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Factors impacting the generation and maintenance of $CD8^+$ tissue-resident memory T cells following respiratory immunization

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By

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2022

Abstract

Factors impacting the generation and maintenance of CD8$^+$ tissue-resident memory T cells following respiratory immunization

By Jenna L. Lobby

Despite the availability of a seasonal vaccine, influenza virus remains a critical burden to human health. The limited efficacy of current influenza vaccine strategies is largely due to the virus' ability to evade established antibody responses. One potential strategy to achieving broad, long-lasting protection is to develop a T-cell based vaccine that recognizes conserved internal epitopes. While cellular immunity cannot prevent influenza infection, pre-existing antiviral T cells can limit viral replication and disease severity. Lung tissue-resident memory CD8$^+$ T cells (CD8$^+$ T_{RM}) are a subset of memory T cells that remain localized within the lung tissue, where they provide rapid protection against viral challenge. However, CD8$^+$ T_{RM} in the lung gradually decline over time, correlating with a decline in cellular immunity to respiratory infection, including influenza. Currently, our knowledge of the mechanisms governing the maintenance of tissue-resident memory CD8$^+$ T cells in the lung remains limited.

The data presented in this thesis identify mechanisms driving generation and maintenance of lung-resident CD8$^+$ T_{RM} in the context of two common vaccination strategies known to promote T cell immunity: recombinant adenovirus and live attenuated influenza vaccine (LAIV). Firstly, studies using adenovirus expressing influenza nucleoprotein (AdNP) have shown that NP antigen persists in immunized animals, resulting in protective CD8$^+$ lung T_{RM} being maintained long-term. Using a lineage tracing approach, we identify alveolar macrophages as the cellular source of persistent NP antigen in the lungs of AdNP-immunized mice. Importantly, depletion of alveolar macrophages following AdNP immunization results in significantly reduced numbers of NP$^+$ CD8$^+$ lung T_{RM}.

LAIV elicits both humoral and cellular immune memory in children, but its efficacy is limited in adults. We hypothesized that pre-existing immunity from past infections and/or immunizations prevents LAIV from boosting or generating *de novo* CD8$^+$ T_{RM}. Using a series of mutated LAIV strains with varying capabilities of escaping pre-existing immunity, we determine that both pre-existing humoral and cellular immunity can limit the effectiveness of LAIV.

Combined, these studies identify several mechanisms that impact the generation and durability of cellular immunity in the lung. Together, the findings presented here will guide future cell-mediated vaccine strategies against respiratory pathogens.

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Acknowledgements

I would first like to thank my mentor, Dr. Jacob Kohlmeier, for all his guidance and encouragement. He is a wonderful mentor who always brings out the best of his students, and I have enjoyed every second of my time as a graduate student in his lab. I would also like to thank all the members of the Kohlmeier lab, past and present, for their advice, support, and camaraderie over the years. I was privileged to work with some of the most dedicated students in our program and I look forward to seeing where their goals take them.

The work in this dissertation was supported by many resources. First, I would like to thank the members of my thesis committee, Jeremy Boss, Anice Lowen, Frances Eun-Hyung Lee, and Luisa Cervantes Barragan, for their advice on my thesis work. I would like to thank Laurel Lawrence for her excellent maintenance of our mouse colony (and supply of delicious baked goods) over the years. A huge thank you must also go to the staff of the Division of Animal Resources for their support in maintaining our mice and equipment. Thank you to the NIH Tetramer Core Facility, Emory University School of Medicine Flow Cytometry Core, and Emory University Pediatric Flow Cytometry Core. A special thank you goes to Aaron Rae and Lisa Bixby for all their help with our cell sorting experiments. Thank you to the members of the Boss and Scharer labs for their help with library preparation and analysis for sequencing experiments. And thank you to the members of the Lowen lab for generating our influenza viruses.

Finally, I would like to thank all my friends and family for their endless love and support. Thank you to my IMP cohort mates, Vincent, Brian, Karla, Devon, Jakob, Tim and Troy, for their friendship, encouragement, and wonderful memories. Lastly, a huge thank you to my parents, Jean and Harry, and my brother, Harry, for always supporting me in my endeavors no matter how far from them it took me. Without them, none of this work would have been possible.

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Chapter I: Introduction

Influenza & human health

Despite the availability of a seasonal influenza vaccine, millions worldwide are infected with the virus each year, with annual deaths reaching into the hundreds of thousands[1]. Improved vaccine strategies that result in cross-protective immunity against a range of influenza virus strains are therefore greatly needed. Influenza virus is a negative-sense single-stranded RNA virus that typically causes acute infection of the respiratory tract. Symptoms of influenza infection range in severity and include local inflammation, as well as a sudden onset of fever, cough, headache, sore throat, runny nose, and/or muscle and joint pain. Although most people recover from symptoms within 1-2 weeks without requiring medical care, high risk individuals (e.g., elderly over the age of 65, young children, pregnant women, individuals with chronic illness, and immunocompromised individuals) are still at risk for severe illness. Occasionally, primary influenza infection results in the development of viral or bacterial pneumonia and can even lead to death. According to the World Health Organization (WHO), seasonal influenza epidemics are responsible for an estimated 3 to 5 million cases of severe illness and approximately 290,000 to 650,000 deaths[2]. In addition to the potential loss of life, annual influenza outbreaks pose a threat to global economies and overall wellbeing due to the cost of healthcare and a loss of productivity from missed work & school.

Influenza virology

There are four types of seasonal influenza virus: Type A, Type B, Type C, and Type D. Influenza A and B viruses circulate within the human population and are responsible for seasonal influenza epidemics. Type C influenza is detected far less frequently within the human population.

To date, Type D influenza virus has not been known to infect humans, although its inability to do so has not been conclusively proven. All four types of influenza viruses are enveloped negative-sense single-strand RNA viruses with a segmented genome[2]. Influenza A and influenza B viruses contain eight RNA segments that encode for 16 proteins (influenza C and influenza D viruses each contain seven segments) [3]. The polymerase segment codes for the viral polymerase proteins PA, PB1, and PB2. The nonstructural gene segment encodes NS1 and NS2. NS1 antagonizes the host antiviral defense by blocking activation of the dsRNA-activated protein kinase R (PKR), thereby allowing viral gene transcription to occur[4]. NS2, also known as nuclear export protein (NEP), mediates export of assembled influenza virus ribonucleoprotein complexes from the nucleus[5]. The matrix gene segment encodes three proteins: the matrix protein M1, the ion channel protein M2, and the protein M42[6]. The nucleoprotein (NP) encapsulates the influenza virus genome, helping to maintain its single-stranded structure.

The hemagglutinin (HA) and neuraminidase (NA) viral glycoproteins are located on the influenza virus outer capsid and help mediate viral entry and release, respectively. Influenza gains entry to permissive cells (e.g., epithelial cells lining the respiratory tract) via the binding of its HA to sialic acid residues and the target cell surface. After binding to sialic acid, HA must be cleaved to induce membrane fusion and result in a productive infection[3]. NA mediates the release of nascent influenza virions by cleaving sialic acid residues on the epithelial cell surface. Influenza A viruses are further classified into subtypes according to the specific combination of HA and NA glycoproteins on the virions' cell surface. Currently, influenza A(H1N1) and A(H3N2) are circulating within the human population[2].

Influenza HA and NA are the main targets of protective antibodies made by B cells against influenza virus. Antibodies are typically directed against the head domain of the HA glycoprotein

and provide protection by preventing virus from binding to the receptors on target cells. However, due to the segmented nature of influenza's RNA genome structure, antigenic drift and antigenic shift (e.g., reassortments) often occur within the genome[7-9]. Therefore, the HA and NA proteins are highly variable. Antigenic drift occurs when mutations accumulate in the virus' RNA genome during replication because of the error-prone nature of the viral polymerase[10]. Mutations due to antigenic drift typically occur within the head region of the HA glycoprotein, where the antibody binding sites for anti-HA antibodies are located, thereby rendering pre-existing antibodies made during a previous infection less effective against mutated strains when they enter circulation[9]. Because these random mutations typically occur within the head region of the HA protein, antibody-mediated immune responses exert selection pressure on the circulating seasonal influenza strains[11, 12]. Influenza strains with variant HA head sequences can therefore be selected for at high frequencies in the population, consequently making individuals more at risk for re-infection during subsequent influenza seasons.

Whereas antigenic drift describes escape mutations that accumulate gradually over time, antigenic shift is less common and results in a more dramatic, sudden change in the virus' genome[8, 10]. Antigenic shift occurs when two different influenza strains infect the same host cell. This allows for potential reassortment, or the interchange of RNA segments, to occur between influenza virus strains of the same type (e.g., between two influenza A viruses or between two influenza B strains). What results is a new subtype of influenza virus that has a combination of HA and NA surface proteins derived from the original strains. This new subtype is different from previously circulating strains, and therefore, cannot be neutralized by pre-existing antibodies already established within the human population[13-15]. Pandemics, such as the 2009 H1N1 swine influenza, are a serious consequence of the combined results of antigenic drift and shift. For example, the 2009 H1N1

influenza virus arose from swine strains independently circulating throughout North America and Eurasia. Multiple reassortments and mutations within porcine hosts resulted in a novel strain that was transmissible to and between humans and could evade pre-existing antibodies[16].

The immune response to influenza virus

Innate recognition of influenza infection

Influenza A virus (IAV) infects epithelial cells lining the respiratory tract. Human influenza A viruses preferentially bind receptor sialyl sugar chains that have SAα2,6Gal sequences[17, 18]. This restriction helps limit the infection to the respiratory tract. Epithelial cells, as well as dendritic cells (DCs) and alveolar macrophages, detect the presence of virus through recognition of pathogen-associated molecular patterns (PAMPs) by their pattern-recognition-receptors (PRRs) [19]. One major class of PRRs that recognize respiratory viruses are the Toll-like receptors (TLRs): TLR3, 7, and 9 are located on late endosomes and recognize dsRNA, ssRNA, and unmethylated CpG DNA, respectively[20-23]. Each of these TLRs leads to transcription of interferon (IFN)-inducible genes which help to control the infection[24]. In addition, viral nucleic acids are recognized in the cytosol by retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). Signaling through these pathways leads to activation of IFN regulatory factor (IRF) and nuclear factor-κB (NF-κB) [25].

When PRRs recognize pathogen-associated molecular patterns (PAMPs) on the virus, a cascade of cytokines and chemokines are released by alveolar macrophages, DCs, and epithelial cells that help drive recruitment of innate immune cells into the infected lung. Type I interferons (IFN-α and IFN-β) are early mediators of the antiviral response in the lung and are largely produced by plasmacytoid DCs (pDCs) and alveolar macrophages[26-28]. IFN α/β signal to nearby

cells to release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6[29, 30]. These pro-inflammatory cytokines signal to other cells to produce inflammatory chemokines such as CCL2, CCL5, CXCL8, and CXCL10 to recruit innate immune cells such as neutrophils, natural killer (NK) cells, and monocytes to the inflamed lung[31]. During a primary IAV infection, DCs produce waves of chemokines that predominantly recruit neutrophils and NK cells to the lung[32]. NK cells directly recognize IAV-infected cells when their activating receptor, NCR1, interacts with influenza HA[33]. This results in cytolysis of the infected cells and increased IFN-ɣ production by the NK cells[34-36]. Furthermore, the early inflammatory milieu in the lung helps promote maturation and trafficking of DCs to the draining lymph nodes. This process is dependent on expression of the chemokine-receptor CCR7 by the DCs to facilitate entry into lymph nodes[37-39]. Trafficking of DCs from the lung to lymphoid tissues is vital to establishing the adaptive response to respiratory infections.

Dendritic cells and the initiation of the adaptive response

Although the innate immune response to influenza successfully dampens the early stages of infection, it is the adaptive response that ultimately eliminates the virus[40]. Following influenza infection, lung resident DCs that lie close to the airway epithelium and within the lung parenchyma (e.g., interstitium) encounter virus or viral antigens and become activated[38, 39]. In the context of influenza infection, most DCs present antigen after being directly infected by the virus. However, they can also acquire antigen by phagocytosing infected cells or virions[41]. These activated DCs upregulate expression of molecules involved in antigen presentation, such as MHC-II and T cell costimulatory molecules CD80, CD86, and CD40, and they then migrate from the lung through the draining lymphatics to secondary lymphoid organs (mainly the mediastinal lymph node

(MLN)) by following a gradient of CCL21 in a CCR7-dependent manner[37-39, 42, 43]. The migration of mature respiratory DCs carrying IAV antigens from the respiratory tract to the MLN is crucial for initiating the T cell response against influenza infection.

There are several subsets of DCs that are important for mediating the adaptive response against influenza infection. Four subsets of $CD11c^{+}$ Siglec F^{-} DCs that have been identified in the respiratory tract based on MHC expression and other surface markers include: (1) CD103+ DCs, (2) $CD11b^{hi}$ DCs, (3) monocyte derived DCs, and (4) the previously mentioned plasmacytoid DCs (pDCs) [42]. Within the first few days of infection, CD103+ DCs, which mainly reside within the airway mucosa, are the most efficient at priming influenza specific $CD4^{+}$ and $CD8^{+}$ T cells. $CD11b^{hi}$ DCs preferentially localize to the submucosa and lung parenchyma and continually present influenza antigens at later timepoints[44]. It has been shown that both $CD103^{+}$ and $CD11b^{hi}$ DCs are minimally represented in the mediastinal lymph node under steady state conditions but accumulate in the lymph node following influenza infection[42].

T cell response to influenza

After migrating to the lymph node, DCs present influenza antigens to naïve $CD8^{+}$ and $CD4^{+}$ T cells via MHC class I (MHCI) and MHC class II (MHCII), respectively. The majority of the CD8+ T cell response is directed towards internal proteins including NP, PA, PB1, and M1. In contrast, $CD4^{+}$ T cells recognize epitopes derived from both external proteins, HA and NA, and the internal proteins NP, PA, and PB1[45-52]. During an acute influenza infection, effector T cells (T_{eff}) proliferate approximately every 6-8 hours, resulting in a substantial increase in their population size[53, 54]. Following activation, effector T cells also upregulate expression of adhesion molecules and begin migrating towards the infected respiratory tract. Effector T cells are first

observed in the lung 6-7 days post-infection with influenza. Their migration depends on several different chemokines and chemokine receptors. For example, the chemokine receptor CXCR3 plays a large role in directing both CD4+ and $CD8^+$ T cells to the lung[55, 56]. In addition, $CD4^+$ influenza-specific effector T cells increase expression of CCR4 after they are activated by DCs. This facilitates their homing to the infected lung by binding MCP-1, MIP-1α, and CCL5[57]. Studies of the human lung have also demonstrated that CCR6 and CXCR3 may be important for trafficking of $CD4^+$ effector T cells to the lung[58]. $CD8^+$ effector T cells upregulate surface expression of CXCR4 and follow a gradient of CXCL12 secreted by neutrophils recruited during the initial stages of infection[59]. $CD8^+$ T cells have also been shown to require CCR5 to migrate from the airways to the lung parenchyma[60]. Once in the inflamed lung tissue, effector T cells need to see their cognate antigen again (either in the context of infected cells or antigen presenting cells (APCs)) to gain full effector function. This secondary antigen exposure is partially mediated by CXCL10, which directs effector T cells to antigen depots in the lung via CXCR3[61].

There are several mechanisms by which effector T cells clear influenza infection. The main function of $CD8^+$ effector T cells during infection is to kill influenza-infected cells. Cytotoxic $CD8^+$ T lymphocytes (CTLs) mediate cell killing by either initiating apoptosis via engagement of the surface cell death receptors Fas-FasL and TRAIL-DR5, or by perforin/granzyme B-dependent granule exocytosis[62-65]. When infected cells are lysed, additional PAMPs and danger-associated molecular patterns (DAMPs) are released that further recruit the innate branch of the immune system. $CD8^+$ effectors also produce a variety of pro-inflammatory cytokines, including IFN-ɣ, TNF-α, and IL-2, which help control the infection[66, 67]. Interestingly, studies have shown that $CD8^+$ T cell function depends on the strength of TCR signaling. For example, strong TCR signaling

favors cytokine production, whereas weaker TCR signal strength correlates with cytolytic activity[68-70].

Antigen-experienced CD4$^+$ T cells also produce pro-inflammatory cytokines and predominantly adopt a Th1 phenotype during influenza infection, although a small portion differentiates into Th17 cells, which have been shown to be particularly effective against a high-dose influenza virus challenge[70]. In addition, CD4$^+$ effector T cells can adopt a T follicular helper (Tfh) phenotype or become a "killer CD4$^+$" T cell. Tfh cells are crucial for the generation and maintenance of germinal center responses. Like cytotoxic CD8$^+$ T cells, cytotoxic CD4$^+$ T cells can directly kill virus-infected cells via perforin and granzyme B[66, 71]. CD4$^+$ T cell differentiation is influenced by cytokine (e.g., IL-21 promotes Tfh development, whereas IL-2 favors formation of cytotoxic CD4$^+$ T cells) and TCR-MHCII affinity (e.g., high affinity favors Th1 development) [72, 73]. Combined, these effector functions result in a rapid decline in lung viral load beginning at day 7 post-infection. Influenza virus is typically cleared from the lung by day 10 post-infection (this coincides with when effector T cell numbers are at their peak in the lung tissue) [74, 75].

While pro-inflammatory cytokines such as IFN-ɣ, TNF-α, IL-1β, IL-6, and IL-17 help to control influenza infection, they also inflict collateral damage to the lung tissue. An overwhelming production of pro-inflammatory cytokines, also known as "cytokine storm," is typical in patients with extreme cases of influenza infection (e.g., during pandemic outbreaks). Damage to the respiratory tract is also caused by the cytolytic granules released by cytotoxic lymphocytes in their effort to eliminate infected cells. Luckily, in addition to pro-inflammatory cytokines, both CD4$^+$ and CD8$^+$ effector T cells produce anti-inflammatory cytokines that help minimize tissue damage[66, 67]. For example, influenza-specific CD8$^+$ effector T cells are major producers of IL-10

during acute influenza infection. Importantly, eliminating IL-10 signaling results in pulmonary inflammation that increases mortality in mice following infection with IAV[67].

T cell memory

Following clearance of influenza virus, the majority (~90-95%) of the expanded effector T cell population dies by apoptosis. What remains is a heterogeneous pool of long-lived, antigen-experienced memory T cells. Memory T cells are divided into three subsets based on effector function(s), migration pattern, and proliferative capacity[76]. Central memory T cells (T_{CM}) are mainly found in peripheral circulation and secondary lymphoid organs (SLO) such as the spleen and lymph nodes. T_{CM} express high levels of CD62L (L-selectin) and CCR7, both cell surface markers known to facilitate entry into lymph nodes[77, 78]. T_{CM} produce IL-2 and proliferate extensively upon antigen re-encounter. Effector memory T cells (T_{EM}) are also found in the blood, spleen, and lymphoid organs. However, they can also traffic through peripheral tissues such as the lung, liver, kidney, GI tract and reproductive tract. Migration into the lung depends on several adhesion molecules, including the integrin VLA-1 and chemokine receptors CXCR3 and CCR5[61, 79]. This is discussed in further detail below. T_{EM} do not express CD62L or CCR7 and are therefore found in much lower numbers in the lymph nodes compared to T_{CM}[76, 80, 81]. Although T_{EM} are much less proliferative compared to T_{CM}, they rapidly produce IFN-ɣ upon antigen re-encounter in the peripheral tissues. A third subset of memory T cells, the tissue-resident memory T cells, will be discussed in more detail below.

Development of T cell memory during influenza A virus infection

The ability of an effector T cell (KLRG1^{lo} CD127(IL-7Rα)^{lo}) to transition into a memory cell precursor (KLRG1^{lo} CD127^{hi}) is determined early during T cell priming and depends on several factors, including cytokine/chemokine exposure, transcription factor expression, and costimulatory molecule signaling[82, 83]. Memory T cell precursors express a unique transcription factor profile that includes Eomes, ID3, Bcl-6, STAT3, Tcf1, Hobit, and Runx3[84, 85], and epigenetic modifications can also impact differentiation into effector and memory T cell subsets[86]. The inflammatory environment within the host also influences the formation of long-lived memory T cells[87]. For example, blocking IFN-ɣ within the lung during IAV infection has been shown to increase accumulation of both effector and memory CD8^{+} T cells in the lung[88]. Furthermore, at later stages of IAV infection, the inflammatory environment of the lung favors memory T cell formation. It follows logically that effector T cells entering the lung over the course of an IAV infection encounter differing levels of inflammation. As previously mentioned, innate effector cells in the lung during the early stages of infection produce pro-inflammatory cytokines, such as IFN-ɣ, in response to virus still present in the tissue[89]. In addition, DCs present antigen to effector T cells, thereby expanding the inflammatory milieu in the inflamed lung[90]. These cytokines promote terminal differentiation of the expanding T_{eff} population, skewing them away from a memory T cell phenotype. However, in addition to pro-inflammatory cytokines, CD8^{+} T_{eff} and regulatory T cells (T_{regs}) within the lung also produce regulatory cytokines such as IL-10[67, 91, 92]. IL-10 has been shown to induce formation of memory precursors via STAT3 signaling[93]. Therefore, as IAV infection progresses, the cytokine milieu more favorably promotes formation of memory T cells.

CD8⁺ tissue-resident memory T cells

Whereas T_{CM} and T_{EM} populations recirculate through the bloodstream to patrol lymphoid and peripheral tissues, respectively, tissue-resident memory T cells (T_{RM}) do not re-enter circulation, and instead, permanently remain within the tissue. To date, T_{RM} have been identified predominantly in barrier tissues such as the skin, gut, lung, and genital tract[94-97]. At these barrier sites, T_{RM} act as sentinels of the immune system by accelerating control against pathogen re-exposure[94, 98, 99]. Canonically, $CD8^+$ T_{RM} are identified by expression of the surface markers, CD69 and CD103, which both help to promote residence. Cells in the bloodstream typically do not express the C-type lectin, CD69. When T cells are activated via cytokine and TCR stimulation, CD69 is expressed and begins forming a complex with the S1P receptor, S1PR1. Normally, S1PR1 binds the sphingolipid metabolite, S1P, which is found in high concentration in the bloodstream, thereby promoting cell egress from the tissue[100]. However, upon binding with CD69, S1PR1 is internalized and destroyed[101]. As a result, T cells are retained within the tissue. Interestingly, it has recently been demonstrated that the functional relevance of CD69 in the generation and maintenance of $CD8^+$ T_{RM} may vary by tissue location. For example, CD69 had no considerable role in formation of CD8+ T_{RM} in the small intestine but did promote $CD8^+$ T_{RM} generation in the kidney[102]. Furthermore, since CD69 expression occurs in response to TCR stimulation and cytokines, it can be transiently expressed by recirculating T cells[103]. The requirement for expression of CD103 (integrin chain αE) as a marker for residence has also been shown to vary with tissue location. CD103 is encoded by the gene ITGAE and binds E-cadherin expressed on the surface of epithelial cells lining the respiratory tract.

