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Factors that regulate and maintain CD8 T cell memory in the lung & airways

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Factors that regulate and maintain CD8 T cell memory in the lung & airways

By

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B.S., University of Wisconsin-Madison, 2010

Advisor: Dr. Jacob Kohlmeier; PhD

An abstract of
a dissertation submitted to the Faculty of the
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Abstract

Factors that regulate and maintain CD8 T cell memory in the lung & airways

By Sean Ryan McMaster

Respiratory virus infections are a significant source of annual morbidity and mortality, constituting a major human health problem worldwide. Memory T cell responses are critical for quickly limiting viral replication and mitigating unnecessary inflammation responsible for immunopathology. Acting as sentinels capable of quickly organizing a secondary immune response upon pathogen challenge, resident memory CD8 T (T_{RM}) populations are initially primed by microenvironment cues and antigen presenting cell (APC) licensing following acute infection of peripheral tissues. Despite their capacity for heterologous protection against influenza virus challenge, scant knowledge exists as to factors necessary to establish and maintain airway and lung parenchymal (LP) CD8 T_{RM} cells. This body of work focuses on 1) the regulation of CD8 T_{RM} cell establishment and maintenance in the LP and airways and 2) the protection conveyed by airway and LP CD8 T_{RM} cells. In the absence of antibody protection, antigen-specific T cell responses, generated following previous influenza virus infection, reduce murine morbidity and mortality during lethal H7N9 influenza virus challenge. Airway CD8 T_{RM} cells are alone sufficient to mediate protection upon heterologous challenge, working in concert with LP CD8 T_{RM} cells to produce effector cytokines and kill infected cells, respectively. Furthermore, this work demonstrates that, in contrast to mechanisms described for other tissues, lung CD8 T_{RM} cell establishment requires cognate antigen recognition once systemic effector T cells are recruited to the lung by local inflammation. Priming with local antigen and inflammation or native intranasal infection conveys equal protection upon heterologous challenge, while priming with local inflammation alone does not confer protection. This combination of local antigen and inflammation formed long-lasting T_{RM} populations in the LP and airways that highly express the chemokine receptor CXCR6 and adhesion molecule CD49a, which uniquely characterize these two populations. Importantly, we demonstrate that CXCR6 is necessary for robust formation of virus-specific airway and LP CD8 T_{RM} cells. These findings have identified novel mechanisms regulating the establishment and protective efficacy of lung CD8 T_{RM} populations. Applying these findings may aid in the development of a new vaccination strategy for enhanced CD8 T_{RM} cell establishment and protection.

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Chapter 1

Introduction: Evolving the Understanding of T Cell Memory

Adaptive Immune System

Mediating eventual clearance of a pathogen initially controlled by the innate immune system, the adaptive immune system provides subsequent protection from a pathogen expressing cognate antigens for which the immune system has generated a response.

Humoral immunity relies on antibody protection and affinity maturation by B cells which receive help in the form of local activating cytokines and receptor cross-linking by CD4 T cells (1). This process promotes the generation of neutralizing antibodies, which are capable of effectively 1) Inhibiting a pathogen from infecting host cells, in the case of intracellular pathogens; 2) Initiating antibody-dependent cell-mediated cytotoxicity whereby natural killer (NK) cells, macrophages, neutrophils, and eosinophils degranulate for direct cell lysis; 3) Opsonizing a pathogen for eventual phagocytosis by recruited macrophages; 4) Binding the pathogen to permit the activation of complement and assembly of the membrane attack complex to disrupt the membrane polarization of the target pathogen (2).

