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# Persistent antigen harbored by alveolar macrophages enhances the maintenance of lung-resident memory CD8+ T cells

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## 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).

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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*$^{tm14(CAG-tdTomato)Hze}$/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 $2\times10^7$ 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 ($2\times10^6$ 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 ($2\times10^6$ 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.

### 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-$\gamma$ 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.

## 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 (Supplementary Fig. 1). 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.

## 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 (Supplementary Fig. 2). 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.

### 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.

## 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Key points:**

- Alveolar macrophages harbor antigen following intranasal adenovirus immunization
- Depletion of this antigen depot impacts the longevity of respiratory $CD8^+$ $T_{RM}$

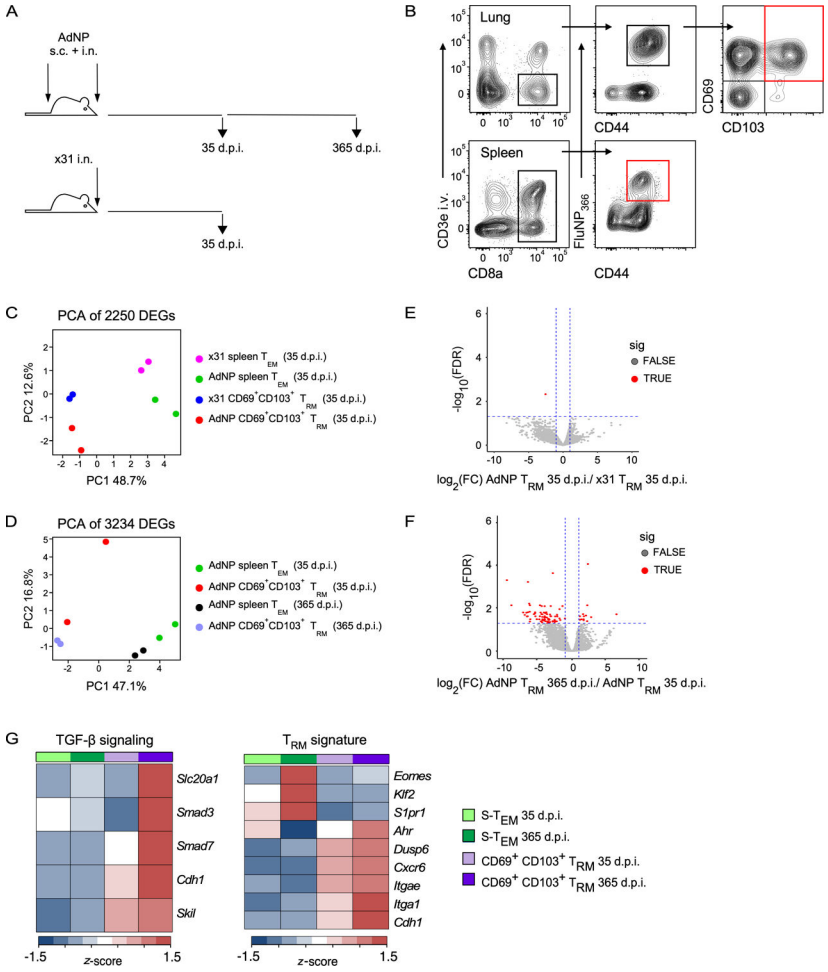


**Figure 1. Immunization with AdNP generates CD8$^+$ $T_{RM}$ that are transcriptionally alike those generated during a primary infection with influenza.**