The unique molecular signature of T_{RM}, including the expression of CD69 and CD103, is controlled by a key set of transcription factors. Expression of CD69 and CD103 is controlled by

elevated levels of Runx3[104]. Expression of the transcription factors Blimp-1 and Hobit is also associated with downregulation of molecules responsible for T cell exit from tissue[105]. During their development, exposure of T_{RM} to the cytokines TGF-β, TNF-α, and/or IL-33 results in downregulation of the transcription factor KLF2 and its target S1P1[106]. As previously mentioned, S1P1 mediates tissue egress. The T_{RM} transcriptional signature is also characterized by downregulation of the T-box transcription factors T-bet and Eomes. TGF-β signaling results in coordinated downregulation of Eomes, which is necessary for formation of $CD103^{+}$ $CD8^{+}$ T_{RM}. Downregulation of T-bet relieves T-bet-mediated repression of CD103[107]. However, low levels of T-bet are required for maintained expression of IL-15R, a receptor for the cytokine IL-15 which is an important for cell survival[108, 109].

Techniques for identifying and studying T_{RM}

Several methods are used to identify and study tissue-resident memory T lymphocytes. The most simplistic method for assessing residency is to look at expression of CD69 and CD103. However, as mentioned previously, the absence of either of these markers does not entirely disqualify a cell from being resident. Thus, this method of identification has significant limitations when used alone. Methods that combine analysis of surface marker expression and migration patterns are much more reliable techniques. Transplantation and parabiosis are two surgical techniques that are commonly used. Transplanting an organ containing a trackable lymphocyte population onto a recipient allows one to examine infiltration of the graft, as well as donor cell egress into the recipient recirculation[99, 110]. However, ease of achieving successful transplantation varies by type of graft and cell migration can be overestimated due to inflammation from the surgery. Parabiotic surgery involves the conjoining of two congenic mice. Within approximately

one week of the surgery, the vascular systems of the mice are shared and the recirculating lymphocyte populations between the parabionts can be assessed; resident cell populations are expected to fail to equilibrate between corresponding tissues in each parabiont. Parabiosis is an extremely useful technique because it allows for lymphocyte migration to be followed throughout the entire animal and for long periods of time.

Both methods are still used today, however, the more common, accepted standard for defining tissue residence is now widely considered to be intravascular staining. This technique was initially described by David Masopust's group and involves injecting mice intravenously (i.v.) with a fluorophore-conjugated antibody against surface markers expressed by the cell type(s) of interest[111]. Mice are then euthanized three to five minutes post-infection, tissues are harvested, and excess antibody is washed away during processing. If the intravital labeling is successful, then cells circulating throughout the bloodstream are labeled with the i.v. antibody. Within the lymph nodes, almost no cells are stained. Interestingly, a portion of cells in the spleen are also not labeled with the i.v. antibody. These cells are mostly found within the white pulp, with labeled cells localizing to the red pulp. Using this method, Masopust's group found that some i.v. label-negative cells in the lung did indeed stain positive for both CD69 and CD103, and that they exhibited lower levels of CD11a expression[111]. Alternatively, depletion antibodies can be used, as any cells outside the vasculature are protected from the depleting antibody (just as they are protected from the i.v. antibody). Intravascular labeling is used throughout the work presented here and in many of the referenced works. Early studies in cell residency were hampered by contamination from the vasculature, and intravascular staining helps remedy this flaw. However, like all techniques, it is not without caveats. T_{EM} move transiently throughout tissues and could therefore be protected from

the antibody and be included in the i.v. negative pool. It is important to take this into consideration when evaluating tissue-resident memory lymphocyte populations.

In recent years, there has been increased attention given to evaluating lung interstitium- vs. airway-resident cells. This is an important consideration to take given the unique characteristics of each environment. Airway cells can be isolated via bronchoalveolar lavage (BAL) [112-114]. If an i.v. antibody is administered prior to euthanasia, then there should be minimal staining in BAL samples since the lung airways are anatomically separate from blood vessels. Blood cell contamination is still a concern, however, if the blood vessels are damaged due to infection (this is seen more frequently at acute timepoints) or poor technique. Lymphocytes in the lung interstitium can be isolated from the tissue by enzymatically digesting lung tissue. Intravascular staining is required to distinguish cells in the interstitium from those in the pulmonary capillary bed (e.g., blood derived cells that contaminate the sample) [111]. BAL must also be performed prior to lung harvest to remove airways cells. Alternatively, CD11a staining may be used to distinguish long-term airway residents from those simply trapped in the airways (discussed in the next section).

Generation & maintenance of $CD8^+$ T_{RM} within the lung & airways

Following infection with IAV, $CD8^+$ (and $CD4^+$) T_{RM} are found within the lung interstitium, airways, and nasal tissue[96, 115-117]. Overall, the route of migration used by T cells to enter the lung is not very well understood. Some chemotactic signals have been shown to correlate with migration of T_{eff} into the inflamed lung tissue. CXCR6, for example, is upregulated on $CD8^+$ T cells isolated from the lung and airways of mice following intranasal immunization against *M. tuberculosis*. Furthermore, mice that lack CXCR6 have reduced protection when challenged with tuberculosis, suggesting that CXCR6 may be important for establishing $CD8^+$ T cells at sites of

infection[118]. It is thought that CD8$^+$ memory T cells in the airways are partially maintained by continual recruitment of memory T cells to the airways. For example, Slutter et al. showed that expression of CXCR3 is necessary for continual recruitment of cells to the airways, and that loss of CXCR3 expression results in accelerated decline of influenza-specific CD8$^+$ T cells from the airways[119]. Additionally, a study from our group showed that CXCR6 regulates steady-state migration of CD8$^+$ T_{RM} to the airways by binding to the ligand CXCL16 which is expressed on the airway epithelium[120].

As previously discussed, expression of CD69 on the cell surface prevents exit of cells from the tissue and into the blood stream by antagonizing S1PR1 signaling. CD103 binds e-cadherin on epithelial cells, thereby maintaining cells within the lung. Approximately 50-70% of lung T_{RM} express CD69[96, 117]. However, although a portion of CD69$^+$ CD8$^+$ T_{RM} in the lung also expresses CD103, the CD4$^+$ lung T_{RM} pool is completely lacking in CD103[121]. CD103 expression is reduced on memory T cells isolated from the airways when compared to memory T cells isolated from the lung interstitium[111, 122]. Both CD4$^+$ and CD8$^+$ T_{RM} express CD49a, which complexes with CD29 to form the integrin, VLA-1. Following infection with influenza, a large portion of influenza-specific CD8$^+$ T cells express VLA-1[79]. VLA-1 binds collagen types I and IV in the respiratory tract[79, 123, 124]. CD4$^+$ T_{RM} constitutively express high levels of CD11a, also known as integrin alpha a, which is a component of the integrin LFA-1. However, CD8$^+$ lung T_{RM} only express high levels of CD11a in the interstitium and downregulate its expression within ~ 40 hours of entering the airways[125, 126]. The combined efforts of these molecules help retain CD8$^+$ T cells within the lung tissue.

There are several cell intrinsic and cell extrinsic factors that are known to impact the maintenance of CD8$^+$ T_{RM} in the lung. Firstly, development of influenza-specific CD8$^+$ T_{RM} in the

lung requires recognition of cognate antigen in the lung tissue. This is most obviously observed when comparing routes of infection/vaccination. In some tissues, such as the skin and female reproductive tract (FRT), local T_{RM} populations can be established via a "prime and pull" method that recruits circulating $CD8^+$ T cells to the mucosa by applying inflammatory stimuli or topically applying chemokines[127, 128]. However, this is not the case in the lung: intranasal (i.n.) administration of virus/antigen elicits robust populations of $CD8^+$ T_{RM} in the lung but non-pulmonary routes do not[129]. Furthermore, our lab and others have shown that simply exposing $CD8^+$ T cells to the lung environment is not sufficient to induce their differentiation into lung T_{RM} [123, 130, 131]. Rather, antigen must be administered to the pulmonary system to induce conversion of circulating $CD8^+$ T cells into lung T_{RM}[123, 130].

Development of $CD8^+$ lung T_{RM} also requires exposure to locally produced TGF-β. Since influenza virus infects and replicates in epithelial cells, the localization of $CD8^+$ T cells adjacent to antigen exposes them to TGF-β produced by nearby epithelial cells. TGF-β has been well characterized as a factor influencing the development of T_{RM} in the intestine and skin, but it has also been implicated in development of lung T_{RM}[132]. Specifically, exposure to TGF-β has been shown to induce expression of CD103[133]. Influenza virus has been shown to transiently activate TGF-β[134, 135]. This transient activation is likely beneficial in the lung environment so as not to induce unnecessary damage to the lung tissue – over-expression of TGF-β has been shown to induce pulmonary fibrosis and lung disease[136]. In addition to cognate antigen and TGF-β, exposure to IL-15 produced by macrophages and DCs in the lamina propria of the lung is necessary for the long-term survival of $CD8^+$ $CD103^+$ lung T_{RM}[108]. Furthermore, lung T_{RM} express the IFN-inducible transmembrane protein, IFITM3, which has been shown to confer broad antiviral

resistance. This allows lung T_{RM} cells to survive within the lung microenvironment, especially during secondary viral infection[122].

It has been shown that the number of influenza virus-specific $CD8^+$ T_{RM} within the lung interstitium and airways steadily declines over time[137]. Airway $CD8^+$ T_{RM} are susceptible to the nutrient poor environment of the airways and undergo high levels of apoptosis[137]. Within the lung interstitium, $CD8^+$ T_{RM} are likely susceptible to the effects of subsequent respiratory infections, but further studies are needed. Furthermore, T_{RM} and T_{EM} have been found to localize to distinct compartments within the lung interstitium. T_{EM} are more sparsely dispersed throughout the tissue[123]. $CD8^+$ T_{RM} tend to develop around inducible bronchus-associated lymphoid tissue (iBALT), while $CD4^+$ T_{RM} form clusters within iBALT structures. Lung T_{RM} also tend to associate with areas of tissue repair and regeneration following injury due to infection[123, 138, 139]. During respiratory infection, cytolysis along with the harsh anti-viral functions of $CD8^+$ T_{eff} cause tissue damage. Consequently, various cell types accumulate at sites of damage and help mediate repair of the tissue. This results in the formation of special niches in the lung that have been shown to be required for the establishment and maintenance of $CD8^+$ T_{RM}[123, 140, 141]. These sites have been termed repair-associated memory depots (RAMD) [123]. RAMD structures can be identified as cytokeratin-expressing cell aggregates and differ from iBALT in that the $CD8^+$ T_{RM} in RAMD do not form organized lymphoid structures[123, 141, 142]. Since $CD8^+$ T_{RM} in the lung localize to RAMD structures, it is therefore likely that they play a role in protecting areas undergoing repair within the lung following infection[143].

It is also possible that cell-intrinsic factors contribute to the longevity of $CD8^+$ T_{RM} within the lung. For example, it has been shown that $CD8^+$ memory T cells that have encountered antigen multiple times displayed improved longevity compared to memory cells generated from naïve

$CD8^+$ T cells[144]. Furthermore, T_{RM} cells derived from memory cells more readily occupy lung T_{RM} niches compared to T_{RM} derived from naïve cells[144]. These results suggest that memory-derived $CD8^+$ T_{RM} may be capable of receiving additional signals that induce or enforce transcriptional programs facilitating their formation into T_{RM} in RAMD, or that there is simply a higher frequency of T_{RM} precursors (KLRG1^{lo} effector T cells) among memory-derived $CD8^+$ T_{RM}[145]. In addition, it has been shown that $CD4^+$ T cell help in the lung can influence longevity of $CD8^+$ T_{RM}. IFN-ɣ derived from $CD4^+$ T cells induces downregulation of T-bet expression by $CD8^+$ T cells. As previously mentioned, reduced T-bet expression is needed to allow for expression of CD103. $CD4^+$ T cell help has also been shown to transcriptionally modulate the metabolism of $CD8^+$ T cells in the lung such that they can better survive as memory T cells by maintaining a higher spare respiratory capacity[146].

Effector functions of $CD8^+$ T_{RM}

Tissue-resident memory T cells are important mediators of immune responses against reinfection in mucosal and barrier tissues and serve as a critical line of defense against influenza virus. $CD8^+$ T_{RM} rapidly protect against invading pathogens by limiting replication, inflammation, and tissue damage. When present in sufficient numbers, $CD8^+$ T_{RM} mediate protection primarily through the production of cytokines and chemokines to recruit circulating immune cells and through direct lysis of infected cells[94, 96, 98, 99, 127, 128, 130, 147-150]. For example, local encounter of cognate antigen by $CD8^+$ T_{RM} in the female reproductive tract results in the rapid recruitment of circulating immune cells to the infected area[94]. In addition, $CD8^+$ T_{RM} in peripheral tissues, such as the small intestine, are highly cytotoxic due to constitutive expression of granzyme B[96, 97]. Within the lung, $CD8^+$ T_{RM} have been shown to be highly protective against respiratory pathogens.

In the human lung, influenza-specific $CD8^+$ T_{RM} are highly polyfunctional and express gene profiles consistent with a rapid anti-viral response[94, 98]. Furthermore, our group has shown that intra-tracheal transfer of influenza-specific $CD8^+$ T_{RM} into naïve mice provides sufficient protection against respiratory virus challenge in an antigen-dependent manner[99]. Interestingly, the heterologous protection conferred by intra-tracheally transferred $CD8^+$ T_{RM} was dependent on production of IFN-ɣ, as IFN-ɣ deficient T cells were less efficient at limiting viral replication when transferred[97].

$CD8^+$ lung T_{RM} and influenza infection

It is well established that cross-reactive memory T cells significantly improve the immune response to influenza challenge[94, 96-99, 147]. In both human and animal models, it has been shown that increased numbers of cross-reactive memory T cells significantly lower viral loads and improve clinical outcomes[148, 149]. Moreover, studies have demonstrated that the number of $CD8^+$ T_{RM} in the lung correlates with the efficacy of cellular immunity to heterologous influenza challenge[96, 151]. However, while studies of the skin, gut, and FRT indicate that $CD8^+$ T_{RM} remain stable and provide long-lasting protection, $CD8^+$ T_{RM} populations within the lung have been shown to decline, as we and others have shown that the number of virus-specific $CD8^+$ T_{RM} in the lung and airways steadily decreases over time[113, 152-154]. This finding complements ample experimental evidence that cellular immunity to respiratory viral infections declines over time due to loss of virus-specific memory T cells in the lung[94, 96-99, 128, 147-149]. Heterosubtypic immunity against influenza virus declines within 4-6 months post-initial infection, the same amount of time it takes for $CD8^+$ T_{RM} in the lungs and airways to decline to nearly undetectable levels following establishment[96, 151]. The mechanisms behind this loss of $CD8^+$ T_{RM} within the lung have not been

fully elucidated. Interestingly, T_{RM} have been shown to persist long-term in many non-lymphoid tissues. For example, VSV-specific T_{RM} can be detected in the brain for as long as 120 days post-infection, while infection of mice with cutaneous herpes simplex virus results in formation of skin T_{RM} that can be detected for the lifetime of the animal[155, 156]. Exactly why T_{RM} do not persist long-term in the lung to the extent that they do in other tissues is not known. However, a recent study did show that $CD8^+$ T_{RM} in the airways are distinct from those in the interstitium and that airway T_{RM} adopt a transcriptional program in response to their nutrient poor environment that ultimately promotes their cell death[137]. This provides one potential explanation for why the decline in number of virus-specific $CD8^+$ lung T_{RM} over time.

Vaccination strategies against respiratory pathogens

Current influenza vaccine strategy

Studies estimate that the annual influenza vaccine reduces the risk of illness by 40-60%. Currently, the influenza vaccine available in the United States consists of three (trivalent) to four (quadrivalent) strains of inactivated influenza virus that are predicted to be the most common during the upcoming season. Quadrivalent influenza vaccines consist of an influenza A (H1N1) virus, an influenza A (H3N2) virus, and two influenza B viruses. Trivalent vaccines only contain one influenza B virus[157]. The annual influenza vaccine is designed to induce neutralizing antibodies targeted against the viral HA and NA surface proteins. Therefore, because of the genomic changes that occur due to antigenic drift and shift, it is necessary to reformulate the influenza vaccine every year using viral surveillance data obtained during the previous season[158, 159]. Unfortunately, methods of predicting the predominant circulating influenza strains are not always accurate, resulting in vaccines that are poorly matched against seasonal strains.

Furthermore, the high mutation rates of the HA and NA influenza proteins often allow for the virus to escape preexisting humoral immunity[160].

Strategies to enhance T_{RM} in the lung

It is now widely accepted that the number of memory $CD8^+$ T cells, including tissue-resident memory, correlates with the quality of cell-mediated protection against secondary infection. In contrast to the variable antibody response against influenza virus, CD8+ T cells recognize epitopes that are located within internal viral proteins such as NP, PA, PB1, and matrix protein. These epitopes are often conserved across different strains of influenza virus, thereby allowing the T cell compartment to provide cross-reactive immunity against multiple strains of the virus [161-164]. However, the fact that the number of virus-specific $CD8^+$ T_{RM} in the lung interstitium and airways steadily declines over time presents a unique challenge to achieving sustained cross-protection against respiratory challenge. Because T_{RM} serve as critical mediators of cross-protection against influenza infection, considerable efforts are underway to develop vaccines that induce pulmonary T_{RM} and support their maintenance.

Successful T-cell based influenza vaccines must elicit a robust memory T cell response in the relevant location. Since recognition of cognate antigen in the respiratory tract is necessary for formation of lung CD8+ T_{RM}, any successful vaccination must therefore deliver antigen intranasally. Thus far, several animal studies have demonstrated that intranasal immunization with influenza peptide or protein alone, loaded onto DCs, or coupled onto monoclonal antibodies that target antigen to DCs in the respiratory tract results in the generation of CD8+ lung T_{RM} that are highly protective against heterologous influenza challenge [115, 123, 131, 143, 165, 166]. Recently, nanoparticles have also proven an effective method for delivering antigen to the respiratory tract:

delivery of nanoparticles containing influenza nucleoprotein via a single intranasal dose generated NP-specific T_{RM} that conferred protection against influenza challenge [167-169].

Interestingly, despite the proven importance of cognate antigen recognition for optimal lung T_{RM} formation, studies have shown that intranasal delivery of the adjuvant zymosan can bypass this requirement [170]. Studies determined that zymosan stimulated antigen-independent formation of lung T_{RM} via signaling through a dectin-1 receptor, and that when combined with an injectable influenza vaccine, significantly boosted the population of T_{RM} formed [170]. Although alum, the classical adjuvant used in most vaccines in humans, favors antibody generation over a CD8+ T cell response, considerable efforts are now focused on including additional adjuvants, such as zymosan, that can help harness T cell-mediated immunity post-immunization [171]. One way this could be accomplished is by including adjuvants that stimulate a cytokine profile that promotes T_{RM} formation, such as TGF-β, IL-1β, IL-33, and TNFα [172-174]. Additionally, the inclusion of co-stimulatory molecules in influenza vaccines is being investigated to enhance generation and maintenance of lung T_{RM} [145, 175, 176]. Lastly, it is important to note that in addition to influenza virus, intranasal but not parenteral immunization has been shown to elicit respiratory tract T_{RM} against SARS-CoV-2, respiratory syncytial virus (RSV), and *M. tuberculosis* [177-179].

Viral vectors as a vaccine platform for influenza

One promising vaccination strategy against respiratory viruses, including influenza, is virus vector-based vaccines. Recombinant adenoviral vectors that are replication-deficient are of particular interest because they can be easily manipulated, are relatively safe, and can induce strong CD8+ T cell responses [180-182]. Prior work has shown that immunization with recombinant adenoviral vectors expressing influenza proteins induces long-lasting cellular immune protection

against a breadth of influenza A strains [181, 183]. Importantly, immunization with an adenovirus serotype 5 expressing influenza nucleoprotein (AdNP) significantly increases the number of influenza NP-specific CD8+ T_{RM} in the lung for up to 12 months post-vaccination [181]. This is in stark contrast to the decline in virus specific CD8+ T_{RM} observed following primary infection with influenza virus and provides us with a tool for examining the currently unknown mechanisms that drive lung T_{RM} longevity post-infection/immunization. Although the exact mechanism behind the enhanced maintenance of $CD8^+$ lung T_{RM} following intranasal immunization with AdNP is not fully elucidated, subsequent studies revealed NP antigen persists in the lungs of immunized mice[184]. In addition to adenovirus, Modified Vaccinia Ankara (MVA) virus vector-based influenza vaccines are currently in development. For example, an MVA-vaccine that expresses CD4+ and CD8+ T cell epitopes from M1 and NP of influenza A virus (MVA-NP+M1) has been shown to induce influenza A virus-specific T cell protection [185-187]. Following immunization with MVA-NP+M1, individuals experienced decreased viral shedding and viral symptoms following challenge with influenza A virus [186].

A third vaccination strategy that is known to elicit both humoral and CD8+ T cell responses (including CD8+ T_{RM}) is the live attenuated influenza vaccine (LAIV) [188, 189]. Commercially known as FluMist, LAIV is administered intranasally as a spray and is attenuated such that it causes no disease. A study from Donna Farber's group demonstrated that intranasal immunization with LAIV generated cross-protective $CD8^+$ lung T_{RM} whereas the inactivated influenza vaccine did not. Furthermore, injection of LAIV failed to generate lung T_{RM}, again highlighting the importance of targeting the respiratory system during delivery [188]. Unfortunately, the T cell responses observed following administration of LAIV in humans has been highly variable [190].

Given its unreliable efficacy, FluMist was not recommended in the United States market for several years and only recently became available again.

Impact of pre-existing immunity on T_{RM} and vaccine efficacy

A key concern regarding use of virus-based vectors for vaccination is pre-existing immunity to the vector. Unlike controlled animal experiments in which the immune landscape can be tailored to the investigator's needs, the immune status of the human population is much more variable. Pre-existing immunity resulting from prior infection(s) and/or immunization(s) can impact the immune system's response to subsequent infections and immunizations. For example, pre-existing immunity to orthopoxvirus has been shown to prevent formation of antigen-specific T cells following MVA-based immunization against influenza virus yet had minimal impact on the induction and boosting of anti-influenza antibodies [191]. Similarly, pre-existing immunity against human adenovirus serotype 5 (HAd5) is widespread in the population, thus compromising the efficacy of HAd5-based vector vaccines [192]. To circumvent this issue, alternative adenovirus serotypes, including those of human and non-human origin such as chimpanzee, are being developed as vaccine platforms [193]. Currently, recombinant adenovirus vectors of human and chimpanzee origin are being clinically evaluated or have been approved for use against the SARS-CoV-2 pandemic [194-197].

In the case of influenza virus, pre-existing memory can bias a secondary response towards a first antigen exposure and prevent formation of neutralizing antibodies against previously unseen influenza strains. This phenomenon is referred to as original antigenic sin and results in repeated boosting of the initial antigen encountered, even when it is no longer a dominant component of subsequent strains [198, 199]. Although this scenario may be beneficial against challenge with an

antigenically similar strain of influenza virus, there is ample evidence that pre-existing humoral immunity may have detrimental effects on subsequent immune responses, particularly on establishing cross-reactive immunity[200, 201]. In terms of evaluating the impact of pre-existing memory T cells on vaccine efficacy, most efforts have focused on the role of CD4+ memory T cells. For example, pre-existing cross-reactive T cells have been shown to suppress naïve T cells and virus-specific T helper cells in response to natural influenza infection[202, 203]. Recently, a study found that a higher proportion of pre-existing SARS-CoV-2 memory CD4+ T cells correlates with a decrease in the quality of the immune response to subsequent SARS-CoV-2 vaccination[204]. However, despite the proven importance of tissue-resident memory T cells in mediating cross-protection against respiratory challenge, little is known about the impact of pre-existing immunity on the development of *de novo* lung T_{RM} responses following vaccination.