Thus, pathogens are under selection pressured to modify surface antigens to escape antibody neutralization and opsonization. Minor alterations in antigenic epitopes of a pathogen's exterior limit the breadth of antibody-mediated humoral immunity (3). For intracellular pathogens, which enter the extracellular space solely to move to another cell or host and continue the infection chain, the cellular arm of the adaptive immune system is overwhelmingly important to mediate protection and limit the spread of infection. While antibodies can recognize both linear and conformational epitopes on a pathogen, the cellular immune system

only recognizes linear peptides in the context of MHC (Major Histocompatibility Complex) (4). These linear sequences can be processed from any protein, intracellular or extracellular, from pathogen or host (self). These processed peptides are presented on MHC molecules for recognition by local T cells (5). MHC-II is expressed on antigen-presenting cells (APCs) largely consisting of dendritic cells and macrophages which process proteins they phagocytose for presentation to CD4 T cells (6). All nucleated host cells express MHC-I, processing and presenting intracellular proteins to CD8 T cells.

Cellular Immunity:

Following αβ-TCR (T cell receptor) rearrangement, thymocytes are educated in the thymus through positive selection to recognize self by being able to bind MHC-I or MHC-II; those that fail to gain TCR stimulation through interaction with MHC, die from neglect. Conversely, T cell progenitors also undergo negative selection where T cell progenitors that recognize self-peptides in the context of MHC are deleted from the T cell repertoire; defects in negative selection can pre-dispose an individual to autoimmunity diseases (7). Upon successfully undergoing selection, these naïve T cells survey the circulation, secondary lymphoid organs, and periphery for the distinct antigen to which their TCR binds.

Recognizing antigen in the context of MHC class II, "helper" CD4 T cells largely work to modulate and coordinate an immune response, optimizing it to best focus on the pathogenic insult at hand through directed cytokine production. Induced by select cytokines, CD4 T cell subsets adopt a profile characterized by expression of a single master regulator transcription factor and a unique cytokine production profile, serving to adapt the immune response to target a specific pathogen class; these subsets are summarized in Table 1.

CD4 T cell subset	Inducing cytokines	Characteristic TF	Target cytokine produced	Beneficial Function
Th1	IL-12	T-bet	IFN-γ, TNF-α	Protect from intracellular pathogens
Th2	IL-4	GATA-3	IL-4, IL-5, IL-13	Protect from multicellular parasites
Th9	TGF-β, IL-4	PU.1	IL-9	Protect from extracellular parasites
Th17	TGF-β, IL-6, IL-23	RORC2	IL-17, IL-21, IL-22, IL-25, IL-26	Protect from extracellular bacteria & fungi
Th22	IL-6, TNF-α	AHR	IL-22	Restrict commensals to their niche
Treg	TGF-β	FOXP3	IL-10, TGF-β	Maintain self-tolerance
TFH	IL-6, IL-21	BCL6	IL-21	B cell help

Table 1: Description of known CD4 T cell subsets; adapted from Geginat, J *et al.* 2014 Frontiers in Immunology & Raphael, I *et al.* 2014 Cytokine (8, 9).

Protection conveyed by cellular immunity is broader in that epitopes recognized by CD8 cytotoxic T lymphocytes (CTLs) are often found in conserved internal structures of the pathogen, enabling memory CTLs to recognize many different strains of a pathogen, such as influenza virus, and limiting the potential for pathogen escape upon secondary challenge (10-12). MHC class I-restricted CTLs play a central role in intracellular pathogen clearance, especially influenza virus clearance during primary infection of the lung (13-16). TCR binding of a MHC-I molecule displaying a foreign peptide permits activated and memory CTLs to recognize when a host cell is infected with an intracellular pathogen. CTLs can selectively kill infected cells through two means (17). Receptor-mediated cell-directed apoptosis occurs when FAS ligand, expressed on the CTL cell surface, binds to FAS receptor expressed on the target cell to induce Caspase 1 cleavage and subsequent apoptosis of the target cell (18). Alternatively, the CTL can form a cytotoxic synapse with the target cell to directly exocytose perforin and granzyme, mediating target cell death; perforin self-assembles to form pores in the target cell membrane, while granzyme, a serine protease, enters the cell to directly activate apoptosis of the infected cell (19). Finally, CTLs produce paracrine and autocrine cytokines, albeit to a lesser extent than CD4 T cells, to control local immune responses.

