our experiments do not allow us to distinguish these possibilities or to understand why the numbers of cells in aged mice when challenged at 3 mo after initial infection were much higher than those in young challenged mice.

Comparisons with Goplen

This report is only the second study examining Trm in aged mice following influenza infection. Our results are consistent with those of Goplen et al. (45), in that both studies found enhanced lung inflammation and elevated numbers of lung Trm in aged mice when assessed by flow cytometry. However, the Goplen study (45) showed that Trm isolated from aged memory mice failed to express molecules involved in TCR signaling and effector function and were functionally impaired. In contrast, we found Trm from aged mice to be functional in vitro. Furthermore, the Goplen study (45) showed that aged memory mice succumbed to heterosubtypic viral challenge, whereas we showed the aged memory mice survived infection and cleared virus with comparable kinetics as young mice, although aged mice had greater weight loss.

There are experimental differences in the two studies that may contribute to the different experimental results. First,
Goplen et al. (45) infected young and aged mice with influenza PR8 and then challenged them with influenza x31, whereas we infected mice with x31 and then challenged them with a sublethal dose of PR8 (45). This may be a key difference, as the response of lung epithelium, specifically the formation of Krt5 pods essential for RAMD formation, important sites of persistence of Trm in the lung, have been shown to vary postinfection with different strains of influenza virus (81). Thus, the induction of RAMD may have been different for the two experimental protocols, although it is unclear how that might contribute to the disparate results. Second, we showed that aged memory mice were able to survive heterosubtypic viral challenge, whereas the Goplen study (45) had the opposite result. An important difference in our protocols was that Goplen et al. (48) treated mice with FTY720 prior to heterosubtypic challenge, to deplete circulating memory T cells in an attempt to ensure that protection was mediated only by lung-resident Trm (82, 83). The finding that the aged, FTY720-treated memory mice failed to respond to heterosubtypic challenge, to deplete circulating memory T cells in an attempt to ensure that protection was mediated only by lung-resident Trm (82, 83). The finding that the aged, FTY720-treated memory mice failed to respond to heterosubtypic challenge and succumbed to infection was interpreted by the authors to mean that Trm were not functional in vivo. In our studies, we did not treat mice with FTY720 prior to challenge. Because one of the functions of Trm is to recruit other memory T cells to the lung (32), we did not want to inhibit that important function of Trm.

In conclusion, these data show that, unexpectedly, the generation, maintenance, and function of Trm in aged mice is similar to that of young mice. In our studies, heterosubtypic challenge of aged memory mice (memory generated when aged) was accompanied by inflammation and a dramatic increase in the number of T cells in the lung and protection from secondary challenge in terms of survival and viral clearance.

DISCLOSURES
The authors have no financial conflicts of interest.

ACKNOWLEDGMENTS
We acknowledge Dr. Larry Johnson for help with statistical analysis, Dr. David Woodland for review of the manuscript, and the animal care facility at Trudeau Institute for skilled care and maintenance of the aged mice.

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well into old age, but memory generated from aged naive cells functions poorly. Proc. Natl. Acad. Sci. USA 100: 15053–15058.


INFLUENZA-SPECIFIC Trm IN AGED MICE


Supplementary Figure. Representative stitched images showing heterogeneity in numbers of airway Trm in young and aged mice. Young and aged female mice were infected intranasally with 3,000 EID$_{50}$ x31 (H3N2) and challenged at 3 months post infection with 1000 EID$_{50}$ of A/PR8 (H1N1) influenza virus. Lungs were harvested at six days post-challenge, following intravital labeling with anti-CD8β. Whole lung sections were imaged (10X) and stitched using the TileScan feature in Leica LAS AF software. Smaller images are at 20X. **A.** Representative image from young mouse. **B.** Representative image from aged mouse.