Summary

Tissue-resident memory $CD8^+$ T cells remain localized within their tissue, where they rapidly respond to invading pathogens. Within the respiratory tract, sufficient numbers of $CD8^+$ T_{RM} can limit viral replication and immunopathology. Unfortunately, influenza-specific $CD8^+$ T_{RM} in the lungs and airways steadily decline over time, resulting in significant loss of cross-protection against influenza viral challenge. Despite the proven importance of $CD8^+$ T_{RM} in mediating protection against respiratory infection, several questions regarding their generation and durability remain unanswered. The data presented in this thesis provide critical insight to several mechanisms governing the generation and maintenance of $CD8^+$ T_{RM} following respiratory immunization and/or infection. In Chapter II, we investigate the enhanced maintenance of $CD8^+$ lung T_{RM} following immunization with a recombinant adenovirus. In Chapter III, we evaluate the impact of

pre-existing humoral and cellular immunity on the ability of a live attenuated influenza vaccine to generate *de novo* $CD8^+$ lung T_{RM}. Combined, the results from both studies will inform future design of T cell-based influenza vaccines by providing valuable insight into the factors driving generation and maintenance of lung T_{RM} and thus, the durability of cellular immunity to respiratory pathogens.

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Chapter II: Persistent antigen harbored by alveolar macrophages enhances the maintenance of lung-resident memory CD8$^+$ T cells

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Modified for thesis from accepted version prepared for The Journal of Immunology

Abstract

Lung tissue-resident memory T cells are crucial mediators of cellular immunity against respiratory viruses; however, their gradual decline hinders the development of T-cell based vaccines against respiratory pathogens. Recently, studies using adenovirus-based vaccine vectors have shown that the number of protective lung-resident $CD8^+$ T_{RM} can be maintained long-term. Here, we show that immunization of mice with a replication-deficient adenovirus expressing influenza nucleoprotein (AdNP) generates a long-lived lung T_{RM} pool that is transcriptionally indistinct from those generated during a primary influenza infection. In addition, we demonstrate that $CD4^+$ T cells contribute to the long-term maintenance of AdNP-induced $CD8^+$ T_{RM}. Using a lineage tracing approach, we identify alveolar macrophages as a cell source of persistent NP antigen following immunization with AdNP. Importantly, depletion of alveolar macrophages following AdNP immunization resulted in significantly reduced numbers of NP-specific $CD8^+$ T_{RM} in the lungs and airways. Combined, our results provide further insight to the mechanisms governing the enhanced longevity of antigen-specific $CD8^+$ lung T_{RM} observed following immunization with recombinant adenovirus.

Introduction

$CD8^+$ tissue-resident memory T cells (T_{RM}) are a distinct subset of memory T cells that are established within barrier tissues such as the lung, skin, and reproductive tract, where they provide a critical line of local defense against pathogen challenge. Canonically defined as extravascular cells that express surface markers known to promote retention (such as CD69 and/or CD103), T_{RM} share a core transcriptional signature that promotes their longevity and further distinguishes them from effector and central memory T cell subsets (T_{EM} and T_{CM}, respectively) [1-5]. Within the lung and airways, $CD8^+$ T_{RM} confer protection against a variety of respiratory pathogens, including influenza virus and SARS-CoV-2 [2-4, 6, 7]. Although they do not provide sterilizing immunity, lung resident T_{RM} have been shown to significantly improve the immune response to heterologous influenza infection by rapidly reducing viral loads and limiting immunopathology [1, 8-11]. However, while studies of T_{RM} populations in the skin, intestinal tract, and reproductive tract indicate that $CD8^+$ T_{RM} remain relatively stable within these tissues and provide long-lasting protection, the number of virus-specific $CD8^+$ T_{RM} in the lung steadily declines over time to nearly undetectable levels [8, 12-16]. The mechanisms behind this loss of T_{RM} are not entirely understood, but it has been well established that the decline in lung T_{RM} greatly diminishes the protective capacity of cellular immunity against influenza virus [17].

Given the demonstrated importance of $CD8^+$ lung T_{RM} in mediating protection against pulmonary challenge, identifying mechanisms governing their formation and longevity within the respiratory tract is of great interest. Despite many gaps in our current knowledge, several key factors such as exposure to TGF-β, IL-15, and recognition of cognate antigen within the lung tissue have been identified as important for the development and long-term survival of $CD8^+$ lung T_{RM} [2, 18-22]. Several studies have also investigated the role of co-stimulatory molecules, such as 4-

1BB/4-1BBL, in the formation and accumulation of T_{RM}, as well as their inclusion in vaccine platforms designed to target influenza virus [23-28]. Virus-based vectors, such as replication-deficient adenoviruses (Ad), are of particular interest as a vaccine platform candidate because they can be easily manipulated and have been shown to induce robust memory $CD8^+$ T cell responses against viral and cancer antigens [29-34]. Most recently, Ad vectors have been utilized in the formulation of vaccines against the SARS-CoV-2 pandemic virus [35-38]. One key feature of Ad vectors that contributes to their success in inducing long-lasting cellular immunity is the ability of the vector to persist *in vivo* [39-41]. For example, a recent study demonstrated that Ad vectors can generate local antigen depots that support generation of local immunity [42]. This finding complements prior work that showed that a combined systemic and local immunization strategy using an adeno-based vector that expresses the influenza A nucleoprotein (AdNP) results in formation of NP-specific $CD8^+$ lung T_{RM} that provide protection against heterologous influenza virus for up to 1-year post-immunization, and that influenza NP antigen persists long-term in the lungs of mice following immunization [31, 43]. This starkly contrasts the dynamics of T_{RM} following infection with influenza virus and could provide critical insight to the mechanisms of T_{RM} generation and maintenance within the respiratory tract.

In this present study, we further investigate mechanisms that contribute to the longevity of $CD8^+$ lung T_{RM} and identify the cellular source of persistent antigen in AdNP immunized animals. Prior findings suggested that circulating $CD8^+$ T cells are pulled into the lung T_{RM} pool in AdNP immunized mice, potentially providing an explanation for the enhanced maintenance of lung T_{RM} and duration of protection [43]. Here, we find that $CD8^+$ lung T_{RM} generated following infection with influenza or immunization with AdNP are transcriptionally similar, indicating that cell-extrinsic factors are promoting T_{RM} longevity. In addition, we find that help from $CD4^+$ T cells is important

for maintaining the T_{RM} pool in the lungs and airways of mice immunized with AdNP. Using a combination of lineage tracing experiments and immunofluorescence microscopy, we identify alveolar macrophages as the cellular source of NP antigen in the lungs following intranasal immunization and confirm that depletion of this cell subset reduces the number of $CD8^+$ lung T_{RM} over time. These results provide further insight into the mechanisms driving enhancement of T_{RM} in the respiratory tract following immunization with replication-deficient adenovirus vectors and will inform future design of vector-based vaccines against respiratory pathogens, including influenza virus.

Materials & Methods

Mice

C57BL/6J (WT) and B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J (Ai14) mice were bred in-house or purchased from Jackson Laboratory and were housed at Emory University under specific pathogen-free conditions. Mice were between 8-12 weeks of age at time of infection, after which they were housed in specific animal biosafety level 2 conditions. Both male and female mice were used for experiments. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of Emory University.

Viral infections

Replication-deficient adenovirus serotype 5 expressing influenza (A/Puerto Rico/8/34) nucleoprotein (AdNP) was produced and titered as previously described [31, 44]. Replication-deficient adenovirus serotype 5 expressing Cre recombinase (Ad-Cre) was obtained from SignaGen Laboratories. Prior to all infections, mice were anesthetized using isoflurane (Patterson Veterinary). For primary influenza infection, mice were inoculated intranasally (i.n.) with 30,000 EID_{50} influenza A/HKx31 (x31) in 30uL volume. For adenovirus immunizations, mice were inoculated with $2x10^7$ plaque-forming units (PFU) of adenovirus via both i.n. and subcutaneous (s.c.) routes each in 30uL volume. For secondary infection experiments, mice received either 500 EID_{50} Sendai parainfluenza virus or 30,000 PFU x31 NP N370Q (x31 NP^-) i.n. in 30uL volume. Control groups for challenge experiments received 30uL i.n. of 1X phosphate buffered saline solution (PBS).

Single cell isolation

To distinguish tissue-resident cells from those in circulation, mice were intravenously (i.v.) labeled via tail vein injection of fluorescent anti-CD3e (1.5 ug) or anti-CD45 antibody (4 ug) in 200uL 1X PBS and rested for 5 minutes. Mice were subsequently euthanized by intraperitoneal (i.p.) injection with Avertin (2,2,2-tribromoethanol) followed by brachial exsanguination. Spleen, lungs, and bronchoalveolar lavage (BAL) were then harvested. Lungs were enzymatically digested in Collagenase D (5g/L, Roche) and DNase ($2x10^6$ U/L, Sigma) for 30 minutes at 37C, with occasional mechanical dissociation. To enrich for lymphocytes, lung samples were centrifuged in a 40%/80% Percoll gradient. For Ad-Cre experiments, lungs were digested using Collagenase D (5g/L), DNase ($2x10^6$ U/L), and Dispase (15U/mL, Sigma) and then passed through a 70um filter without centrifugation over a Percoll gradient. Spleens were mechanically dissociated and then RBC lysed. For cell sorts, $CD8^+$ $CD62L^-$ splenocytes were enriched for using a Miltenyi $CD8a^+$ T cell isolation kit and biotinylated anti-CD62L antibody just prior to staining.

Cell staining and flow cytometry

Single cell suspensions were first F_C blocked using murine 2.4G2 antibody. Samples were then stained with influenza-specific tetramer against $NP_{366-374}D^b$ (provided by the National Institutes of Health (NIH) Tetramer Core Facility at Emory University) for 1 hour at room temperature, followed by extracellular staining for 30 minutes. Cell viability was determined using either Zombie fixable viability dye (BioLegend) or 7-AAD. All samples were run on either a Fortessa X20 or a Symphony A3 (BD Biosciences) flow cytometer. Flow cytometry data were analyzed using FlowJo v.10 software.

RNA-sequencing

For each population, 100-2000 cells were sorted on a FACSAria II (BD Biosciences) directly into RLT buffer (Qiagen) containing 1% 2-Mercaptoethanol and total RNA isolated using the Quick-RNA Microprep kit (Zymo Research). All resulting RNA was used as input for the SMART-seq v4 cDNA synthesis kit (Takara) with 12 cycles of PCR amplification. cDNA was quantitated and 200 pg of material was used with the NexteraXT kit and NexteraXT Indexing primers (Illumina, Inc) in 12 cycles of PCR to generate libraries. Samples were quality checked on a bioanalyzer, quantitated by Qubit fluorometer, pooled at equimolar ratios, and sequenced on a NextSeq500 using 75 bp paired-end chemistry at the University of Alabama, Birmingham Helfin Genomics Core. Raw sequencing reads were mapped to the mm10 version of the mouse genome using STAR v2.5.3a [45] and duplicate reads flagged using PICARD (http://broadinstitute.github.io/picard/) filtered based on the uniquely mappable and non-redundant reads. Reads mapping to exons for all unique ENTREZ genes was summarized using GenomicRanges v1.34.0 [46] package in R v3.5.2 and data normalized using custom R/Bioconductor scripts. Differentially enriched genes (DEG) were determined using edgeR v3.24.3 [47] and genes that displayed an absolute log2 fold change (log2FC) > l and a Benjamini-Hochberg false-discovery rate (FDR) corrected p-value < 0.05 were considered DEG. Principal component analysis was performed using the vegan package v2.5.6 using the indicated set of DEG. The sequencing dataset can be accessed in the GEO repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE198980) under accession no. GSE198980.

$CD4^+$ T cell depletion

To deplete $CD4^+$ T cells, mice were first injected i.p. with 200ug of anti-CD4 monoclonal antibody (clone GK1.5, Bio X Cell) or isotype control in 1X PBS, and then injected with 100ug i.p. every 3-4 days afterwards for a total of 1 month of treatment.

Depletion of alveolar macrophages

High potency anionic liposomal clodronate and empty liposomes were obtained from FormuMax Scientific. Mice were anesthetized via injection with Avertin and then given 2mg in 100uL volume intra-tracheally (i.t.).

Immunofluorescence microscopy

BAL was collected and re-suspended in 1X PBS. 30,000-50,000 cells were then concentrated onto a glass slide using a Thermo Shandon Cytospin 4 cytocentrifuge. Slides were subsequently H&E stained using standard protocols or fixed using 75:25 acetone/ethanol. Fixed slides were blocked using FACS buffer containing 1ug/mL murine 2.4G2 antibody, 10% mouse serum, 10% rat serum, and 10% donkey serum. Staining was done in blocking buffer using anti-mouse CD11c-A594 (clone N418, BioLegend), anti-mouse influenza A nucleoprotein-FITC (clone 431, Abcam), rabbit anti-fluorescein-A488 (Life Technologies), and DAPI. Coverslips were applied using ProLong Gold antifade reagent and samples were imaged the following day using a Zeiss Axio Observer Z1 immunofluorescence microscope with an Axiocam 506 monochromatic camera. Image processing was performed with Zen 2 software.

Statistical analysis

Cell counts were determined either manually using a hemocytometer or with a LUNA-II automatic cell counter (Logos Biosystems). Statistical analyses were performed using the GraphPad Prism Software.

Results

CD8$^+$ lung T_{RM} from influenza infected and AdNP immunized mice are transcriptionally indistinct

To determine whether persistent antigen in AdNP immunized mice has any potential cell-intrinsic effects on the genetic program of lung T_{RM} that result in their enhanced longevity, we performed RNA-sequencing to compare the transcriptional profiles of influenza NP-specific lung T_{RM} (CD8$^+$ i.v. antibody$^-$ NP$^+$ CD69$^+$ CD103$^+$) and splenic T_{EM} (CD8$^+$ CD62L$^-$ NP$^+$) from mice either infected with x31 influenza or immunized with AdNP at 1-month (35 days post-infection (d.p.i.), x31 and AdNP) and 1-year (365 d.p.i., AdNP only) time points (**Fig. 1A, B)**. Principal component analysis (PCA) revealed that T_{EM} and lung T_{RM} cluster separately, as expected, at both 1-month (**Fig. 1C**) and 1-year (**Fig. 1D**) post-infection regardless of whether mice were given influenza or AdNP. Interestingly, we identified very few genes that were differentially expressed (DEGs) between lung T_{RM} from AdNP immunized and x31 infected mice at 1-month, suggesting that there is no significant transcriptional difference between lung T_{RM} formed following influenza infection or AdNP immunization (**Fig. 1E**). In contrast, we identified several DEGs between lung T_{RM} on days 35 and 365 post-immunization with AdNP (**Fig. 1F**). Notably, lung T_{RM} from AdNP immunized mice had similar expression of genes from a known core T_{RM} transcriptional program, including *Itgae*, *Cdh1*, *Klf2*, and *S1pr1*, confirming that these cells are bona fide T_{RM} at both timepoints post-immunization [4, 48] (**Fig. 1G**). However, the DEGs observed at 365 days post-immunization were enriched for TGF-β signaling (including *Slc20a1*, *Smad3*, and *Cdh1*) (**Fig. 1G)**. Nevertheless, overall, we did not identify any transcriptional differences that would suggest the persistence of CD8$^+$ lung T_{RM} in AdNP-immunized mice is due to a distinct genetic program that confers increased durability.

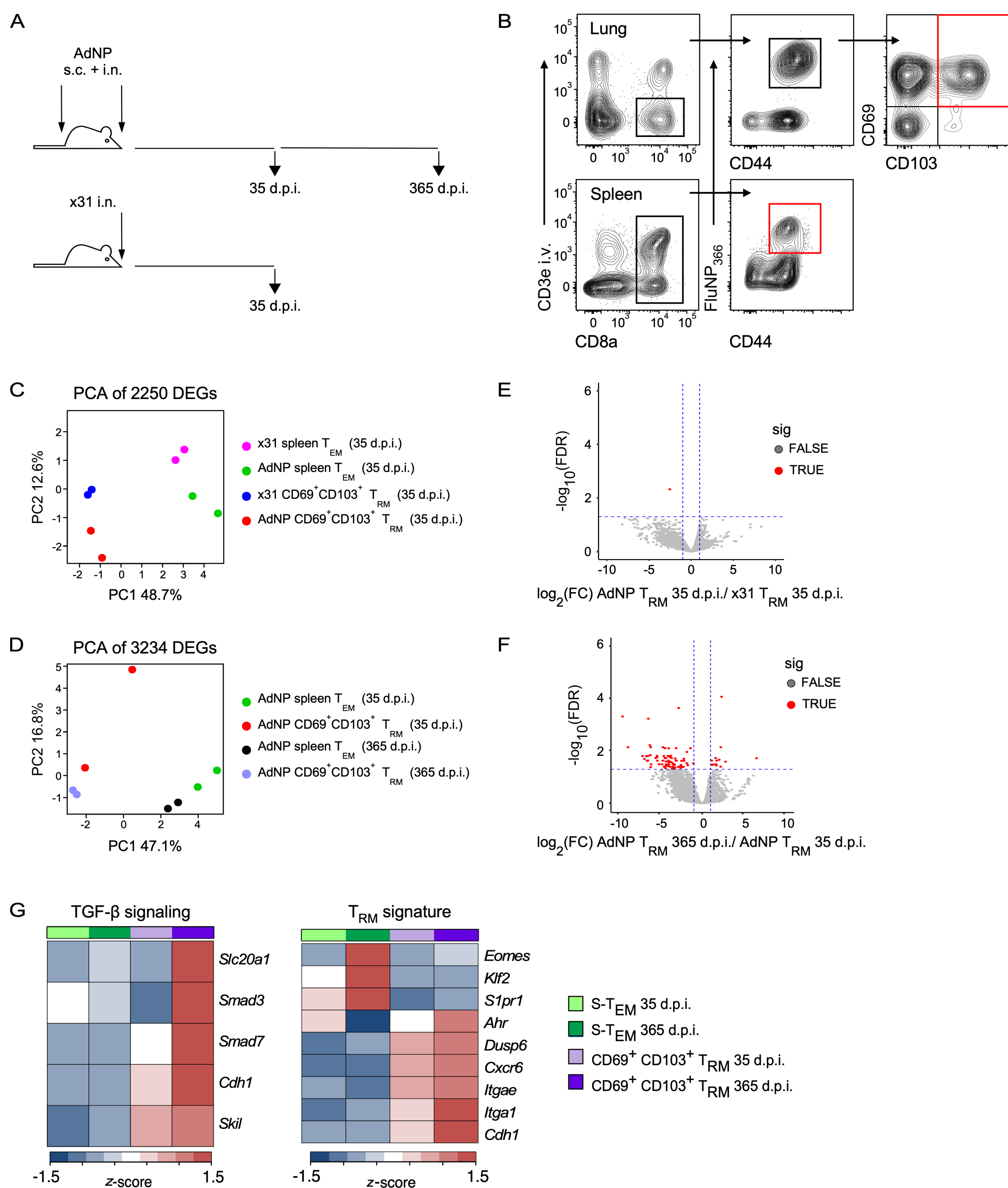

Figure 1. Immunization with AdNP generates CD8^{+} T$_{RM}$ that are transcriptionally alike those generated during a primary infection with influenza. (A) Experimental design. **(B)** Example gating strategy to sort for influenza NP (FluNP$_{366-374}$)-specific splenic T$_{EM}$ and CD69^{+} CD103^{+} lung T$_{RM}$ from mice either infected with x31 influenza or immunized with AdNP. Final

sorted populations are highlighted in red. For x31, n = 10-20 mice per sort, 2 independent sorts. For AdNP, n = 10 mice per sort, 2 independent sorts per timepoint. **(C)** Principal component analysis (PCA) plot of 2250 differentially expressed genes (DEGs) identified in influenza-infected and AdNP-immunized mice on day 35 post-infection. **(D)** PCA of 3234 genes identified on day 35 and 365 post-immunization with AdNP. **(E)** Volcano plot illustrating DEGs identified when comparing $CD69^+$ $CD103^+$ lung T_{RM} from AdNP-immunized mice to those from x31 influenza-infected mice on day 35 post-infection. **(F)** Volcano plot illustrating DEGs between $CD69^+$ $CD103^+$ lung T_{RM} from AdNP-immunized mice on days 35 and 365 post-immunization. **(G)** Heatmaps of selected genes from FluNP-specific splenic T_{EM} and $CD69^+$ $CD103^+$ lung T_{RM} from AdNP-immunized mice related to TGF-ß signaling and a core T_{RM} signature.

$CD4^+$ T cells are important for the maintenance of $CD8^+$ lung T_{RM} following immunization with AdNP

$CD4^+$ T cells are important for proper maintenance and recall of influenza-specific $CD8^+$ memory T cells in the lungs and airways [49, 50]. Furthermore, IFN-γ produced by $CD4^+$ T cells is critical for formation of protective $CD103^+$ $CD8^+$ T_{RM} in the lung following infection with influenza virus [51]. To investigate whether $CD4^+$ T cell-dependent signals are required for long-term maintenance of $CD8^+$ T_{RM} in AdNP immunized mice, we treated mice with anti-CD4 depleting antibody starting 30 days post-immunization **(Fig. 2A)**. After administering depleting antibody for a total of 1 month, we confirmed depletion of $CD4^+$ T cells in all tissues (data not shown) and evaluated the number of influenza NP-specific $CD8^+$ T_{RM} (**Fig. 2B**). As expected, there was no change in the number of $CD8^+$ splenic T_{EM} upon depletion of $CD4^+$ T cells. However,

within the lung and airways, depletion of CD4^{+} T cells resulted in a significant reduction in the number of NP-specific CD8^{+} T_{RM} when compared to mice that received an isotype control antibody. Furthermore, the decrease in the overall number of CD8^{+} T_{RM} in the lungs and airways correlated in both tissues with a reduction in CD69^{+} CD103^{+} NP-specific CD8^{+} T_{RM} (**Fig. 2C, D**). These results show that help from CD4^{+} T cells plays an important role in the long-term maintenance of CD8^{+} T_{RM} in the lungs and airways of mice immunized with AdNP.