During an acute infection, naïve T cells can encounter antigen in the context of MHC-I (pMHC-I) presented on APCs at the site of infection or in the draining lymph node. Binding of TCR to pMHC-I promotes the formation of an immunological synapse and represents signal one of T cell activation (20, 21). Co-stimulation through CD28 on the T cell by either CD80 (B7-1) or CD86 (B7-2) on the APC provides signal two, which is necessary for both sustained effector function and generation of long lived T cell memory (22). Paracrine and autocrine production of IL-2, largely by CD4 T cells, induces a feed forward signaling cascade that enables for proliferation of effector T cells and sustains the acute immune response (23). Upon resolution of the acute infection, the effector T cell compartment contracts until a stable memory T cell number is reached for the target pathogen. These newly minted memory T cells will be maintained within the larger memory T cell compartment through signaling of homeostatic public factors IL-7 and IL-15 in the absence of secondary antigenic stimulation (24, 25).

CD8 Memory T cells:

Following resolution of an acute infection, three types of long lived memory CD8 T cells are generated. Central memory CD8 T (T_{CM}) cells are characterized by their co-expression of CCR7 and CD62L which allow them to home to and reside in secondary lymphoid organs such as lymph nodes and white pulp of the spleen (26). Effector memory CD8 T (T_{EM}) cells lack expression of CCR7 and CD62L but do express markers of antigen experience such as CD127 and CD44, in mice (26). T_{EM} cells are found in the circulation, entering and exiting peripheral tissues transiently during continual surveillance for their cognate antigen. Resident memory CD8 T (T_{RM}) cells are phenotypically similar to T_{EM} cells in that they lack expression of lymphoid homing markers yet express markers of antigen experience; however, unlike the

T_{EM} population, T_{RM} cells, upon entering a peripheral tissue, remain there long-term with most populations maintaining themselves through homeostatic proliferation. Resident memory formation only occurs in peripheral tissues where the acute infection occurs. Secondarily, T_{RM} cells are noted for their expression of CD69 and CD103 (α_E integrin). Information summarizing the memory CD8 T cell subsets can be found in Table 2.

CD8 T cell Subset	Phenotype	Location
T_{CM}	$CD44^{high}$, $CD62L^{+}$, $CCR7^{+}$, $CD127^{+}$, $CD69^{-}$, $CD103^{-}$	Secondary lymphoid organs (lymph nodes, spleen white pulp); blood; bone marrow
T_{EM}	$CD44^{high}$, $CD62L^{-}$, $CCR7^{-}$, $CD127^{+}$, $CD69^{-}$, $CD103^{-}$	Spleen red pulp; blood; lung; liver; intestinal tract; female reproductive tract; kidney; adipose tissue; heart
T_{RM}	$CD44^{high}$, $CD62L^{-}$, $CCR7^{-}$, $CD127^{+}$, $CD69^{+}$, $CD103^{+}$	Skin epithelium; intestinal tract; female reproductive tract; salivary glands; lung parenchyma; lung airways; brain

Table 2: Characteristics of memory CD8 T cell subsets in mice; Central memory (T_{CM}), Effector memory (T_{EM}), Resident memory (T_{RM}); adapted from Mueller, SN *et al.* 2013 Annual Reviews (27)

Upon encountering cognate antigen during a secondary infection, T_{CM} cells proliferate to expand a secondary effector T cell population; T_{EM} cells are already primed for effector function and act to contain the pathogen to the site of initial infection (28). T_{RM} cells are already localized to sites with a high probability of secondary infection; thus, upon encountering cognate antigen, they act as sentinels to organize an adaptive immune response specific to the peripheral tissue, limiting the expansion of the pathogen niche and tempering the duration of the immune response so as to limit immunopathology.

Resident Memory CD8 T Cells:

CD8 T_{RM} cells convey immunologic protection in peripheral tissues (27, 29-31). In addition to residency in the tissue in which they are originally formed, the distinguishing aspects of T_{RM} cells consist of a unique chemokine receptor which allows for homing to the target peripheral tissue and a specific adhesion molecule necessary for extravasating into and remaining in the target tissue.