**(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.

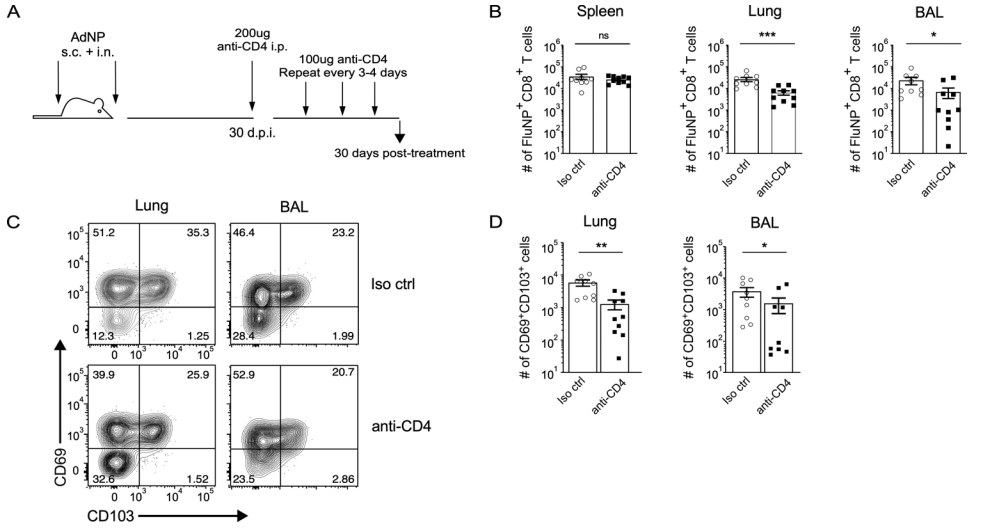


**Figure 2. CD4$^+$ T cells are important for the maintenance of CD8$^+$ T$_{RM}$ following immunization with AdNP.**

**(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.

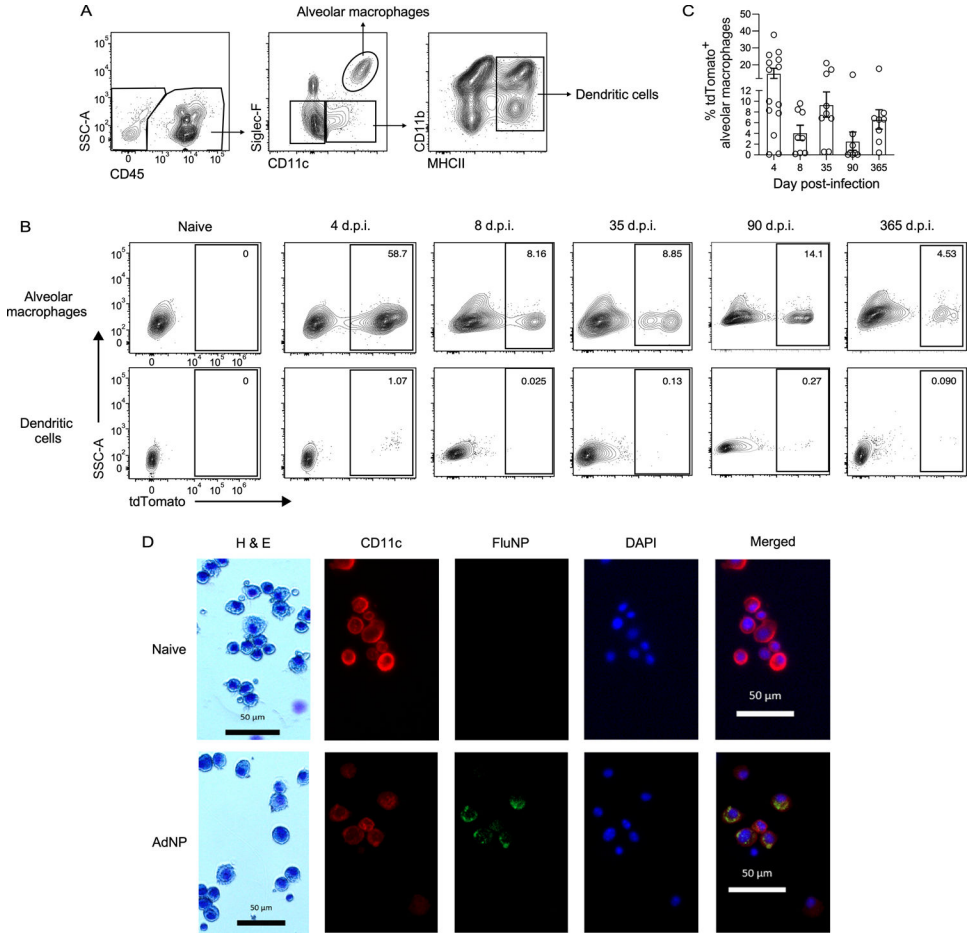


**Figure 3. Alveolar macrophages are the cell source of persistent influenza NP antigen in AdNP-immunized mice.**

**(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.

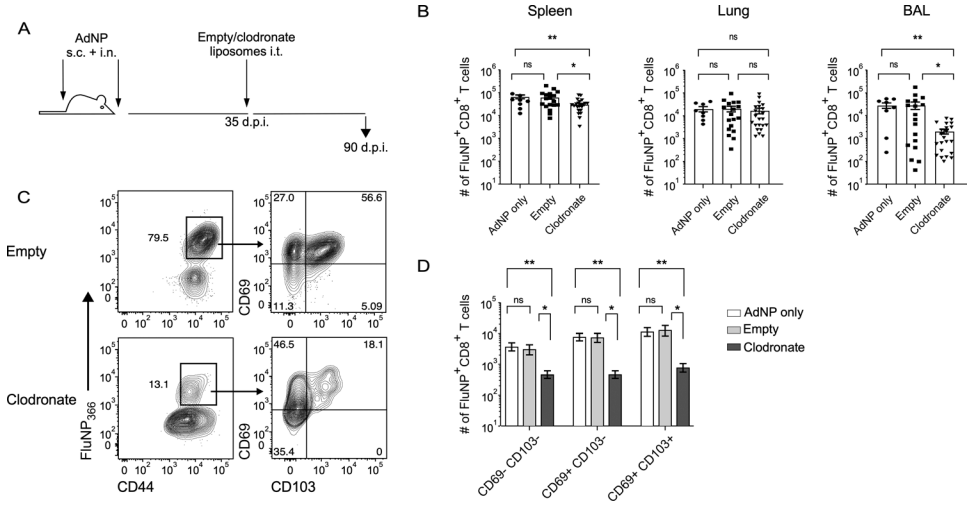