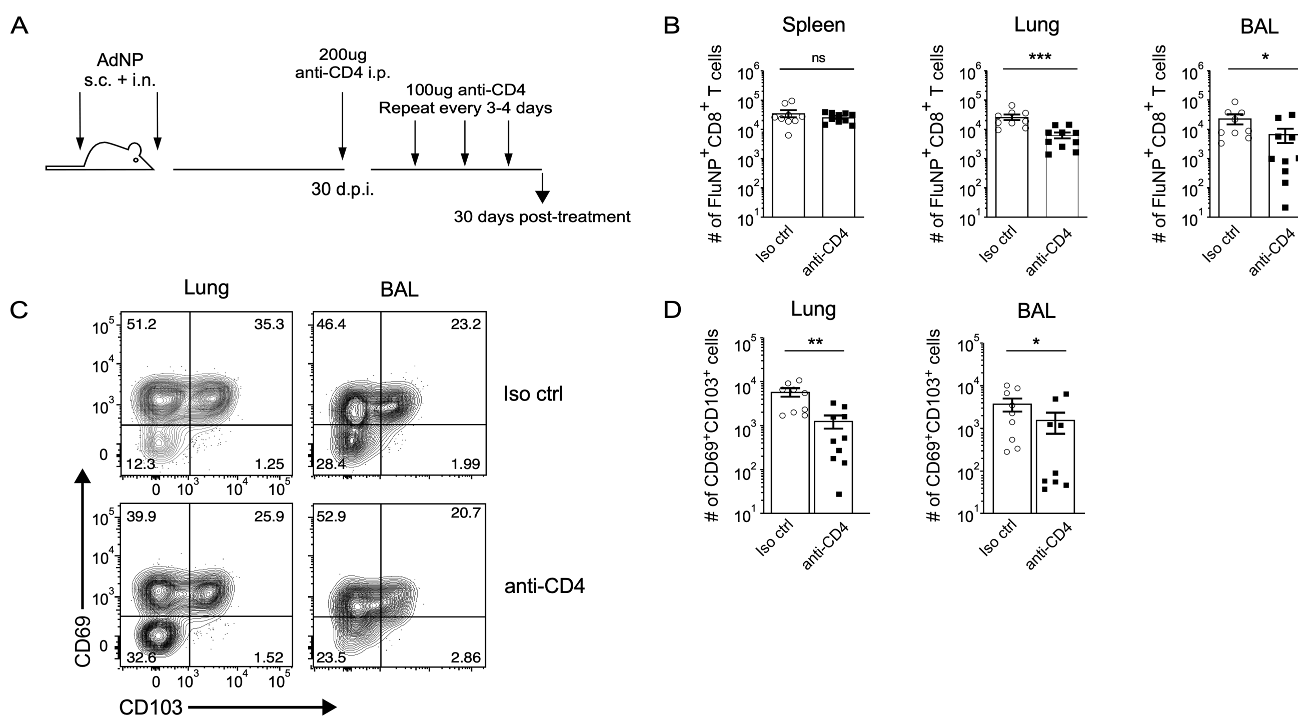

Figure 2. CD4^{+} T cells are important for the maintenance of CD8^{+} T_{RM} following immunization with AdNP. (A) Experimental design. **(B)** Number of FluNP-specific CD8^{+} T_{RM} in the spleen, lung, and bronchoalveolar lavage (BAL) following depletion of CD4^{+} T cells. For isotype control, n = 9 mice total, 2 independent experiments. For anti-CD4, n = 10 mice total, 2 independent experiments. **(C)** Example staining for CD69 and CD103 subsets amongst FluNP-

specific $CD8^+$ T_{RM} in the lung and BAL. **(D)** Number of $CD69^+$ $CD103^+$ FluNP-specific $CD8^+$ T_{RM} in the lung and BAL following depletion of $CD4^+$ T cells. n = 4-5 mice per group, 2 independent experiments. Significance was determined using a Mann-Whitney test. Data represent mean ± SEM. P values are as follows: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, ns= not significant.

Alveolar macrophages harbor persistent influenza NP antigen in AdNP immunized mice

Although it has been established that influenza NP antigen is still present in mice immunized with AdNP for at least several months following immunization, the cellular source of this persistent antigen reservoir has not yet been identified [43]. To investigate this, we used a replication-deficient adenovirus that expresses Cre recombinase protein (Ad-Cre) to immunize Ai14 (tdTomato) reporter mice, in which cells express the reporter protein tdTomato following Cre-mediated recombination. Within the lung, tdTomato fluorescence was predominantly observed in alveolar macrophages up to at least a year post-immunization with Ad-Cre (**Fig. 3A, B)**. Minimal, if any, fluorescence was observed in dendritic cells **(Fig. 3B)**. Even as early as day 4 post-immunization with Ad-Cre, tdTomato fluorescence was mostly limited to alveolar macrophages and was not detected in any other cell type, including fibroblasts, epithelial cells, and monocytes (**Fig. S1**). In addition, the frequency of tdTomato+ alveolar macrophages was varied at all timepoints examined (**Fig. 3C**). To confirm this finding, we obtained a cytospin of BAL samples from naïve and AdNP-immunized mice (90 d.p.i.). Immunofluorescent staining revealed co-localization of CD11c and influenza nucleoprotein at memory following immunization with AdNP (**Fig. 3D**). Combined, these experiments identify alveolar macrophages as the cellular source of persistent influenza NP antigen following intranasal immunization with recombinant adenovirus.

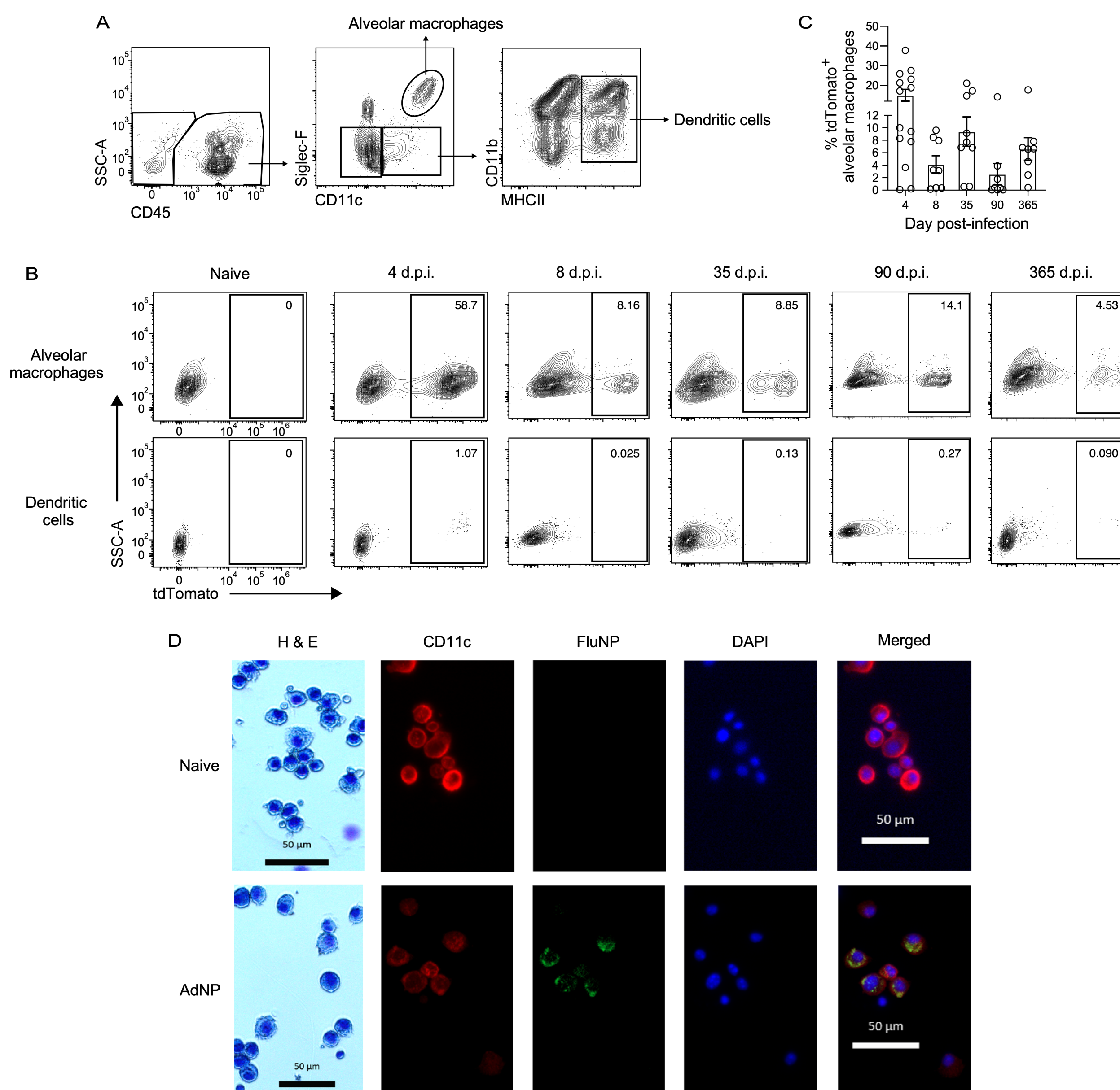

Figure 3. Alveolar macrophages are the cell source of persistent influenza NP antigen in AdNP-immunized mice. (A) Example gating strategy for alveolar macrophages and dendritic cells in the lung of Ai14 reporter mice immunized with Ad-Cre. **(B)** Expression of tdTomato by alveolar macrophages (top row) and dendritic cells (bottom row) from mice immunized with either PBS (naïve) or Ad-Cre at indicated timepoints. **(C)** Frequency of tdTomato^{+} alveolar macrophages at indicated timepoints. n = 3-5 mice per timepoint, 2 experiments per timepoint. **(D)** H&E staining

and immunofluorescence microscopy of BAL samples from mice that were naïve (top row) or 90 days post-immunization with AdNP (bottom row) showing CD11c (red), influenza nucleoprotein (FluNP, green), and DAPI (blue). Original images were taken at 100X magnification.

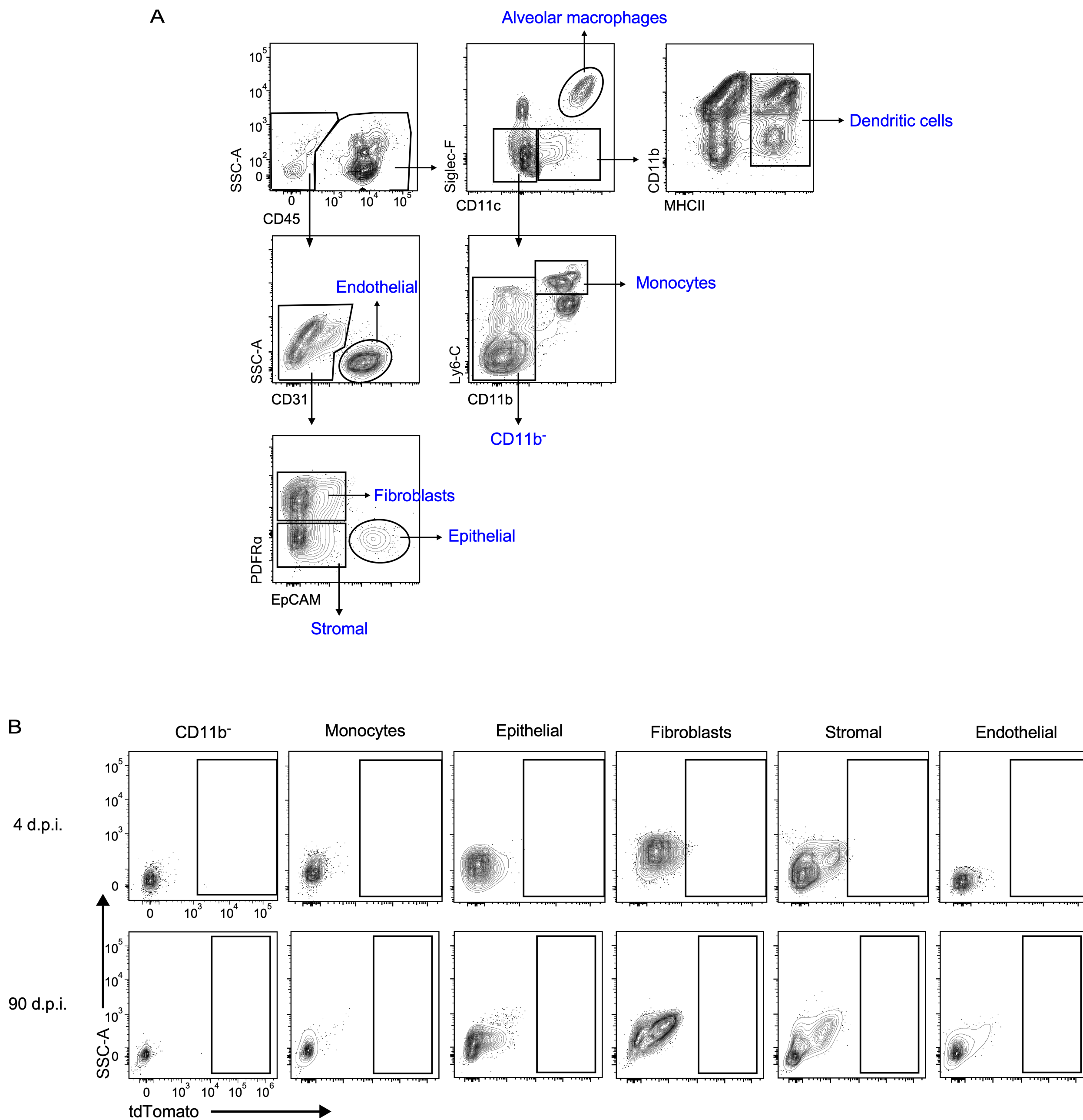

Figure S1. Immunization with Ad-Cre does not result in tdTomato expression in other cell types. (A) Example gating strategy for all cell types analyzed in the lungs of Ai14 reporter mice following immunization with Ad-Cre. **(B)** Example staining for tdTomato in indicated cell types on days 4 and 90 post-immunization with Ad-Cre. n = 3-5 mice per timepoint, 2 experiments per timepoint.

Depletion of alveolar macrophages impairs the longevity of $CD8^+$ T_{RM} in the airways

Alveolar macrophages are among the first responders to influenza infection, and their depletion has been shown to result in increased morbidity and mortality during infection [52, 53]. Given our data shows that alveolar macrophages are a source of prolonged influenza NP antigen following intranasal immunization with AdNP, we hypothesized that depletion of this population would result in decreased maintenance of NP-specific $CD8^+$ T_{RM} over time. We therefore depleted alveolar macrophages by administering liposomal clodronate at 1-month post-immunization with AdNP (**Fig. 4A**). Treatment resulted in a significant reduction of alveolar macrophages in both lung and airways, when compared to injection of empty liposomes or mock treatment (**Fig. S2**). Although depletion of alveolar macrophages had no effect on the number of influenza NP-specific $CD8^+$ T_{RM} in the lung, we did observe a significant decrease in the number of T_{RM} within the airways, including $CD69^-$ $CD103^-$, $CD69^+$ $CD103^-$, and $CD69^+$ $CD103^+$ NP-specific $CD8^+$ T_{RM} (**Fig. 4B, C**). These data further support the observations that alveolar macrophages provide a source of persistent influenza NP antigen in animals following immunization with recombinant adenovirus.

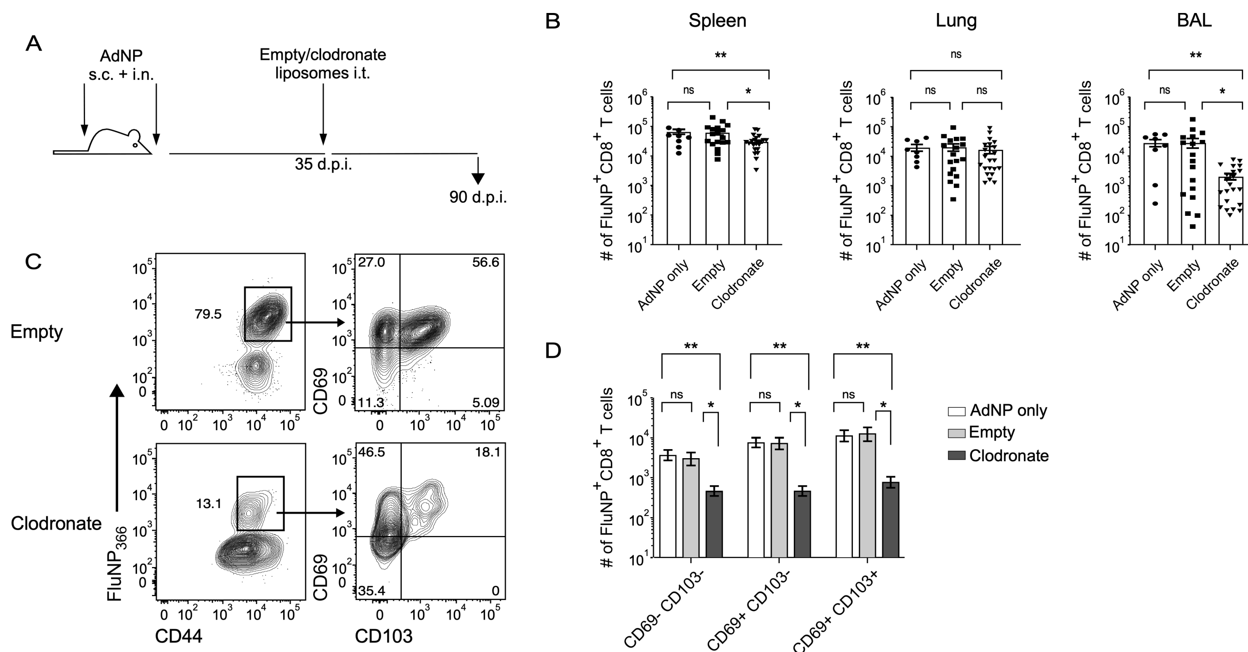

Figure 4. Depletion of alveolar macrophages results in reduced longevity of influenza NP-specific CD8$^+$ T$_{RM}$. (A) Experimental design. **(B)** Number of influenza NP-specific CD8$^+$ T$_{RM}$ in the spleen, lung, and BAL in mice immunized with AdNP and then intra-tracheally administered empty liposomes or liposomes containing clodronate. **(C)** Example staining of influenza NP-specific CD8$^+$ T$_{RM}$ based on expression of CD69 and CD103 from the BAL of mice immunized with AdNP and then treated with empty or clodronate liposomes. **(D)** Number of CD69$^-$ CD103$^-$, CD69$^+$ CD103$^-$, and CD69$^+$ CD103$^+$ influenza NP-specific CD8$^+$ T$_{RM}$ in the BAL. n = 3-8 mice per group, 3 independent experiments. Significance was determined using a Mann-Whitney test. Data represent mean ± SEM. P values are as follows: * = p<0.05, ** = p<0.01, *** = p<0.001, ns= not significant.

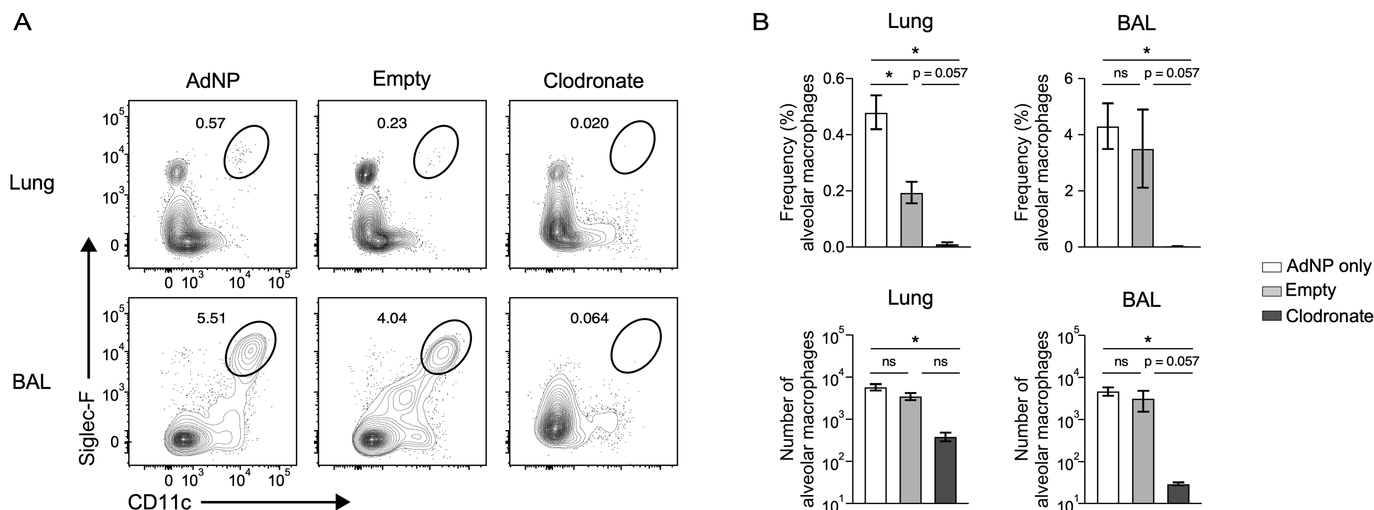

Figure S2. Liposomal clodronate successfully depletes alveolar macrophages in the lung and airways. (A) Example staining for live CD45$^+$ CD11c$^+$ Siglec-F$^+$ alveolar macrophages in the lung and BAL of AdNP-immunized mice 48 hours post-intra-tracheal administration of empty or clodronate liposomes. **(B)** Frequency (top row) and number of alveolar macrophages in the lung and BAL 48 hours after treatment with liposomes. n= 3-5 mice per group, 1 experiment. Significance was determined using a Mann-Whitney test. Data represent mean ± SEM. P values are as follows: * = p<0.05, ** = p<0.01, *** = p<0.001, ns= not significant.

Subsequent respiratory infections impact the longevity of CD8$^+$ T$_{RM}$

Given our identification of alveolar macrophages as an antigen source following intranasal immunization with AdNP, we next investigated the impact of subsequent, antigenically distinct, respiratory infections known to deplete alveolar macrophages on the maintenance of pre-existing NP-specific CD8$^+$ T$_{RM}$. To do so, we first infected AdNP immunized mice with Sendai virus, a murine parainfluenza virus, and then subsequently infected them with an x31 influenza strain that does not present NP antigen on MHC class I (x31 NP$^-$) and would therefore not boost the pre-

existing influenza NP-specific T_{RM} population [54] (**Fig. 5A**). We then examined the impact of these infections on the number of NP-specific $CD8^+$ T_{RM} generated during the initial AdNP immunization. Following initial infection of AdNP immunized mice with Sendai virus, we observed no significant effect on the number of NP-specific $CD8^+$ T_{RM} **(Fig. 5B, D)**. However, the number of NP-specific $CD8^+$ T_{RM} was significantly reduced in the lungs and airways following the second unrelated infection with x31 NP^- influenza when compared to mock infection (**Fig. 5C**). Unsurprisingly, subsequent infection with x31 NP^- had no effect on the number of NP-specific memory $CD8^+$ T cells in the spleen (**Fig. 5C**). Following both Sendai virus and x31 NP^- infections, the number of CD69+ $CD103^+$ NP-specific $CD8^+$ T_{RM} also declined in both the lung and airways (**Fig. 5E**). Lastly, infecting Ad-Cre-immunized reporter mice with Sendai virus and x31 NP^- also resulted in an overall decline in the percentage of $tdTomato^+$ alveolar macrophages when compared to animals that were mock infected (**Fig. 5F**). These findings underscore the importance of alveolar macrophages for the long-term maintenance of NP-specific $CD8^+$ T_{RM} generated following intranasal immunization with AdNP.