Two notable and well-studied T_{RM} populations are found in the skin and gut; gut T_{RM} cells express the chemokine receptor CCR9 and adhesion molecule integrin $\alpha_4\beta_7$; skin T_{RM} cells express the chemokine receptor CCR10 and adhesion molecule cutaneous lymphocyte-associated antigen (CLA), conveying local protection to pathogens such as herpes simplex virus (32-34). For the CD8 T_{RM} population of the female reproductive tract (FRT), the chemokine receptor CXCR3 has been shown to be important for T_{RM} cell formation, while the corresponding adhesion molecule has yet to be elucidated. The T_{RM} population of the lung parenchyma (LP) and airways remains largely uncharacterized; yet, the adhesion molecule CD49a (VLA-1) has been shown to be necessary to generate a protective CD8 T cell response within the LP and airways following a secondary influenza virus challenge (35).

While this chemokine receptor and adhesion molecule pairing definition aids in delineating the T_{RM} population of a specific tissue, it does not provide global characteristics for all T_{RM} populations. An absolute globally defining characteristic of T_{RM} populations is that they are and remain resident within the tissue in which they were initially established. The practice of intravenous (IV) injection of a fluorophore-conjugated antibody immediately prior to organ harvest of experimental animal models has allowed for a finer delineation of true resident populations by both immunofluorescence and flow cytometry (36). Those cells which stain positively for the IV injected antibody have access to the vasculature, while those which stain negatively are resident within the tissue proper, protected from the circulating antibody. While important for all peripheral tissues, this technique was even more critical for heavily vascularized organs such as the lung and liver.

Prior to the application of IV labeling, two widely accepted canonical markers for T_{RM} cells were CD103 and CD69. Binding to E-cadherins which form a belt of adherens

junctions near the basal side of epithelium, CD103 is the integrin α_E which largely complexes with the integrin β_7 in mucosal tissues. CD69 is a C-type lectin marker of activation which is highly upregulated upon T cells quickly after activation; its continual expression on T_{RM} cells promotes retention of the cells within the tissue through its antagonistic effect on sphingosine 1-phosophate receptor-1 (S1P1) (37). Without CD69 expression and subsequent transcriptional down-regulation of S1pr1 (which encodes S1P1), cells could extravasate from the tissue by S1P1 mediated effects and re-localize to lymphoid tissues (38).

However, it should be noted that some T_{RM} populations lack expression of CD103 and/or CD69, yet still demonstrate residency within the tissue. For instance, CD4 T_{RM} cells in the lung, lack the high up-regulation of CD103 which is typically found on CD8 T_{RM} cells in the same tissue. Similarly, CD8 T_{RM} cells of the kidney and brain largely lack CD103 expression. Contrastingly, populations within secondary lymphoid organs, such as the spleen and draining lymph nodes, which exhibit markers of antigen experience and co-express CD69 and CD103, could define yet another population of T_{RM} cells that remain within the lymphoid organs themselves (39). Furthermore, recent evidence from parabiosis experiments show that memory CD8 T cell populations in tissue can lack expression of either CD69 or CD103 but exhibit equal residency as those antigen-specific cells which co-express both CD69 and CD103 (40). Thus, it appears that the quintessential requirement that T_{RM} cells express CD103 and CD69 is not wholly accurate and is insufficient for proper definition of T_{RM} populations.

Therefore, the larger question becomes, "What is the true global phenotype of a T_{RM} population?" Or, perhaps more simply, "Is there a true global phenotype of a T_{RM} population, or is there a unique definition for each specific peripheral tissue?"

Certainly the unique pairing of adhesion molecule and chemokine receptor specific

to T_{RM} cells of particular tissues would provide for a tissue specific phenotype. For a global definition, characterization by transcription factor expression has been suggested, particularly with respect to regulation of KLF2 (Kruppel-like factor 2), a zinc finger, which positively regulates gene expression of CD62L and S1P1 and is found to be down-regulated in T_{RM} cells across all examined tissues (38, 41).