**Figure 4. Depletion of alveolar macrophages results in reduced longevity of influenza NP-specific CD8$^+$ $T_{RM}$**

**(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.

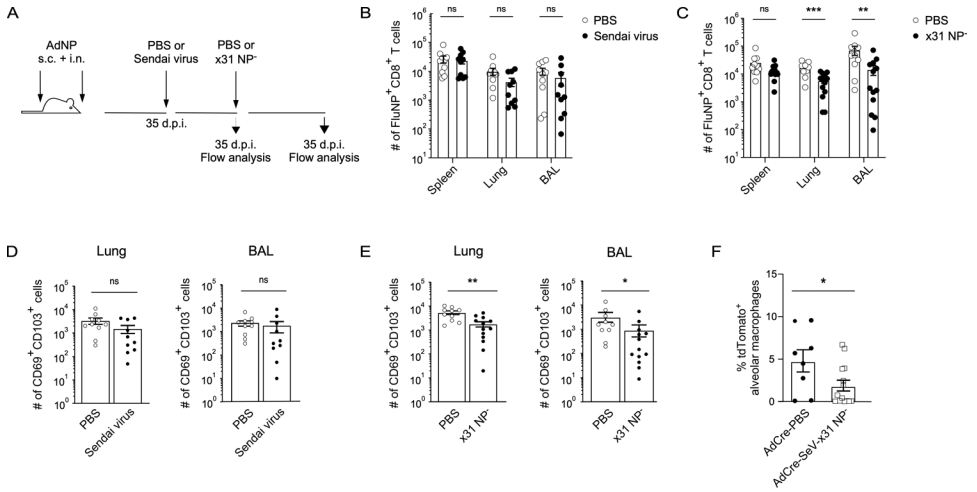


**Figure 5. Subsequent respiratory viral infections impact the maintenance of influenza NP-specific CD8$^+$ $T_{RM}$ in AdNP-immunized mice**

**(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.