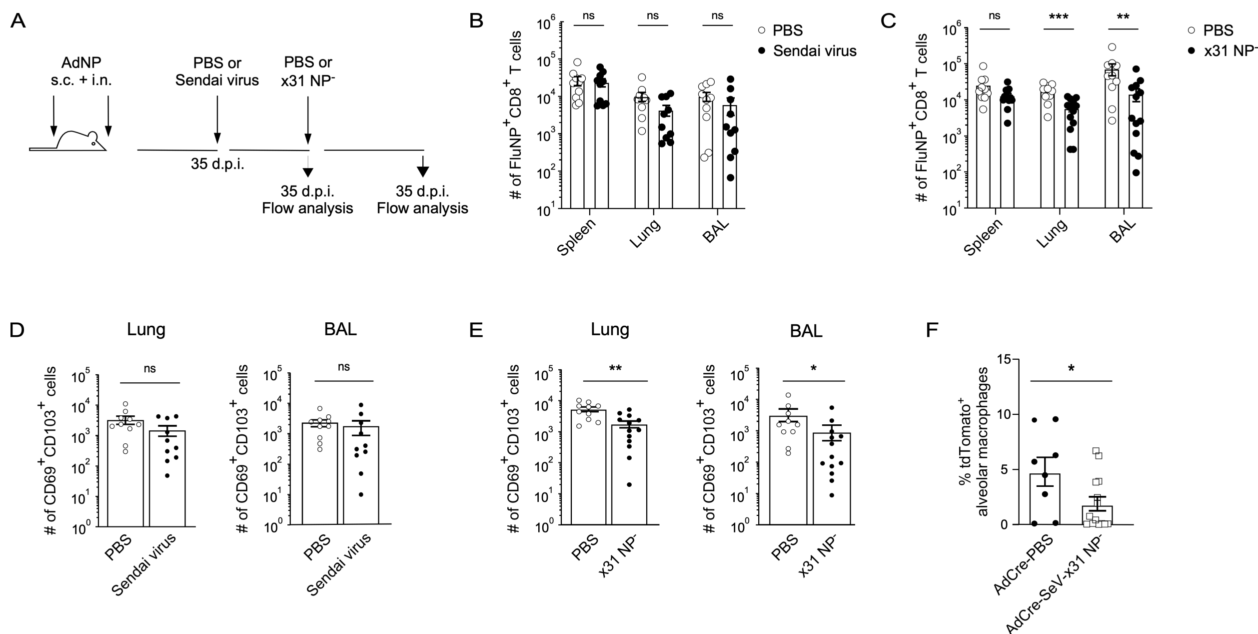

Figure 5. Subsequent respiratory viral infections impact the maintenance of influenza NP-specific CD8$^+$ T$_{RM}$ in AdNP-immunized mice. (A) Experimental design. **(B)** Number of influenza NP-specific CD8$^+$ T$_{RM}$ in the spleen, lung, and BAL of AdNP-immunized mice subsequently infected with Sendai parainfluenza or mock infected with PBS. n = 5 mice per group, 2 independent experiments. **(C)** Number of influenza NP-specific CD8$^+$ T$_{RM}$ in the spleen, lung, and BAL of AdNP-immunized mice subsequently infected with Sendai parainfluenza followed by x31 NP$^-$ influenza. n = 5-8 mice per group, 2 independent experiments. **(D)** Number of CD69$^+$ CD103$^+$ influenza NP-specific CD8$^+$ T$_{RM}$ in the lung and BAL following Sendai parainfluenza infection of AdNP-immunized mice. **(E)** Number of CD69$^+$ CD103$^+$ influenza NP-specific CD8$^+$ T$_{RM}$ in the lung and BAL following infection of AdNP-immunized mice with both Sendai parainfluenza and x31 NP$^-$ influenza. **(F)** Frequency of tdTomato$^+$ alveolar macrophages in Ai14 reporter mice immunized with Ad-Cre and then either mock infected (PBS) or infected with Sendai parainfluenza and x31 NP$^-$ influenza as described in part A. n = 8-14 mice per group, 2 independent experiments. Significance was determined using a Mann-Whitney test. Data represent mean ± SEM. P values are as follows: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, ns= not significant.

Discussion

Establishment of a robust memory T cell response is critical to the success of T-cell based vaccines. However, in the case of respiratory infections, the steady decline of lung resident $CD8^+$ T_{RM} over time presents a concern in generating long-term immunity. Although the mechanism behind this decline is not entirely understood, a recent study has shown that the harsh environment of the lung and airways leads to a high rate of apoptosis amongst $CD8^+$ T_{RM} [55]. However, when mice are immunized with a replication-deficient adenovirus 5 vector that expresses the influenza nucleoprotein (AdNP) using a combination of intranasal and systemic routes, the number of pulmonary $CD8^+$ T_{RM} is maintained long-term [31]. These $CD8^+$ lung T_{RM} are protective for up to at least a year post-immunization and are replenished by circulating T_{EM} being recruited in to the T_{RM} pool after encountering antigen locally within the lung tissue [43]. In this present study, we demonstrate that $CD8^+$ lung T_{RM} generated following immunization with AdNP or infection with influenza virus have similar transcriptional profiles, indicating that immunization with AdNP does not result in cell-intrinsic differences responsible for the improved longevity of $CD8^+$ lung T_{RM}. In addition, we find further support for the model of T_{EM} recruitment to the lungs following immunization with AdNP when we deplete $CD4^+$ T cells post-immunization and observe a significant decrease in the number of $CD8^+$ T_{RM} in the lungs and airways. A prior study showed that $CD4^+$ T cells are required for generation of $CD103^+$ $CD8^+$ lung T_{RM} and promote their migration to the airways via an interferon-γ-dependent mechanism [51]. $CD4^+$ T cell help was also found to be associated with lower expression of the transcription factor T-bet, thereby allowing for TGF-β mediated induction of CD103 [51]. We hypothesize a similar mechanism is occurring in our model. Lastly, lineage tracing using an adenovirus vector expressing Cre recombinase identified alveolar macrophages as a primary cell source of antigen in the respiratory tract. We

were able to confirm this finding by observing colocalization of CD11c and influenza nucleoprotein in airway cells isolated from mice immunized with AdNP. Combined with prior findings, our data support a model in which an antigen reservoir maintained in long-lived alveolar macrophages helps promote differentiation of $CD8^+$ lung T_{RM} from the circulating effector memory T cell pool.

It has been well established that anatomic location directly impacts the development and maintenance of T_{RM} [56]. Since generation of $CD8^+$ T_{RM} in the lung is largely dependent on recognition of local cognate antigen, establishing a reliable antigen depot within the tissue can be critical to the success of immunization against respiratory pathogens. Interestingly, a recent report showed fibroblastic stromal cells in the lung can also serve as long-lived antigen depots following intravenous administration of an Ad5-based vector and support inflationary memory $CD8^+$ T cells in an IL-33-dependent manner [42]. Although this seemingly contrasts our finding, we believe both studies emphasize the importance of the route of immunization in defining unique mechanisms and cell types that can support the maintenance of lung T_{RM} by acting as antigen depots. For example, alveolar macrophages may be shielded from infection by blood-borne vectors, whereas intranasal delivery of the vectors does not allow for efficient infection of cells within the lung parenchyma, such as fibroblastic stromal cells. In addition to lung FSCs, IL-33 is produced by activated macrophages in both humans and mice and has been shown to influence $CD8^+$ T_{RM} formation by downregulating expression of KLF2 and inducing expression of CD69 and CD103 [57-60]. It is therefore conceivable that IL-33 could also be playing a role in our system. Importantly, intramuscular injection remains the most popular route of vaccination and is currently in use for the mRNA- and Ad-vector-based SARS-CoV-2 vaccines, but it can induce suboptimal mucosal immune responses [61]. Alternatively, intranasal administration is widely accepted as the ideal route

for targeting the respiratory tract since it most accurately mimics the natural route of infection. However, prior work showed that combined intranasal and subcutaneous injections of AdNP were superior compared to intranasal immunization alone in establishing a long-lived $CD8^+$ lung T_{RM} population, suggesting that local antigen supply on its own is not sufficient for maximal T cell responses [31].

As some of the first immune cells to encounter pathogens within the airways, alveolar macrophages have long been appreciated as important players in respiratory immunity, with their depletion resulting in increased viral load, pulmonary damage, and mortality following infection with influenza virus [52, 53, 62, 63]. They have also been shown to be important for the establishment and reactivation of memory $CD8^+$ T cells in the lung [21, 64, 65]. One likely reason why intranasal immunization with AdNP results in prolonged maintenance of the $CD8^+$ lung T_{RM} pool is the longevity of alveolar macrophages [66, 67]. In both humans and mice, macrophage populations are maintained for several months to years following formation [68-71]. However, following infection with influenza virus, alveolar macrophages undergo high levels of cell death [72, 73]. In contrast, replication-deficient adenovirus vectors are capable of transducing macrophages without causing their elimination [74-80]. Indeed, we show here that Ad-Cre transduced alveolar macrophages persist in the lung at varying frequencies for up to a year post-immunization. Furthermore, loss of alveolar macrophages through depletion using liposomal clodronate resulted in a significant decline in the number of $CD8^+$ T_{RM} in the airways, despite no significant change in the number of $CD8^+$ T_{RM} within the lung tissue. We hypothesize that this is likely due to alveolar macrophages residing predominantly within the airway spaces. Another possibility is that a small frequency of interstitial macrophages harbors persistent antigen and support maintenance of $CD8^+$ T_{RM} within the lung interstitium, however, we are not able to distinguish this using our system. Lastly, it is also

important to note that although the use of liposomal clodronate is widely considered the standard method of depleting alveolar macrophages, its effects are not exclusive to this population and is known to target dendritic cells as well. Although we cannot ensure that dendritic cells were not impacted during our depletion experiments, our Ad-Cre data clearly demonstrates that dendritic cells are transduced by Ad-Cre at very low frequency and do not persist over time.

Recombinant adenovirus vectors (rAd) display broad tissue tropism and have been shown to transduce a variety of immune and non-immune cell types both *in vitro* and *in vivo.* Although murine macrophages do not express the classical Coxsackie and adenovirus receptor (CAR), rAd vectors including human Ad5 and Ad26, two vectors currently being developed as vaccines for SARS-CoV-2, have been shown to transduce macrophages using scavenging receptors [38, 81-85]. Less is known about the entry methods utilized by non-human rAd vectors; nevertheless, given the ease of altering rAd vectors, targeting adenovirus vectors to specific tissues and cell types is possible and an important consideration for future vaccine design [86]. However, given the sensitive nature of the lung tissue, careful consideration must also be taken when designing any immunization strategy that creates a persistent antigen source. Firstly, although i.n. + s.c. immunization with AdNP results in prolonged protection against challenge with influenza virus, we show here that subsequent unrelated infection(s) also result in a decline in the number of NP-specific $CD8^+$ T_{RM}. This presents a potential limitation to targeting antigen to alveolar macrophages, since recurrent respiratory infections from diverse pathogens are likely to deplete the reservoir. In addition, the possibility of prolonged inflammation and immunopathology that accompany persistent antigen must be more thoroughly evaluated.

In summary, our results identify alveolar macrophages as a persistent cellular source of antigen following intranasal immunization with a recombinant Ad5-based vector that expresses

influenza nucleoprotein. Transduced alveolar macrophages are maintained for at least a year post-immunization and are essential for continual replenishment of the $CD8^+$ T_{RM} pool. Furthermore, we show that persistent antigen does not induce T cell-intrinsic changes that account for the longevity of $CD8^+$ T_{RM}. These results further define mechanisms that promote $CD8^+$ lung T_{RM} generation and maintenance and have important implications for the design of T-cell based vaccines against respiratory pathogens.

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Chapter III: Both humoral and cellular immunity limit the ability of live attenuated influenza vaccines to promote T cell responses

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Modified for thesis from version submitted for publication

Abstract

Live attenuated influenza vaccine (LAIV) elicits both humoral and cellular immune memory in children, but its efficacy is limited in adults. One potential advantage of LAIV is the ability to establish virus-specific tissue-resident memory T cells (T_{RM}) in the respiratory mucosa; however, it is hypothesized that pre-existing immunity from past infections and/or immunizations prevents LAIV from boosting or generating *de novo* $CD8^+$ T cell responses. To determine if we can overcome this limitation, we generated a series of drifted influenza A/PR8 LAIVs with successive mutations in the hemagglutinin (HA) protein, allowing for increasing levels of escape from pre-existing antibody. We also inserted a $CD8^+$ T cell epitope from the Sendai virus nucleoprotein (SeV NP) to assess both generation of a *de novo* T cell response and boosting of pre-existing influenza-specific $CD8^+$ T cells following LAIV immunization. While increasing escape from antibody increased boosting of pre-existing T_{RM}, we were unable to identify *de novo* SeV NP^+ $CD8^+$ T_{RM} following LAIV immunization in PR8 influenza-immune mice, even with LAIV strains that can fully escape pre-existing antibody. As these data suggested a role for cell-mediated immunity in limiting LAIV efficacy, we investigated several scenarios to assess the impact of pre-existing LAIV-specific T_{RM} in the upper and lower respiratory tract. Ultimately, we found that deletion of the immunodominant influenza $NP_{366-374}$ epitope was sufficient to escape pre-existing $CD8^+$ T_{RM} and to establish *de novo* $CD8^+$ T_{RM}. Combined, these studies demonstrate that both pre-existing humoral and cellular immunity can limit the effectiveness of LAIV, which is an important consideration for future design of vaccine vectors against respiratory pathogens.

Introduction

Despite the availability of a yearly vaccine, achieving effective protection against influenza infection remains a challenge. Current vaccination methods primarily focus on generating a strong antibody response against the viral surface proteins hemagglutinin (HA) and neuraminidase (NA). However, these proteins vary between influenza strains and are prone to mutation. For that reason, vaccine strategies that also generate a $CD8^+$ T cell response, which is directed against epitopes within internal viral proteins such as nucleoprotein (NP) and matrix protein and are conserved across influenza strains, are likely to improve efficacy [1-4]. Following clearance of influenza infection, tissue-resident memory $CD8^+$ T cells (T_{RM}) do not re-enter blood circulation and instead remain situated along the respiratory tract where they act as sentinels by rapidly responding to virus re-encounter. Although they alone cannot prevent infection with influenza virus, $CD8^+$ T_{RM} are important for limiting early viral replication and immunopathology and have been shown to play a vital role in mediating protection against a variety of respiratory pathogens in addition to influenza, such as RSV and SARS-CoV-2 [5-9]. Given their demonstrated importance in the cell-mediated response to influenza infection, $CD8^+$ T_{RM} are prime targets (in addition to antibody) for designing vaccines that offer broad protection against a variety of influenza strains.

One influenza vaccine platform that can elicit both antibody and $CD8^+$ T cell responses, including protective $CD8^+$ T_{RM}, is the live attenuated influenza vaccine or LAIV [10, 11]. LAIV is administered as a nasal spray, thus mimicking a natural infection. However, due to its attenuation, it does not replicate effectively in the lower respiratory tract and generates only mild clinical symptoms [12]. Unfortunately, despite its promising ability to engage both arms of the immune system, LAIV has historically performed poorly in adults and has not been recommended for use

in recent years. Interestingly, LAIV did show marked efficacy in children when compared to adults [13-16]. It is hypothesized that this is due to pre-existing influenza immunity in individuals resulting from either prior infection and/or immunization. The implication that pre-existing immunity can impact the ability of a vaccine to "take" in individuals is an important consideration for future design of vaccine regimens, especially against respiratory viral infections. However, the relative contributions of pre-existing humoral and cellular immunity in limiting vaccine efficacy, specifically the resulting virus-specific T cell response, have not been thoroughly investigated.

In the present study, we use LAIV to investigate the impact of pre-existing humoral and cellular immunity on the dynamics of established and *de novo* $CD8^+$ T_{RM} in the respiratory tract. We find that vaccine escape from anti-HA antibodies on its own is not sufficient to generate a *de novo* antigen specific $CD8^+$ lung T_{RM} population in mice that have immunity from a prior influenza infection. However, we do find that LAIV immunization successfully boosts pre-existing influenza specific $CD8^+$ lung $T_{RM,}$ with efficacy increasing as escape from anti-HA antibodies is achieved. Interestingly, LAIV immunization of mice with fewer pre-existing $CD8^+$ lung T_{RM} also failed to generate a *de novo* $CD8^+$ T_{RM} population, possibly demonstrating the important role for virus specific $CD8^+$ T_{RM} within the nasal cavity. Lastly, we show that pre-existing T_{RM} specific for a single immunodominant $CD8^+$ T cell epitope in LAIV is sufficient to prevent generation of *de novo* $CD8^+$ T_{RM} in the respiratory tract following immunization. Combined, these results show that although intranasal immunization with LAIV can boost already existing $CD8^+$ lung T_{RM}, its ability to generate a novel antigen specific $CD8^+$ lung T_{RM} population is hindered by these same pre-formed $CD8^+$ memory T cells. These finding support a previously unrecognized role for T cell-mediated immunity in limiting LAIV efficacy and are important considerations for future design of vaccines against respiratory viral pathogens.

Materials & Methods

Mice

C57BL/6J mice (male and female) were bred in-house or purchased from Jackson Laboratory. All animals were housed at Emory University under specific pathogen-free conditions. Mice were between 8-12 weeks of age at time of infection, after which they were housed in specific animal biosafety level 2 conditions. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of Emory University.

Generation of live attenuated influenza virus

Influenza A viruses used in this study were derived from influenza A/Puerto Rico/8/34 (H1N1) virus (PR8). The wildtype genome sequence of this virus is available in GenBank with accession numbers (AF389115-AF389122). The following amino acid changes were introduced into this PR8 background to confer a live attenuated, cold-adapted phenotype: PB2 N265S, PB1 K391E, PB1 E581G, PB1 A661T and PB1 L319Q. The use of this genotype was based on published reports establishing the attenuating nature of the mutations in a murine model[17, 18]. This strain is referred to herein as PR8 LAIV.

We further modified PR8 LAIV to introduce a foreign epitope from Sendai virus. Using commercial gene synthesis, the nucleotide sequence of the SeV nucleoprotein peptide FAPGNYPAL (5' ttcgcacctggaaattaccctgcacta 3') was inserted into the PR8 influenza A virus neuraminidase (NA) gene at the site corresponding to position 43-44 of the NA stalk domain. The modified NA gene was then cloned into the pDP reverse genetics vector[19] and combined with plasmids encoding the remaining seven gene segments of PR8 LAIV for reverse genetics-based recovery of PR8 LAIV SeV NP virus (referred to as LAIV-SeV NP in the main text).

A further set of virus variants was produced in the PR8 LAIV background, carrying the SeV NP epitope in NA and mutations in the viral hemagglutinin (HA) protein that disrupt known antibody epitopes. The antigenic changes introduced into HA are summarized in Table 1 and described by Das et al. [20]. pDZ[21] reverse genetics plasmids encoding svHA-3, svHA-6, svHA-10 and svHA-12 HA gene segments were kind gifts of Jonathan Yewdell and Christopher Brooke. Finally, an additional amino acid change, N370Q, was introduced into the PR8 LAIV SeV NP svHA-12 virus to disrupt the native T cell epitope present in the NP protein of PR8 (svHA-12 N370Q).

Table 1. Amino acid changes introduced into the PR8 HA sequential variant (sv) viruses

Sequential Variant	Changes in HA, relative to PR8 wild type
svHA-WT	none
svHA-3	G159S, N129D, R78G
svHA-6	G159S, N129D, R78G, R224K, S145N, K163E
svHA-10	G159S, N129D, R78G, R224K, S145N, K163E, E156K, E119K, G173R
svHA-12	G159S, N129D, R78G, R224K, S145N, K163E, E156K, E119K, G173R, E70G, R48K, D225G

Viruses were generated by reverse genetics as previously described[22, 23]. In brief, 293T cells were transfected with reverse-genetics plasmids and then, after a 16–24 h incubation at 37°C, collected and injected into the allantoic cavity of 10-11 day old embryonated chicken's eggs. Eggs were incubated at 33°C for 40–48 h and then chilled overnight prior to harvesting of allantoic fluid. Clarified allantoic fluid was aliquoted and stored at -80°C and virus therein was titrated by plaque

assay on MDCK cells. The presence of introduced mutations was confirmed by Sanger sequencing of viral cDNA corresponding to the modified genome segments or by viral whole genome sequencing on an Illumina myseq platform.

Infections

Prior to all infections, mice were anesthetized using isoflurane (Patterson Veterinary). For primary A/Puerto Rico/8/34 (PR8) influenza infections, mice were infected either intranasally (i.n.) with 20 plaque forming units (PFU) in 30uL volume or via intraperitoneal (i.p.) injection with 10^6 PFU in 200uL volume. For adenovirus infections, mice were inoculated via both i.n. and footpad (s.c.) injections with $2x10^7$ PFU of a replication-deficient adenovirus serotype 5 expressing influenza (A/Puerto Rico/8/34) nucleoprotein (AdNP) (described previously [24, 25]). For LAIV immunizations, mice were given 20,000 -500,000 PFU i.n. in 30uL volume. For protection studies, mice were challenged with either 300,000 EID_{50} (50% egg infectious dose) x31 H3N2 influenza virus or 3,000 EID_{50} Sendai parainfluenza virus i.n. in 30uL volume. For EdU incorporation experiments, mice were dosed i.p. with 1mg of 5-ethynyl-2'-deoxyuridine (EdU, Cayman Chemical) in 200uL volume of sterile 1X PBS.

Single cell isolation

To distinguish tissue-resident cells from those in circulation, mice were intravenously (i.v.) labeled via tail vein injection of fluorescent anti-CD3e (1.5 ug) or anti-CD45.2 antibody (4 ug) in 200uL 1X PBS. Mice were euthanized 5 minutes later by intraperitoneal (i.p.) injection with Avertin (2,2,2-tribromoethanol) followed by brachial exsanguination. Spleen, lungs, bronchoalveolar lavage (BAL), and nasal cavity (NC) were then harvested. Lungs were enzymatically digested in

Collagenase D (5g/L, Roche) and DNase (2x10^6 U/L, Sigma) for 30 minutes at 37C, with occasional mechanical dissociation. To enrich for lymphocytes, lung samples were centrifuged in a 40%/80% Percoll gradient. Nasal cavities were digested in a mixture of Collagenase D (5g/L), DNase (2x10^6 U/L), and Dispase (15U/mL, Sigma) at 37C with mechanical dissociation for a total of 30 minutes. Nasal cavities were then passed through a 70um filter prior to centrifugation. Spleens were mechanically dissociated and then RBC lysed.

Cell staining and flow cytometry

Single cell suspensions were first F_C blocked using murine 2.4G2 antibody. Samples were then stained for 1 hour at room temperature with tetramers against influenza $NP_{366\text{-}374}D^b$, $PA_{224\text{-}233}D^b$, and Sendai parainfluenza $NP_{324\text{-}332}K^b$ (provided by the National Institutes of Health (NIH) Tetramer Core Facility at Emory University). Extracellular staining was then performed for 30 minutes. Cell viability was determined using Zombie fixable viability dye (BioLegend). For experiments utilizing EdU, samples were additionally stained using the Click-iT Plus EdU Flow Cytometry kit (Invitrogen) per the kit's standard protocol. All samples were run on either a Fortessa X20 or a Symphony A3 (BD Biosciences) flow cytometer. $FluNP_{366}^+$ or Sendai NP^+ $CD8^+$ T_{RM} were gated as: singlets, lymphocytes, live cells, $CD4^-CD8\alpha^+$, i.v. label-, $CD44^{hi}$, tetramer$^+$. T_{RM} were additionally gated on CD69 and CD103. For splenic T_{EM}, the i.v. label gate was omitted and all live $CD4^-CD8\alpha^+$ T cells were gated for $CD44^{hi}$ tetramer$^+$. Flow cytometry data were analyzed using FlowJo v.10 software. For experiments evaluating LAIV immunization of PR8-immune or AdNP-immunized mice, a limit of detection was set at $\leq$ 10 cells within the final SeV NP^+ gate.

Plaque assays

Following x31 influenza or Sendai parainfluenza challenge of LAIV-immunized mice, lung viral titers were determined as previously described [26].

Statistical analysis

Cell counts were determined either manually using a hemocytometer or with a LUNA-II automatic cell counter (Logos Biosystems). Statistical analyses were performed using the GraphPad Prism Software.

Results

Live attenuated influenza vaccine elicits protective antigen specific CD8$^+$ lung T$_{RM}$

We first generated an LAIV strain by modifying wildtype (WT) A/PR8 (H1N1) influenza to contain a set of known attenuating point mutations within the polymerase proteins PB1 and PB2 [17, 27]. To identify *de novo* CD8$^+$ memory T cells following immunization, we further modified our LAIV to include the immunodominant epitope from the Sendai parainfluenza nucleoprotein (SeV NP), resulting in our final LAIV-SeV NP strain. We confirmed successful attenuation and generation of epitope-specific CD8$^+$ splenic effector memory T cells (T$_{EM}$) and lung and airway CD8$^+$ T$_{RM}$ following intranasal LAIV-SeV NP immunization *in vivo* (**Fig. 1A-C**). Importantly, CD69$^+$ CD103$^+$ SeV NP$^+$ CD8$^+$ T$_{RM}$ were present in the lungs and airways for up to at least 90 days post-immunization with LAIV-SeV NP (**Fig. 1D-F**).

To determine whether immunization with LAIV-SeV NP confers protection against heterosubtypic influenza challenge, we first immunized animals with 1X PBS, WT PR8 (H1N1), or LAIV-SeV NP and then challenged them with x31 (H3N2) influenza (**Fig. 1G**). Compared to mock immunized mice, mice that were infected with WT PR8 or immunized with LAIV-SeV NP experienced minimal weight loss following x31 influenza challenge (**Fig. 1H**). Mice infected with WT PR8 or immunized with LAIV-SeV NP also had significantly lower lung viral titers by day 4 post-challenge (4 d.p.c.) (when compared to mock immunized mice) and had cleared most virus from the lung tissue within 6 d.p.c. (**Fig. 1I**). Furthermore, we observed no significant difference in the protection conferred by infection with WT PR8 or immunization with LAIV-SeV NP (**Fig. 1H, I**). To assess the protectiveness of SeV NP$^+$ CD8$^+$ memory T cells formed following immunization, we compared weight loss and lung viral titers following Sendai parainfluenza challenge between groups of mice immunized with either LAIV-SeV NP or a version of our LAIV

that lacks the SeV NP epitope (**Fig. 1G**). At 4 d.p.c., we observed no significant difference in morbidity or lung viral titers (**Fig. 1J, K**). However, mice that had been immunized with LAIV-SeV NP did have significantly lower viral lung titers at 6 d.p.c. compared to those that were immunized with an LAIV that does not contain the SeV NP epitope (**Fig. 1K**). Combined, these results show that our LAIV-SeV NP is successfully attenuated and generates protective epitope-specific $CD8^+$ T_{RM} in the lungs and airways following i.n. administration.

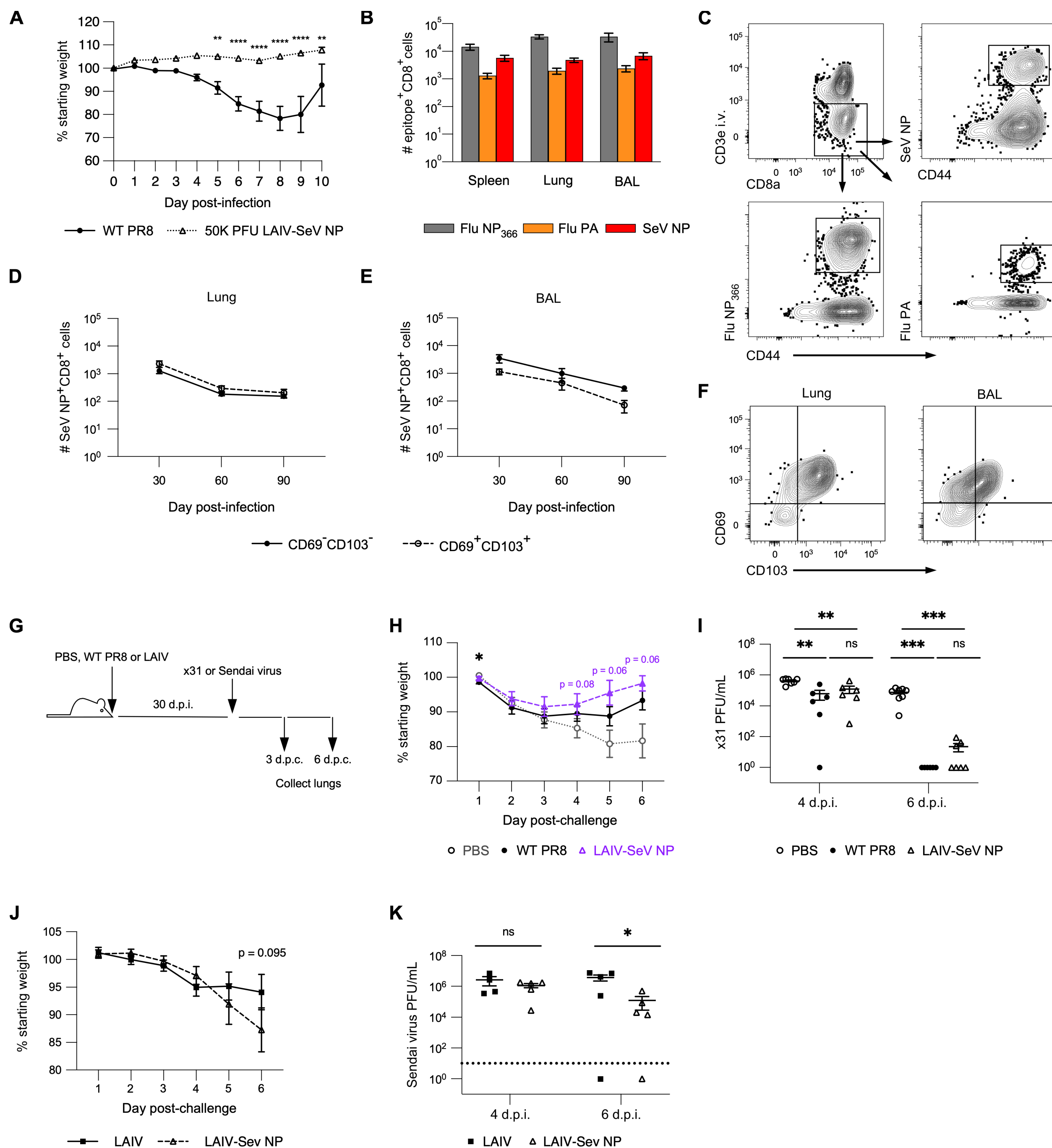

Figure 1. Live attenuated influenza vaccine elicits protective antigen specific CD8⁺ lung T$_{RM}$. **(A)** Percent original weight following i.n. infection with WT PR8 or immunization with LAIV-SeV NP. **(B)** Number of FluNP$_{366}^{+}$, FluPA^{+}, or SeV NP^{+} CD8^{+} T cells in the spleen, lung, and BAL 30 d.p.i. with LAIV-SeV NP. **(C)** Example flow staining for epitope-specific lung T$_{RM}$ in mice 30 d.p.i. with LAIV-SeV NP. **(D, E)** Number of CD69^{-} CD103^{-} or CD69^{+} CD103^{+} SeV NP^{+}

$CD8^+$ T_{RM} in the lung (D) and BAL (E) on indicated d.p.i. with LAIV-SeV NP. **(F)** Example flow staining for CD69 and CD103 amongst SeV NP^+ $CD8^+$ T_{RM} in lung and BAL. **(G)** Experimental design. **(H)** Percent original weight following challenge of mock infected (PBS), WT PR8-infected, or LAIV-SeV NP immunized mice with x31 influenza. **(I)** Lung viral titers on days 4 and 6 post-challenge (d.p.c.) with x31 influenza in mice initially mock infected or given WT PR8 or LAIV-SeV NP. **(J)** Percent original weight following challenge of mice immunized with LAIV +/- SeV NP with Sendai parainfluenza virus. **(K)** Lung viral titers in mice immunized with LAIV +/- SeV NP and then challenged with Sendai parainfluenza virus. p values are as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Immunization with LAIV-SeV NP strains capable of escaping pre-formed antibody fails to generate a *de novo* antigen specific $CD8^+$ T_{RM} population in mice with pre-existing immunity

To assess the impact of pre-formed anti-HA antibody on the efficacy of LAIV-SeV NP immunization, we generated a series of five additional LAIV-SeV NP strains that incorporate previously identified HA mutations that allow for each strain to have increasing ability to escape polyclonal anti-PR8 HA antibodies [20] (**Fig. 2A**). We termed these new LAIV-SeV NP “switch variant” strains based on their original publication: svHA-WT, svHA-3, svHA-6, svHA-10, and svHA-12 [20]. Antigenically, svHA-WT is the same as LAIV-SeV NP and has an HA sequence identical to WT PR8 influenza, differing only by incorporation of the attenuating point mutations in PB1 and PB2, and the SeV NP epitope incorporated into the neuraminidase stalk. In contrast, svHA-12 is the most antigenically distinct from WT PR8 and is fully capable of escaping anti-PR8 HA antibodies (**Fig. 2A**). We first used this series of switch variants to immunize mice that had

been previously infected i.n. with WT PR8 influenza. As expected, svHA-WT immunization generated SeV NP^+ $CD8^+$ lung T_{RM} in naïve animals but not in PR8-immune mice, with most samples in PR8-immune mice falling below the level of detection (**Fig. 2B-D**). Surprisingly, immunization of PR8-immune mice with svHA-12 also failed to generate *de novo* SeV NP^+ $CD8^+$ lung T_{RM} (**Fig. 2E-G**). Both immunizations also failed to generate *de novo* $CD8^+$ T_{RM} in the airways (**Fig. S1**). In fact, none of our switch variant LAIV-SeV NP strains successfully generated SeV NP^+ $CD8^+$ T_{RM} in the lungs or airways of mice that had pre-formed immunity to WT PR8 (**Fig. S2**). All together, these results demonstrate that immunization with LAIV-SeV NP is incapable of generating a *de novo* $CD8^+$ T_{RM} population in mice with pre-existing immunity, even when the pre-formed humoral response is evaded.

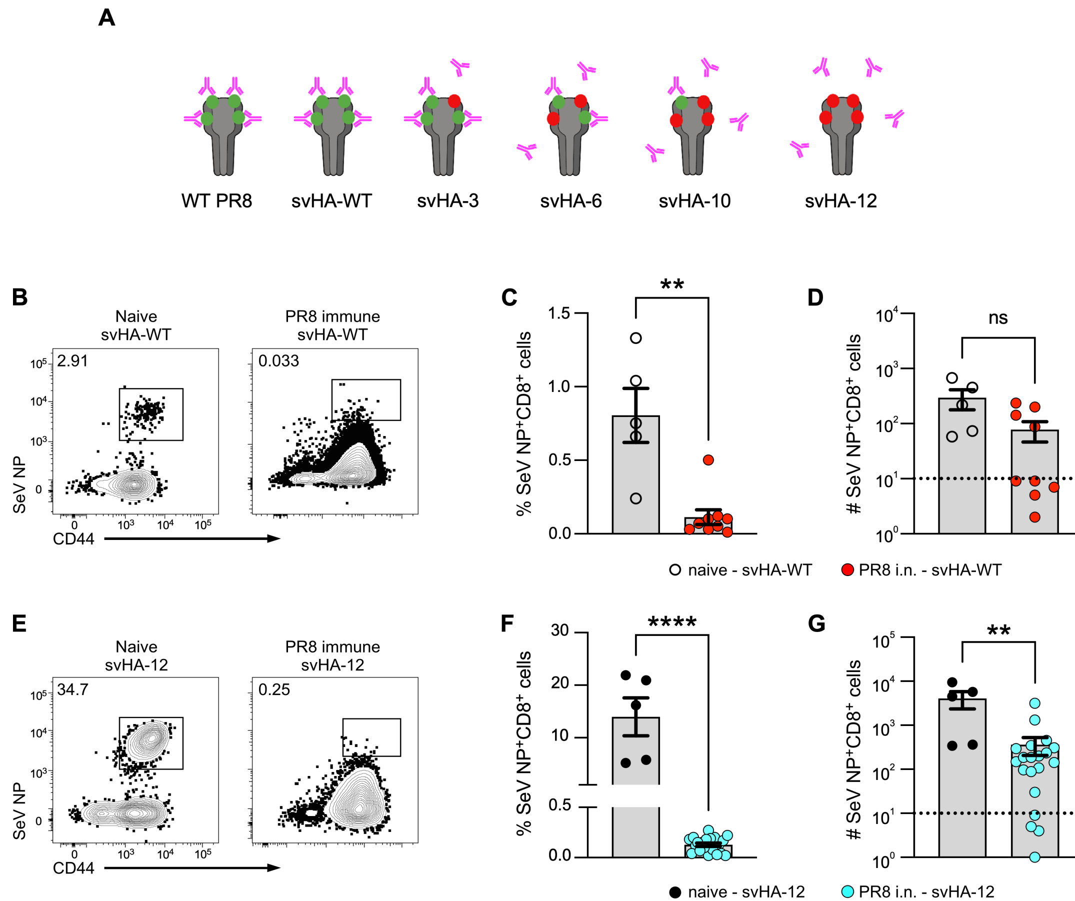

Figure 2. Immunization with LAIV-SeV NP strains capable of escaping pre-formed antibody fails to generate a *de novo* antigen specific CD8⁺ T_{RM} population in mice with pre-existing immunity. (A) Design of switch variant (sv) drifted LAIV-SeV NP strains that have either a WT HA sequence (svHA-WT) or a mutated HA sequence (svHA-3, svHA-6, svHA-10, svHA-12) with point mutations that allow for increasing escape from pre-existing anti-HA antibodies. **(B)** Example flow staining for SeV NP^+ $CD8^+$ T_{RM} in the lung of naïve (left plot) or PR8-immune (right plot) mice subsequently immunized with LAIV svHA-WT. **(C, D)** Frequency (C) and number (D) of SeV NP^+ $CD8^+$ lung T_{RM} 30 d.p.i. of naïve or PR8-immune mice with LAIV svHA-WT. **(E-G)** Same as B-D except with LAIV svHA-12 immunization. p values are as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Limit of detection (LOD) indicated with dotted line at 10^1.

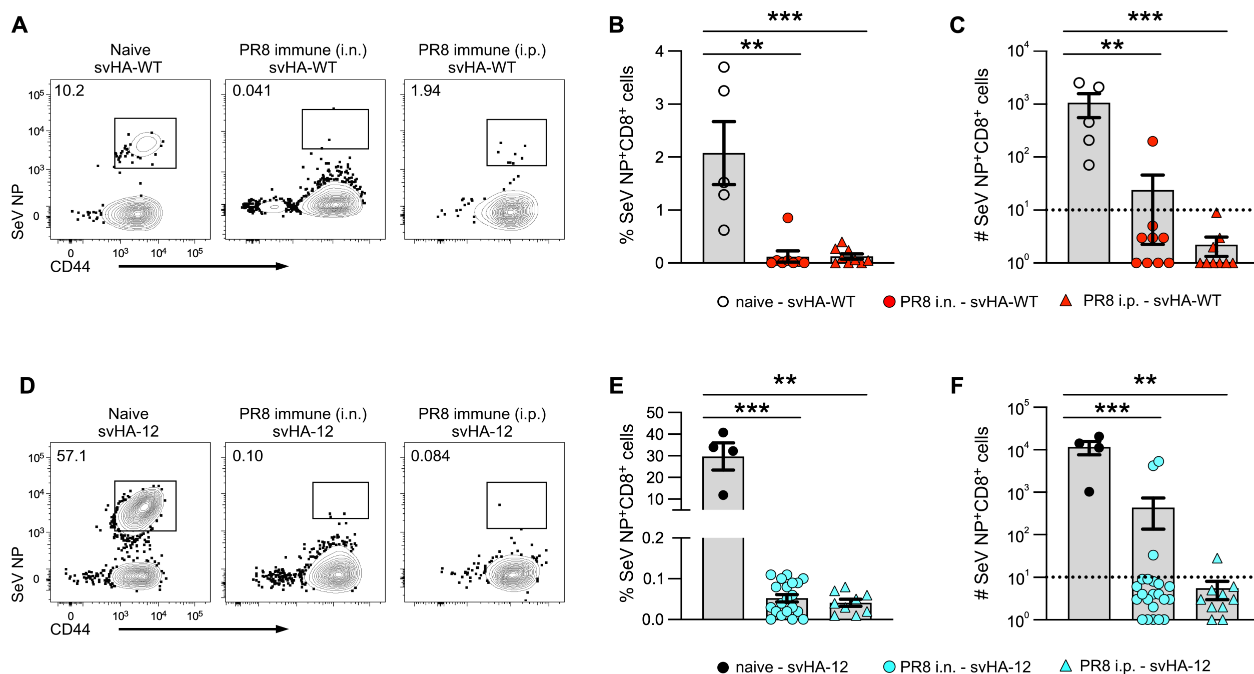

Figure S1. LAIV immunization of PR8-immune mice fails to generate *de novo* CD8$^+$ T$_{RM}$ in the airways. (A) Example staining for SeV NP$^+$ CD8$^+$ T$_{RM}$ in the BAL of naïve or PR8-immune (i.n. or i.p. administration) mice 30 d.p.i. with svHA-WT. **(B, C)** Frequency (B) and number (C) of SeV NP$^+$ CD8$^+$ BAL T$_{RM}$ in naïve or PR8-immune mice 30 d.p.i. with svHA-WT. **(D-F)** Same as A-C except with svHA-12. p values are as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

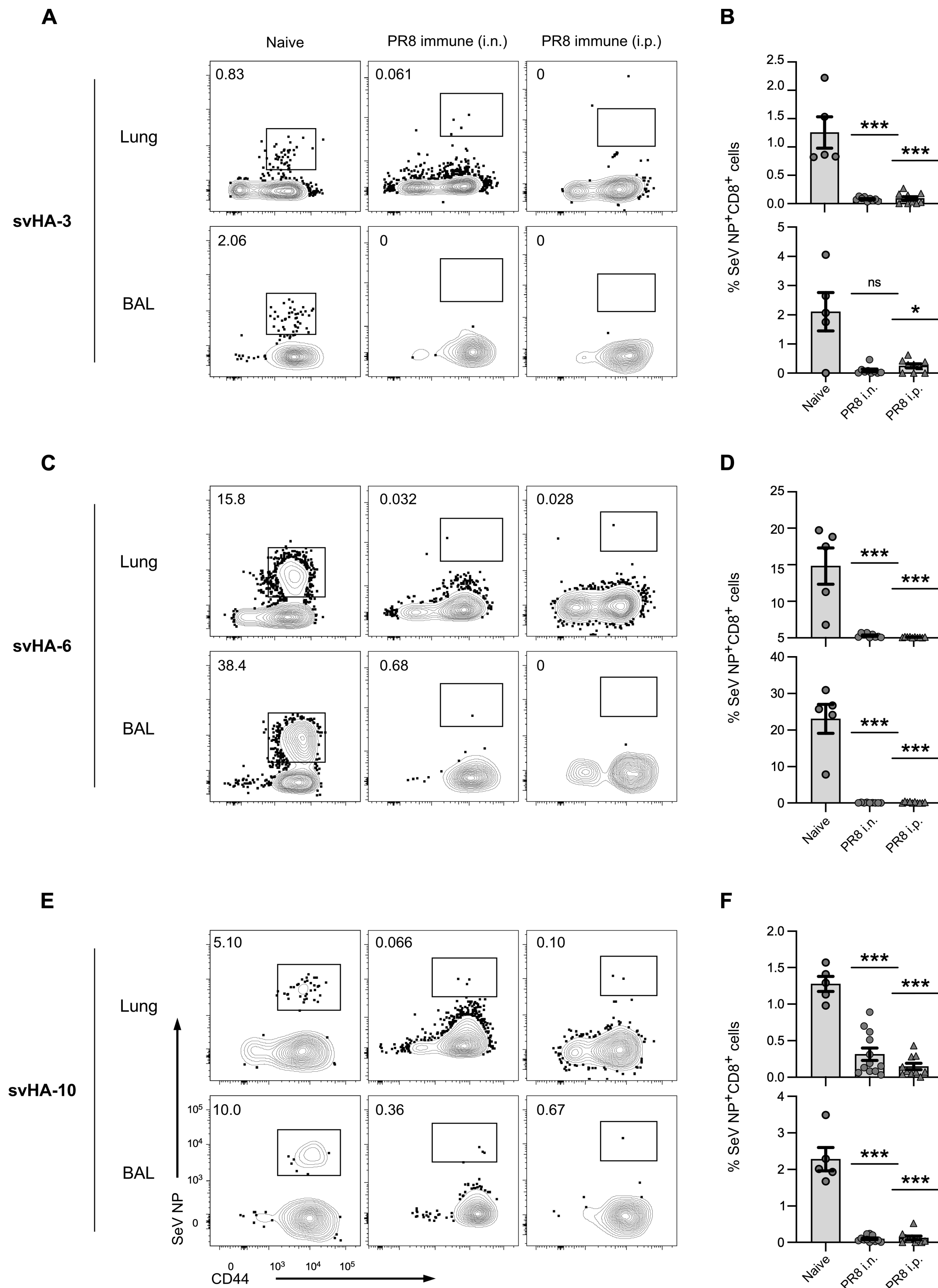
A
Naive
PR8 immune (i.n.)
PR8 immune (i.p.)
svHA-3
Lung
BAL
0.83
0.061
0
2.06
0
0
B
% SeV NP+CD8+ cells
***
***
ns
*
Naive
PR8 i.n.
PR8 i.p.
C
svHA-6
15.8
0.032
0.028
38.4
0.68
0
D
E
svHA-10
5.10
0.066
0.10
10.0
0.36
0.67
SeV NP
CD44
F

Figure S2. Drifted LAIV immunization of PR8-immune mice fails to generate *de novo* $CD8^+$ T_{RM}. (A) Example staining for SeV NP^+ $CD8^+$ T_{RM} in the lung and BAL of naïve or PR8-immune mice 30 d.p.i. with svHA-3. **(B)** Frequency of SeV NP^+ $CD8^+$ T_{RM} in the lung and BAL of naïve or PR8-immune mice 30 d.p.i. with svHA-3. **(C-F)** Same as A & B except with svHA-6 (C, D) and svHA-10 (E, F). Indicated significance is for comparison to naïve mice. p values are as follows: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Pre-existing $CD8^+$ T_{RM} in the respiratory tract undergo expansion upon immunization with LAIV-SeV NP

Following the discovery that LAIV-SeV NP immunization of PR8-immune mice fails to generate a novel antigen-specific $CD8^+$ lung T_{RM} response, we sought to determine whether the pre-existing $FluNP_{366}^+$ $CD8^+$ memory T cells responded to immunization and whether this was influenced by escape from pre-existing antibody. To accomplish this, we examined incorporation of EdU by $FluNP_{366}^+$ $CD8^+$ T_{RM} in PR8-immune mice following mock immunization (PBS) or immunization with svHA-WT or svHA-12 (**Fig. 3A**). At 7 days post-immunization, EdU^+ $FluNP_{366}^+$ $CD8^+$ T_{RM} were clearly identifiable in both the lung interstitium and airways (**Fig. 3B**). Within the lung, a significantly higher frequency and number of $FluNP_{366}^+$ $CD8^+$ T_{RM} incorporated EdU following immunization with either svHA-WT or svHA-12 when compared to samples from mock immunized mice (**Fig. 3C, D**). Importantly, significantly more $FluNP_{366}^+$ $CD8^+$ lung T_{RM} in mice immunized with svHA-12 incorporated EdU when compared to those from mice immunized with svHA-WT, indicating that the ability of LAIV-SeV NP immunization to induce T cell responses increases as anti-HA antibody is avoided (**Fig. 3C, D**). Furthermore, the overall

frequency and number of $FluNP_{366}^{+}$ $CD8^{+}$ lung T_{RM} increased upon immunization with svHA-12 (**Fig. 3E, F**). Immunization with either svHA-WT or svHA-12 also resulted in increased numbers of $FluNP_{366}^{+}$ $CD8^{+}$ T_{RM} as well as incorporation of EdU by these cells within the airways (**Fig. 3G-J**). These results confirm that although immunization of PR8-immune mice with LAIV-SeV NP fails to generate *de novo* $CD8^{+}$ T_{RM} within the respiratory tract, it does succeed in boosting pre-existing lung $CD8^{+}$ T_{RM} populations more effectively when it escapes the anti-HA response.

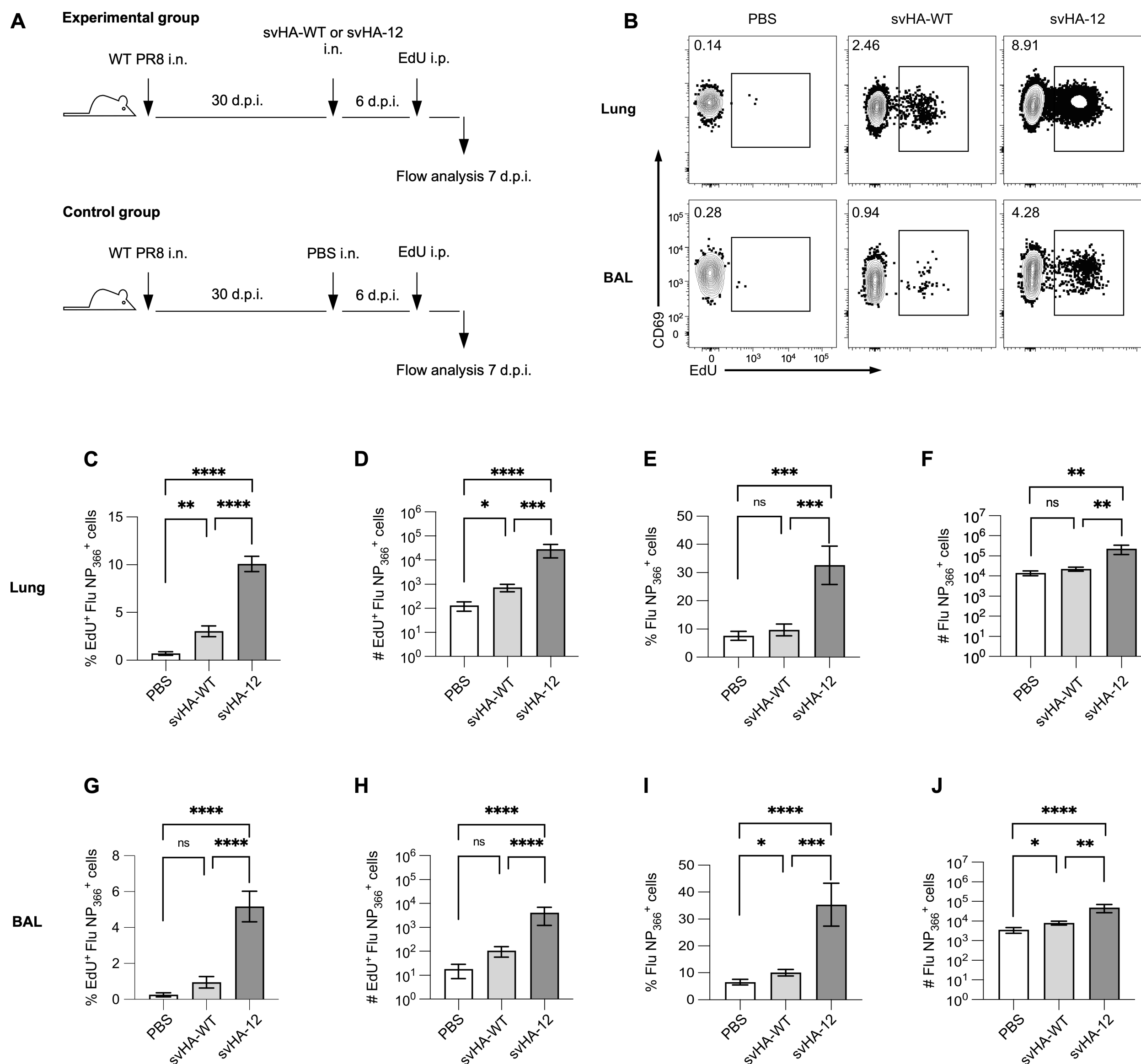

Figure 3. Pre-existing CD8⁺ T_{RM} in the respiratory tract undergo expansion upon immunization with LAIV-SeV NP. (A) Experimental design. **(B)** Example EdU staining amongst $FluNP_{366}^{+}$ $CD8^{+}$ T_{RM} in the lung and BAL following immunization of PR8-immune mice with PBS, svHA-WT, or svHA-12. **(C, D)** Frequency (C) and number (D) of EdU^{+} $FluNP_{366}^{+}$ $CD8^{+}$ lung T_{RM} following svHA-WT or svHA-12 immunization. **(E, F)** Total frequency (E) and number (F) of $FluNP_{366}^{+}$ $CD8^{+}$ lung T_{RM} following svHA-WT or svHA-12 immunization. **(G-J)** Same as C-F but in BAL. p values are as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Route of prior infection does not alter the ability of LAIV to establish *de novo* CD8$^+$ T$_{RM}$

Given that our results suggest the cellular immune response on its own is enough to prevent LAIV-SeV NP immunization from generating a novel CD8$^+$ T$_{RM}$ population, we repeated our immunization experiment, but infected mice with WT PR8 via i.p. injection rather than the i.n. route. Previous studies have shown that a non-pulmonary infection route results in significantly fewer CD8$^+$ T$_{RM}$ in the lungs and airways [28], while generating similar numbers of circulating CD8$^+$ T$_{EM}$. However, neither svHA-WT nor svHA-12 immunization generated *de novo* SeV NP$^+$ CD8$^+$ T$_{RM}$ in the lung (**Fig. 4A-F**). The immunizations did, however, boost the pre-existing FluNP$_{366}^+$ CD8$^+$ T cells in the lung (**Fig. 4G-J**). We also confirmed boosting of the pre-existing FluNP$_{366}^+$ CD8$^+$ T cells in the airways, spleen, and nasal cavity (**Fig. S3**). Apart from the frequency of EdU$^+$ FluNP$_{366}^+$ T$_{RM}$ in the lung, immunization with svHA-12 had significantly higher efficacy when compared to immunization with svHA-WT (**Fig. 4G-J**). Though this result was somewhat surprising, we hypothesize that it can be explained by the presence of CD8$^+$ T$_{RM}$ within the nasal cavity, which is the primary site of LAIV replication. The fold change decrease of CD8$^+$ T$_{RM}$ in the lungs and airways is much greater than that seen in the nasal cavity when comparing i.n. versus i.p. infection routes (**Fig. 4K**).

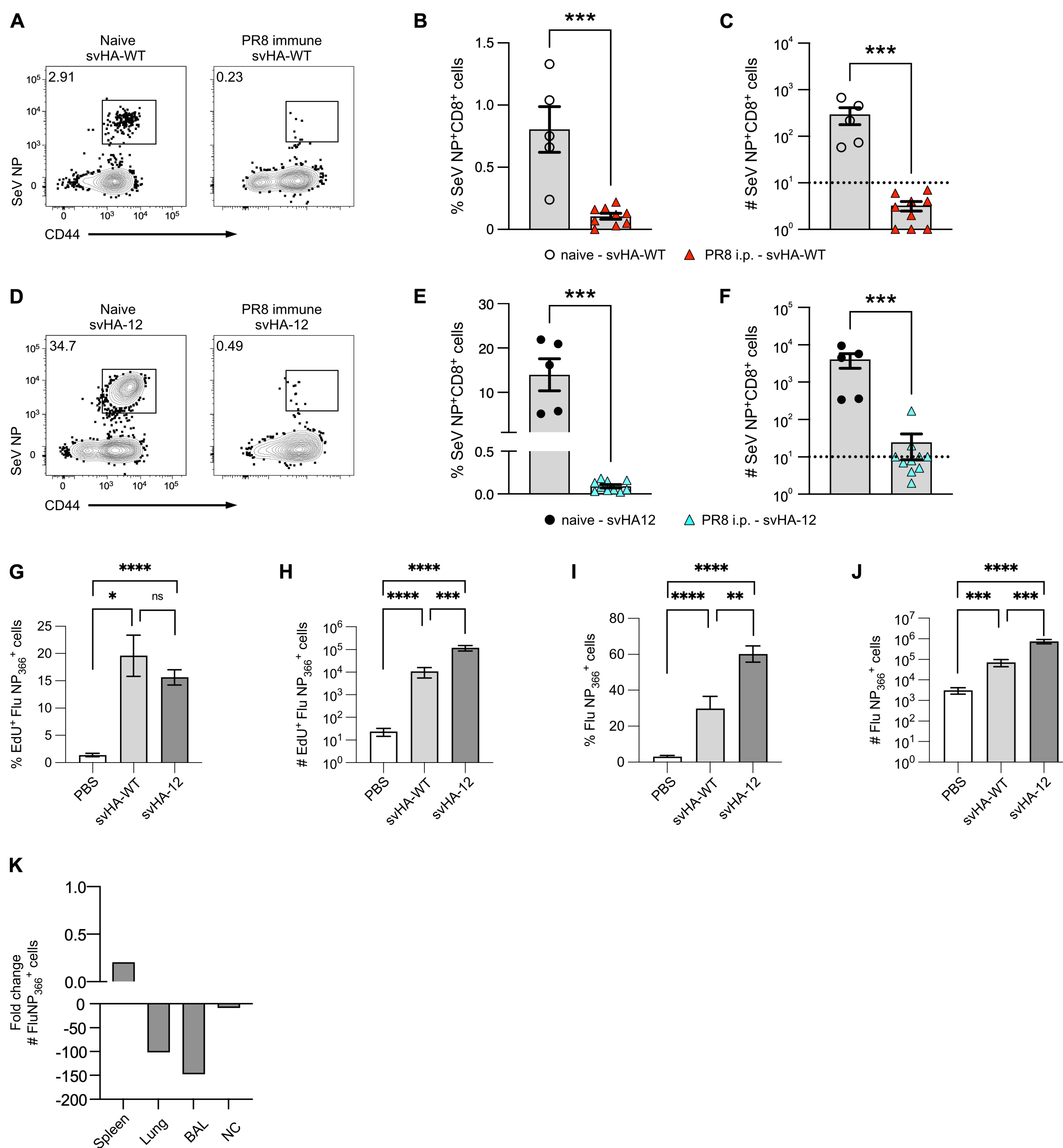

Figure 4. Route of prior infection does not alter the ability of LAIV to establish *de novo* CD8⁺ T_{RM}. (A-C) Example staining (A), frequency (B) and number (C) of SeV NP⁺ CD8⁺ lung T_{RM} following svHA-WT immunization (30 d.p.i.) of naïve mice or mice infected via i.p. injection with WT PR8. **(D-F)** Same as A-C but with svHA-12. **(G, H)** Frequency (G) and number (H) of EdU⁺ FluNP$_{366}$⁺ CD8⁺ lung T_{RM} following indicated immunization. **(I, J)** Total frequency (I) and number

(J) of $FluNP_{366}^{+}$ $CD8^{+}$ lung T_{RM} following indicated immunization. **(K)** Fold change in the number of $FluNP_{366}^{+}$ $CD8^{+}$ T cells in indicated tissues when comparing PR8 infection via i.n. or i.p. route. p values are as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. LOD indicated with dotted line at 10^1.

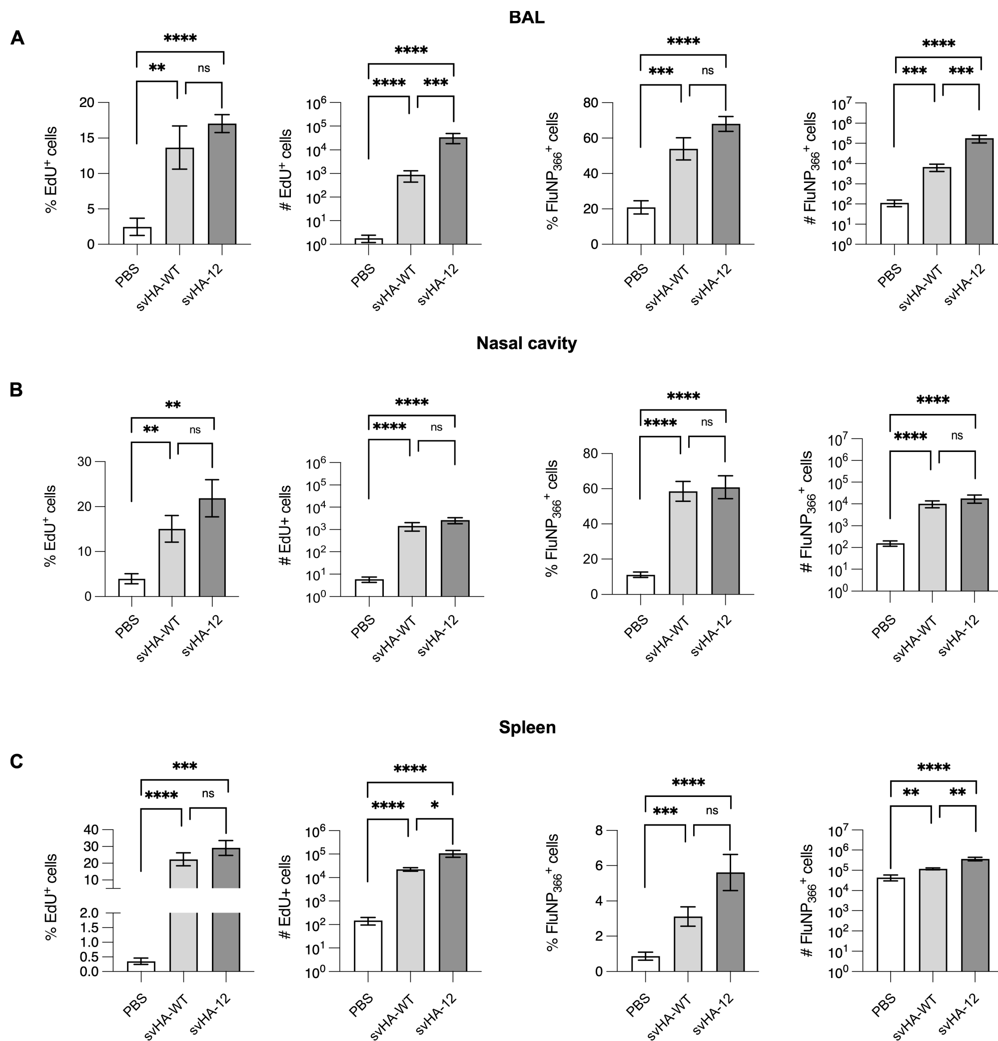

Figure S3. Pre-existing $CD8^{+}$ T_{RM} in other tissues undergo expansion upon LAIV immunization. Frequency and number of EdU^{+} $FluNP_{366}^{+}$ $CD8^{+}$ T cells and total frequency and number of $FluNP_{366}^{+}$ $CD8^{+}$ T cells in the BAL (A), nasal cavity (B), or spleen (C) of PR8-immune

mice (i.p. injection) 30 d.p.i. with svHA-WT or svHA-12. p values are as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Pre-existing $CD8^+$ T_{RM} are sufficient to prevent the generation of *de novo* $CD8^+$ T_{RM} following LAIV immunization

To investigate whether evasion of the influenza-specific $CD8^+$ T cell response enables formation of a novel $CD8^+$ lung T_{RM} population, we generated a new version of svHA-12 that includes a point mutation, N370Q, within the nucleoprotein sequence that prevents presentation of NP antigen on MHC class I [29] (**Fig. 5A**). Inclusion of this point mutation thereby eliminates the immunodominant $NP_{366-374}$ $CD8^+$ T cell epitope from our vaccination strain and allows the immunization to circumvent the pre-existing $FluNP_{366}$-specific $CD8^+$ T cell response. To test whether the presence of T_{RM} specific for a single immunodominant $CD8^+$ T cell epitope alone is sufficient to prevent LAIV immunization from generating a *de novo* T cell response, we first infected mice using a replication-deficient recombinant adenovirus vector that expresses PR8 NP (AdNP). These mice do not generate any antibodies against influenza HA and will only generate influenza-specific $CD8^+$ memory T cells to the $FluNP_{366}$ epitope. We then immunized these mice with either svHA-12 or svHA-12 N370Q (**Fig. 5B**) and looked one month later for SeV NP^+ $CD8^+$ T_{RM} in the respiratory tract (**Fig. 5C**). Like our results in PR8-immune mice, immunization of AdNP-immune mice with svHA-12 did not generate SeV NP^+ $CD8^+$ T_{RM}. However, we did observe a significantly higher frequency and number of SeV NP^+ $CD8^+$ T_{RM} in the lung, BAL, and nasal cavity when AdNP-infected mice were immunized with svHA-12 N370Q (**Fig. 5D, E**). We also confirmed that these SeV NP^+ $CD8^+$ T_{RM} expressed CD69 and CD103 (**Fig. 5F**). Combined,

these results demonstrate that CD8$^+$ T cells specific for a single immunodominant epitope can prevent LAIV-SeV NP immunization from generating *de novo* antigen-specific CD8$^+$ T_{RM}.

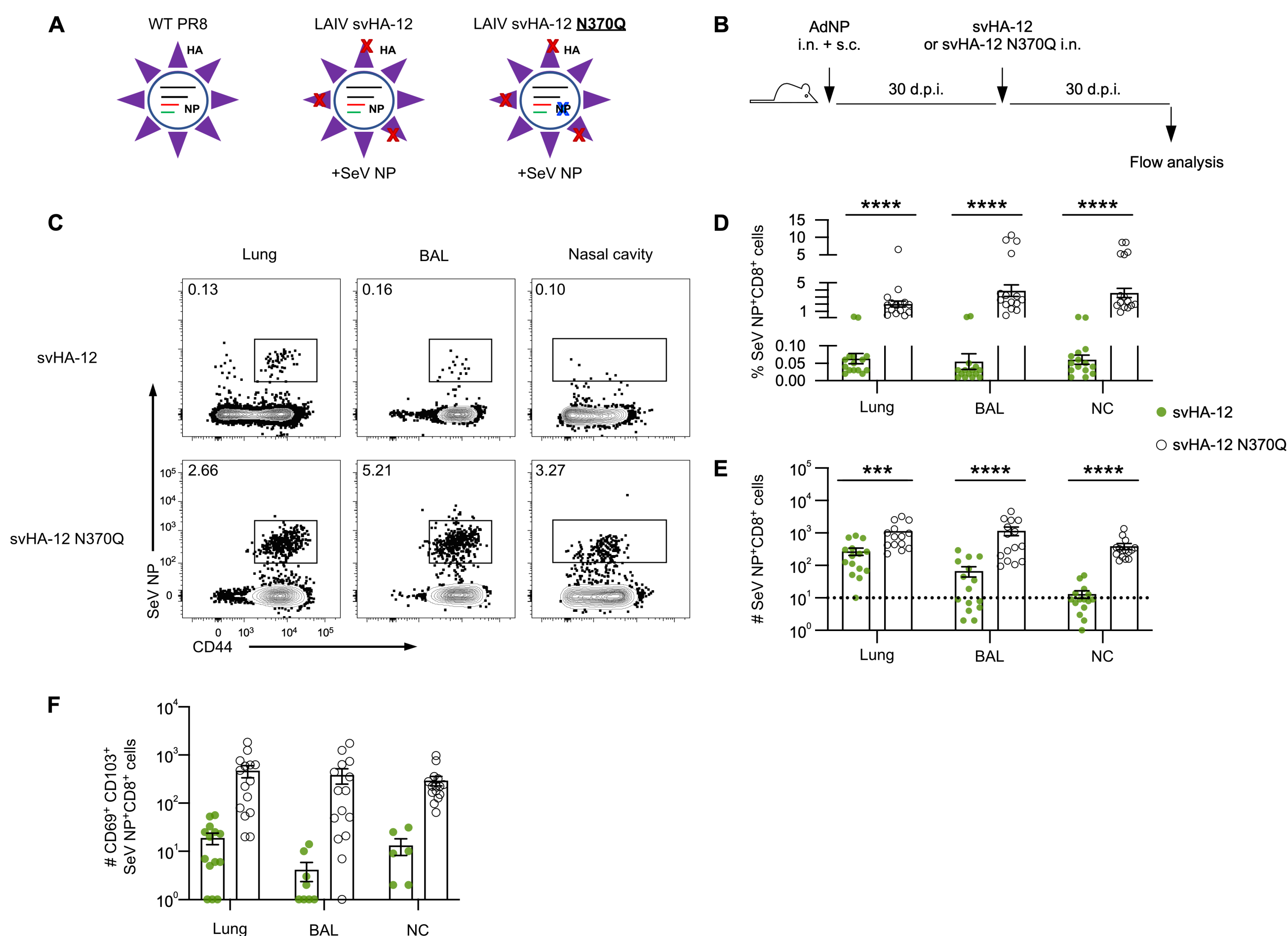

Figure 5. Pre-existing CD8$^+$ T_{RM} are sufficient to prevent the generation of *de novo* CD8$^+$ T_{RM} following LAIV immunization. (A) Schematic illustrating the design of LAIV strain svHA-12 N370Q. **(B)** Experimental design. **(C)** Example staining for SeV NP$^+$ CD8$^+$ T cells in indicated tissues in AdNP-immunized mice 30 d.p.i. with svHA-12 or svHA-12 N370Q. **(D, E)** Frequency (D) and number (E) of SeV NP$^+$ CD8$^+$ T cells in indicated tissues 30 d.p.i. with svHA-12 or svHA-12 N370Q. p values are as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. LOD indicated with dotted line at 10^1.

Discussion

Despite ongoing research efforts, achieving long-term protection against many respiratory pathogens through vaccination remains a challenge. In the case of influenza, the virus's capacity to rapidly mutate its surface proteins results in its ability to evade the antibodies generated during a primary immune response. Influenza-specific $CD8^+$ memory T cells, however, target conserved internal viral proteins that are shared across different subtypes of influenza virus, thereby making them crucial for cross-reactive protection [1-4]. $CD8^+$ T_{RM}, a specialized subset of memory T cells that remain embedded along the respiratory tract and do no re-enter the circulation, have been shown to be particularly important for mediating protection against influenza virus challenge [5, 6]. Several vaccination strategies developed against influenza virus have been shown to be effective at inducing a $CD8^+$ T cell response, including live attenuated influenza vaccine (LAIV). However, while LAIV has been effective in children, it has shown minimal efficacy and variable T cell responses in adults [13-16, 30]. We therefore hypothesized that LAIV's ability to "take" is impacted by pre-existing influenza immunity due to prior infections and/or immunizations.

In the present study, we assess the impact of pre-existing humoral and cellular immunity on the ability of LAIV to generate *de novo* antigen-specific $CD8^+$ T_{RM} in the respiratory tract. Not surprisingly, we observed that LAIV-SeV NP immunization can boost pre-existing influenza NP-specific $CD8^+$ T_{RM} with increasing efficacy the further as escape from anti-HA antibodies increased. However, LAIV-SeV NP immunization failed to generate *de novo* SeV NP $CD8^+$ T_{RM} even when the immunization strain is capable of fully escaping anti-HA antibodies. Intriguingly, immunization with an LAIV-SeV NP strain fully capable of escaping antibodies (svHA-12) still failed to generate *de novo* $CD8^+$ T_{RM} when fewer pre-existing virus-specific $CD8^+$ T_{RM} were present in the lung and airways. This finding is particularly interesting to us, as we believe it

suggests that $CD8^+$ T_{RM} within the nasal cavity are sufficient at preventing LAIV-SeV NP immunization from stimulating formation of $CD8^+$ T_{RM} against novel antigens. In contrast to lung T_{RM}, nasal cavity T_{RM} are formed independently of local cognate antigen recognition and are relatively stable over time [31]. This explains why intraperitoneal infection, which does not deliver antigen to the lungs, results in similar numbers of T_{RM} in the nasal cavity when compared to intranasal administration. Given that LAIV is temperature sensitive and predominantly replicates in the upper respiratory tract, we believe this is a plausible explanation for why LAIV-SeV NP immunization still failed to generate *de novo* lung T_{RM} when significantly fewer influenza-specific T_{RM} were present in the lower respiratory tract, although further experiments are needed to confirm this hypothesis [27]. Lastly, immunization with an LAIV-SeV NP strain that fully escapes antibodies and does not present the immunodominant NP_{366} epitope (svHA-12 N370Q) did succeed in generating *de novo* $CD8^+$ T_{RM} in the lungs and BAL of mice that only have pre-existing NP_{366}^+ T_{RM}. Taken together, our findings suggest that escape from both the pre-existing humoral and cellular responses is necessary to generate a novel epitope-specific $CD8^+$ lung T_{RM} population following immunization with LAIV.

There has been longstanding interest in broadening our understanding of how pre-existing immunity impacts susceptibility to influenza infection [32]. Original antigenic sin, a phenomenon whereby the immune system preferentially recalls existing memory cells made in response to a previous antigen encounter, is a well described impediment to generating *de novo* protection against influenza virus [33]. The process has been best described in relation to antibody responses and is typically not observed during subsequent exposures to distantly related or entirely unrelated antigens [34]. It has been proposed that altered antigen trafficking and prevention of LAIV replication in the nasal epithelia post-vaccination are potential mechanisms by which pre-existing antibodies

reduce the overall effectiveness of LAIV, where effectiveness was measured as strong serum antibody levels and protection against homologous and heterologous strains [35]. Although B cell responses to LAIV were not a focus of this study, our data suggest that pre-existing memory T cells in the upper respiratory tract may also contribute to original antigen sin. The ability of T_{RM} in the respiratory tract to rapidly eliminate virus-infected cells may limit the absolute amount and duration of viral antigen production, further biasing the antibody response toward pre-existing memory B cells. Additional studies are needed to determine the impact of pre-existing cellular immunity on LAIV's ability to stimulate *de novo* B cell responses.

Overall, our results provide critical insight to the roles that both pre-existing humoral and cellular immunity play in restricting the ability of LAIV immunization to promote antigen-specific T cell responses, and in particular the ability of pre-existing T_{RM} to limit the development of *de novo* T cell responses. Recently, it's been shown that prior immunity impacts the immune response to vaccination against SARS-CoV-2. While individuals who are recovered from SARS-CoV-2 infection tend to experience a broader and stronger antibody response after booster vaccinations, a larger proportion of naïve T cells was found to be required for superior quality $CD4^+$ T cell responses post-vaccination [36]. In addition, LAIVs and other viral vectors, such as recombinant adenoviruses and orthopoxviruses, have been proposed as delivery platforms for vaccine antigens or for gene therapy [24, 37-41]. Our data suggest that vector-specific memory T cell responses may also limit the efficacy of these platforms and should be considered in their design. In the case of recombinant adenovirus vaccines, vectors built from serotypes of non-human species are being evaluated to circumvent this issue [42]. While pre-existing antibodies and memory T cells are known to shape the establishment of novel antigen responses, their impact can vary depending on the

specific pathogen and vaccination platform used, and additional research is needed to better inform vaccine design.

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Chapter IV: Discussion

Following clearance of respiratory infection, distinct subsets of memory CD8$^+$ T cells that provide cell-mediated protection are established. Two subsets, the effector and central memory T cell populations, continually migrate through the blood stream to patrol lymphoid and peripheral tissues. The tissue-resident T cell compartment, however, does not re-enter the circulation and instead remains localized within the tissue. Canonically, CD8$^+$ T_{RM} are identified by expression of the cell surface markers CD69 and CD103, which both help retain cells in the tissue. CD69 is a C-type lectin that associates with the S1P1 receptor on the T cell surface thereby preventing its binding to the blood metabolite S1P[1-3]. CD103, also known as integrin αEβ7, binds to E-cadherin on epithelial cells[4]. In addition to their surface markers, T_{RM} can be identified by intravital labeling to distinguish cells not within the blood circulation. Uniquely positioned within barrier tissues such as the respiratory tract, skin, intestinal tract, and female reproductive tract, T_{RM} act as sentinels and rapidly respond to pathogen re-encounter by producing cytokines to recruit other immune cells and/or directly killing infected cells[5-9].

CD8$^+$ T_{RM} have been shown to be critical mediators of immunity against many respiratory pathogens including influenza virus, respiratory syncytial virus (RSV), SARS-CoV-2, and tuberculosis. Although they alone do not provide sterilizing immunity, when present in sufficient numbers, CD8$^+$ T_{RM} help mitigate early viral burden, resulting in limited immunopathology and improved lung function. However, following acute infection with influenza virus, cellular immunity steadily declines over time[5, 10]. Our group and others have shown that this loss of cellular immunity is due to the loss of influenza-specific CD8$^+$ T_{RM} within the lungs and airways; while the number of virus-specific T_{EM} in circulation remains steady over time, the number of CD8$^+$ T_{RM} within the lung interstitium and airways decline to nearly undetectable levels within 7 months post-

infection. The exact mechanisms behind this loss in $CD8^+$ T_{RM} have not been fully elucidated, but a recent study from our lab showed that the airway environment induces transcriptional changes in airway $CD8^+$ T_{RM} that lead to their decline via apoptosis[11]. This is one potential explanation for why airway T_{RM} decline and demonstrates how the tissue environment can help shape the kinetics of T_{RM} loss.

The data within this thesis provide further insight to the mechanisms impacting generation and maintenance of antigen-specific $CD8^+$ lung T_{RM} in the context of two vaccination strategies against influenza virus. Recent studies showed that immunization with a recombinant adenovirus serotype 5 that expresses the influenza nucleoprotein (AdNP) via i.n. and s.c. routes results in formation of protective influenza NP-specific $CD8^+$ T_{RM} populations that are maintained in the lungs and airways for up to 1-year post-immunization[12]. This is in stark contrast to the gradual loss of $CD8^+$ T_{RM} observed following a primary influenza infection. Subsequent studies determined that these T cells continue to encounter their cognate influenza NP antigen locally within the respiratory tract in AdNP-immunized mice. Additionally, parabiosis studies suggested a model in which circulating T_{EM} migrate through the lungs and airways where they encounter persistent NP antigen and differentiate into T_{RM}.

In Chapter II, we used RNA-sequencing analysis to show that the NP-specific $CD8^+$ T_{RM} generated following AdNP immunization have a transcriptional signature like that of $CD8^+$ T_{RM} generated during influenza infection. Gene set enrichment analysis revealed T_{RM} from both AdNP immunized mice and influenza infected mice express a core set of genes from a known T_{RM} signature confirming that both populations are bona fide T_{RM}. However, very few DEGs were identified when comparing 1-month post-AdNP or x31 influenza. This indicates that AdNP immunization does not induce cell intrinsic changes that account for the enhanced longevity of

$CD8^+$ T_{RM} and therefore supports the model that circulating T_{EM} differentiate into T_{RM} upon cognate NP antigen encounter in the lung.

To identify the cell types important for maintaining NP^+CD8^+ lung T_{RM} long-term following AdNP immunization, we first depleted $CD4^+$ T cells following AdNP immunization and found that the number of NP^+ $CD8^+$ T_{RM} in the lungs and airways significantly declined. This aligns with prior work from Susan Kaech's group showing that $CD4^+$ T cells promote the generation of $CD103^+$ lung T_{RM} and their migration into the airways in an IFNγ-dependent manner [13]. The Kaech study proposed a mechanism in which $CD4^+$ T cell help leads to TGF-β-mediated induction of CD103 surface expression, and we hypothesize a similar mechanism may be occurring to promote differentiation of $CD8^+$ lung T_{RM} following immunization with AdNP. By immunizing lox-STOP-lox fluorescent reporter mice with a recombinant adenovirus vector expressing Cre recombinase we also identified alveolar macrophages as the primary cell source of persistent NP antigen in the respiratory tract and confirmed that these cells persisted in the animal for at least 1-year post-immunization. We subsequently confirmed this finding by observing colocalization of influenza NP protein within alveolar macrophages collected from the airways of AdNP immunized mice 90 days post-immunization using immunofluorescent microscopy. In summary, these findings demonstrate that alveolar macrophages provide a long-lived NP antigen depot following intranasal immunization with a recombinant adenovirus serotype 5, and that this depot helps maintain high numbers of protective NP-specific $CD8^+$ T_{RM} for up to 1-year following immunization.

In Chapter III, we explore the impact of pre-existing immunity on the generation of *de novo* antigen-specific $CD8^+$ lung T_{RM} following live attenuated influenza vaccination (LAIV). Since the surface proteins of influenza virus differ between virus subtypes and are prone to high

rates of mutation, humoral responses generated following one influenza infection are often suboptimal against subsequent exposures[14, 15]. The $CD8^+$ T cell response, however, targets epitopes that are highly conserved across different strains of influenza virus[16, 17]. As such, focus on generating $CD8^+$ memory T cells is at the forefront of designing a vaccination strategy that provides broad, long-lasting protection against re-infection with influenza. Immunization with LAIV successfully generates an anti-influenza $CD8^+$ T cell response, including protective $CD8^+$ T_{RM}[18]. However, its overall efficacy has historically been low in human adults when compared to children[19-22]. We hypothesized that pre-existing immunity due to natural infection(s)/immunization(s) prevent LAIV's ability to "take" in individuals and sought to evaluate the role of established humoral and cellular immunity on the generation of *de novo* $CD8^+$ lung T_{RM} following immunization.

To evaluate the impact of pre-existing humoral immunity on the efficacy of LAIV, we first generated a series of drifted LAIV strains each with an NP epitope from Sendai parainfluenza virus (SeV NP) and increasing ability to escape anti-HA antibodies. Using SeV NP as a readout for novel antigen-specific T_{RM}, we determined that LAIV immunization fails to generate *de novo* $CD8^+$ lung T_{RM} in PR8 influenza-immune mice, even when they were immunized with svHA-12 LAIV, an LAIV strain fully capable of escaping anti-HA antibodies. Interestingly, LAIV immunization did succeed in boosting the number of pre-existing influenza NP_{366}^+ $CD8^+$ T_{RM}, with efficacy increasing as the anti-HA antibody response was escaped. Combined, these data suggest that escape from the pre-formed antibody response alone is not sufficient to generate *de novo* $CD8^+$ T_{RM} and that the pre-existing cellular immune response also plays a role in preventing LAIV immunization from "taking."

Since local recognition of cognate antigen is a prerequisite for formation of $CD8^+$ lung T_{RM}, infections administered via a non-respiratory route establish significantly fewer $CD8^+$ T_{RM} within the lower respiratory tract. We therefore repeated our LAIV immunization using PR8-immune mice that had received a primary WT PR8 infection by i.p. injection. To our surprise, we were still unable to detect any SeV NP^+ $CD8^+$ lung T_{RM} following LAIV immunization, even with the decreased numbers of pre-existing influenza NP_{366}-specific $CD8^+$ T_{RM} in the lungs and airways. We hypothesize that $CD8^+$ T_{RM} within the nasal cavity are preventing LAIV immunization from generating novel $CD8^+$ T_{RM} and this possibility is discussed in more detail below.

Given that our data indicates the $CD8^+$ memory T cell response is enough on its own to prevent generation of *de novo* antigen-specific $CD8^+$ T_{RM} following immunization, we next sought to evaluate whether escape from this pre-existing cellular response is sufficient for generating novel antigen-specific $CD8^+$ T_{RM}. We therefore generated a new version of our LAIV immunization strain that fully escapes anti-HA antibodies and includes a point mutation within the nucleoprotein sequence that prevents presentation of the immunodominant $CD8^+$ T cell epitope, NP_{366}, via MHCI. This LAIV strain was named svHA-12 N370Q. To test whether the presence of T_{RM} specific for a single immunodominant $CD8^+$ T cell epitope alone is enough to prevent LAIV immunization from generating a *de novo* T cell response, we immunized mice that had been first given AdNP with svHA-12 and svHA-12 N370Q. Interestingly, svHA-12 N370Q immunization of AdNP-immunized mice successfully generated *de novo* SeV NP^+ $CD8^+$ T_{RM} in the lungs, airways, and nasal cavity, but svHA-12 immunization did not. Combined, these data demonstrate that escape from both pre-existing humoral and $CD8^+$ T cell responses is necessary to generate *de novo* antigen-specific $CD8^+$ lung T_{RM} following immunization with LAIV.

Overall implications for vaccine design

Combined, the data presented in this thesis further inform our knowledge of the mechanisms driving generation and maintenance of $CD8^+$ T_{RM} following respiratory infection or immunization. Unlike T_{RM} in other peripheral tissues such as the intestine and genital tract, virus-specific $CD8^+$ T_{RM} within the lungs and airways steadily decline over time following their establishment following infection[23-25]. Better understanding of the factors that help generate and maintain virus-specific $CD8^+$ T_{RM} is needed to inform future design of T cell-based immunization strategies against respiratory infection; however, many questions remain unanswered. Although several factors have been identified as important mediators of $CD8^+$ T_{RM} generation, specific requirements can vary by tissue and vaccine vector. Furthermore, to date, relatively few studies have evaluated maintenance of T_{RM} in the context of pre-existing immunity and/or subsequent infections.

Location of antigen and T_{RM}

Local recognition of cognate antigen within the lung tissue is one necessary factor for formation of $CD8^+$ lung T_{RM}, so targeting antigen to the respiratory tract is key to achieving protection. Targeting of antigen to specific APCs is known to polarize the subsequent T cell responses and has been utilized for subunit vaccines[26, 27]. Additional studies are needed to determine exactly how the persistent NP antigen depot established in alveolar macrophages described in Chapter II sustains the $CD8^+$ lung T_{RM} population in AdNP-immunized mice. Presumably, NP^+ alveolar macrophages present the antigen directly to circulating T_{EM} and induce their differentiation into T_{RM}; however, this would need to be confirmed experimentally. A recent study demonstrated that fibroblastic stromal cells in the lung can also serve as long-lived antigen

depot following intravenous delivery of recombinant adenovirus, and that this depot supports maintenance of inflationary $CD8^+$ memory T cells in an IL-33-dependent mechanism. Since alveolar macrophages are also known producers of IL-33, it is possible that they support $CD8^+$ lung T_{RM} in a similar cytokine-dependent manner in addition to presenting cognate antigen.

One disadvantage of targeting antigen to alveolar macrophages is that these cells undergo high levels of cell death following acute respiratory infection with influenza virus[28, 29]. Even though recombinant adenovirus does not initiate alveolar macrophage cell death following transduction, we showed in Chapter II that this population could be eliminated following subsequent unrelated respiratory infections. Importantly, the loss of alveolar macrophages post-immunization with AdNP correlates with a significant decline in the number of NP-specific $CD8^+$ T_{RM}. This is an important consideration for future design of vaccines intended to target antigen to innate cell types known to be impacted by acute respiratory infection.

Indeed, our data with AdNP immunization supports a model in which alveolar macrophages provide a persistent source of cognate antigen within the lungs that promotes continual recruitment of circulating T_{EM} into the T_{RM} population. Alveolar macrophages are known to be long-lived under steady-state conditions and can be transduced by recombinant adenovirus vectors via scavenging receptors on the cell surface [30-37]. For these reasons, alveolar macrophages may be ideal candidates to target to establish a long-lived antigen depot despite the disadvantages noted above. In addition to alveolar macrophages, dendritic cells and fibroblastic stromal cells could serve as potential antigen targets following immunization with adenovirus vectors or other vaccine strategies[38, 39].

In Chapter III, we show data that indicates $CD8^+$ memory T cells in the nasal cavity prevent the ability of LAIV vaccination to generate *de novo* T_{RM}. In general, nasal cavity T_{RM} are not as

well described as lung and airway T_{RM} but are likely to play a large role in mediating protection against respiratory infection. To date, studies have shown that nasal cavity T_{RM} are generated independently of local antigen stimulation and TGF-β signaling[40]. Furthermore, the number of nasal cavity T_{RM} has been shown to decay minimally over time[40]. These characterizations suggest nasal cavity T_{RM} are more alike T_{RM} populations in other tissues than they are to the lungs and airways. However, additional studies are needed to fully assess other factors, such as cytokine signaling, required for generation & maintenance of nasal cavity T_{RM}.

Of note, virus-specific $CD8^+$ T_{RM} in the nasal cavity can prevent transmission of virus down to the lower respiratory tract by limiting viral replication within the upper respiratory tract[40]. We believe this explains why LAIV immunization fails in PR8-immune mice, since the temperature-sensitive vector predominantly replicates at lower temperature found in the upper respiratory tract[41-43]. Since nasal cavity T_{RM} are so successful at preventing spread of virus to the lower respiratory system, then developing methods for depositing T cells within the nasal tissue could provide protection against respiratory viral infection. Furthermore, the enhanced longevity of nasal cavity T_{RM} may make them ideal candidates over lung T cells for establishing sustained protection. Lastly, the role of virus-specific $CD8^+$ T_{RM} within the nasal cavity in preventing transmission of virus between individuals stills needs to be investigated.

Impact of prior (and future) infections & immunizations

LAIV is a promising platform for immunization against influenza virus because it can establish a cross-protective $CD8^+$ T cell response. However, as demonstrated in Chapter III, its effectiveness is substantially limited by both pre-existing humoral and T cell immunity. In Chapter II, we comment on the utility of recombinant adenovirus vectors as an alternative vaccine platform.

Although this approach can induce long-lasting protective T cell responses against viral antigens, its efficacy is also impacted by pre-existing immunity within the human population due to natural adenovirus infection[44].

Prime-boost vaccination strategies have also been shown to increase the number of antigen-specific memory $CD8^+$ T cells through repeated antigen exposure and have been used to successfully improve the efficacy of cellular immunity against pathogen challenge[45-48]. Furthermore, repeated boosting of pre-existing memory T cells can lead to improved memory T cell responses on a per cell basis, suggesting that secondary or tertiary memory lung T_{RM} may have intrinsic properties that enhance their longevity[49-51]. Recently, a comparison of primary and quaternary $CD8^+$ T cell memory showed that lung T_{RM} from repeated influenza infections were resistant to apoptosis in the lung tissue and persisted at much larger numbers[52]. These studies suggest that repeated stimulation may enforce an epigenetic and transcriptional program that renders lung T_{RM} more resistant to gradual decay.

Although repetitive boosting has been shown to result in sustained memory T cell responses against respiratory infections, our data in Chapter III suggest careful consideration must be taken when designing the vaccine regimen; the number of pre-existing $CD8^+$ T cell epitopes may be increased upon boosting, but possibly at the detriment of not generating T cell responses against novel antigens. Ongoing studies in our group also seek to understand the cell intrinsic changes and environmental effects governing $CD8^+$ lung T_{RM} following a prime-boost vaccination strategy against influenza virus. Based on our findings in Chapter III, it is conceivable that *de novo* $CD8^+$ lung T_{RM} will be undetectable after a certain amount of boosting has occurred, likely due to the immunity generated against the early vaccination strains. In addition, the impact of subsequent unrelated respiratory infections on the maintenance of $CD8^+$ lung T_{RM} needs to be further

evaluated. It was recently shown that T_{RM} highly express the purinergic receptor, P2RX7, and that this receptor's recognition of danger-associated molecular patterns (DAMPs) can lead to cell death of T_{RM} following respiratory infection[53]. Interestingly, TCR stimulation was also shown to protect $CD8^+$ T_{RM} from P2RX7-mediated cell death, suggesting a mechanism by which T_{RM} capable of recognizing the invading pathogen are maintained[53]. Perhaps selectively eliminating pre-existing T_{RM} of certain antigen specificities may be beneficial to achieving protection against novel antigen epitopes.

T_{RM} beyond respiratory infection

Tissue-resident memory T cells have been implicated in many diseases beyond viral and bacterial respiratory infection. For example, skin T_{RM} are known players in several autoimmune diseases, such as vitiligo and psoriasis[54]. Studies suggest autoreactive $CD8^+$ T cells are recruited to diseased skin sites in vitiligo patients in a CXCR3-dependent manner and that the subsequent T_{RM} that are formed are resistant to treatment with JAK/STAT inhibitors[55, 56]. In psoriasis patients, relapse following treatment preferentially occurs in skin areas with high numbers of $CD69^+$ $CD103^+$ T_{RM}[57]. Thus, the persistence of skin T_{RM} over time is a challenge to achieving permanent results with treatment against these diseases. Further studies on how autoreactive T_{RM} in the skin can be suppressed are needed to fully evaluate the potential of T_{RM} as therapeutic targets.

$CD69^+$ $CD103^+$ $CD8^+$ T_{RM} are present in many types of solid tumors and associate with improved responses to immunotherapy. Recently, a mouse model demonstrated that mice lacking functional T_{RM} markers such as CD69, CD103, and CD49a are more susceptible to transplantable melanoma when compared to WT mice[58-60]. However, the exact role of tumor-infiltrating T_{RM} and the mechanisms behind their generation and maintenance have not been adequately described.

Some studies suggest TGF-β, which is often present at high levels within solid tumor microenvironments, may promote differentiation of $CD103^{+}$ T_{RM} in tumors[61, 62]. In addition, $CD69^{+}$ $CD103^{+}$ $CD8^{+}$ T_{RM} within human lung, breast, and colon tumors express several markers associated with antigen exposure or exhaustion such as PD-1, TIM-3, and CD39, suggesting these cells may become dysfunctional under certain conditions, but further studies are needed[63-65].

In summary, tissue-resident memory T cells provide protection across many diverse tissue environments and are implicated in various disease states. Lung tissue-resident memory T cells are crucial mediators of cellular immunity against respiratory pathogens such as influenza viruses, but the number of these cells in the lung tissue gradually declines over time. This thesis identified several mechanisms that impact the generation and durability of cellular immunity in the lung. Together, the findings presented here will guide future cell-mediated vaccine strategies against respiratory pathogens.

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