A second way of potentially defining global T_{RM} populations could be by their ability to mediate protection. Direct transfer experiments would aid in demonstrating sufficiency of protection, as has been done for the airway CD8 T_{RM} population (42). But such experiments present distinct hurdles for execution given the difficulties in efficiently isolating T_{RM} cells from peripheral tissues in any appreciable numbers and then ensuring their correct localization upon transfer into a naïve animal (40). Recent studies on the T_{RM} populations of the FRT and lung airways have demonstrated both populations mediate protection through antigen-specific production of effector chemokines and cytokines to help establish an anti-viral state following secondary challenge. Further studies in this nascent area of research will hopefully elucidate a more universal definition for T_{RM} cells.

Establishment, Maintenance & Recall of T_{RM} Cells:

Upon primary infection, effector T cells enter effected peripheral tissues, migrating along gradients established by innate immune cells in response to inflammation and pathogen-associated molecular patterns (PAMPs) (43). The number of effector T cells entering the peripheral tissue wanes with resolution of the local infection and inflammation, but a population of T cells, which eventually become a T_{RM} population, traffic to the target peripheral tissue, maintain themselves locally, and do not return to the systemic circulation (44, 45). It is understood that T_{RM} populations are generated through an imprinting or tissue

licensing process whereby memory T cells gain a unique pairing of chemokine and adhesion molecules that enable access to and establishment in the target peripheral tissue where the primary infection occurred.

Understanding how T_{RM} populations are established is currently a large gap in the field, as no overarching postulate appears to widely apply to most T_{RM} populations. Recently, different strategies of "pulling" systemic effectors to areas where resident memory formation is desired have been employed to tease out the stimuli necessary for the formation of these populations.

For example, a recent study found that a FRT T_{RM} population could be merely established through transient chemokine signaling of systemic effectors to the site in the absence of antigen and inflammation (46, 47). Additionally, other studies have shown that a long-lived T_{RM} population can be established in the skin through an antigen-independent manner where local inflammation from a non-infectious insult induces migration of systemic effectors to the site; this generated population is also able to convey protection (48). However, the necessary stimuli for inducing the LP and airway T_{RM} population are largely unknown; greater insight to the establishment and maintenance of the airway and LP T_{RM} population is further discussed in Chapter 4.

Lung & Airway Microenvironment:

The lung is a highly structured organ whose continued optimal function is extremely important for quality of life; yet, it is a very fragile environment assaulted with potential pathogens, allergens, and irritants with every breath (49). Thus, it is necessary that any inflammation in the lung be tightly controlled, permitting effective pathogen protection and functional repair capacity without an overzealous response (50). The presence of airway CD8

T_{RM} cells is one way to quickly control the local spread of a pathogen and mitigate an undesired and potentially deleterious wide-spread inflammatory response.

Furthermore, the lung airways represent a harsh microenvironment with poor nutritional content, including glucose concentrations an order of magnitude lower than is found in the blood (51, 52). Corresponding with the known down-regulation of cellular proteins, including CD11a, autophagy may be an auxiliary survival means during the ten day lifespan of T_{RM} cells in the airways. Additionally, the high oxygen tension, as experienced in the airways, limits CD8 T cell cytolytic function, yet can be recovered if cells are returned to an environment at physiological oxygen tension (53). While important for the physiological function of the lung, surfactant also suppresses T cell proliferation in a dose dependent manner (54, 55). Despite these challenges, the airway T_{RM} population still plays a critical role upon heterologous challenge.

Respiratory Viral Infections:

Respiratory virus infections such as those caused by influenza and parainfluenza viruses are a significant source of morbidity and mortality, presenting a major health obstacle for the United States and the world (56). Annual epidemics of influenza virus are responsible for an average of 30,000 deaths and 200,000 hospitalizations, as the virus exacerbates other comorbidities including genetic defects (e.g. Cystic Fibrosis), environmental predispositions (e.g. asthma), and age-related illnesses (e.g. chronic obstructive pulmonary disease) (57-62). Additionally, mortality from these viral pathogens has increased significantly within the last two decades with the economic burden of the yearly influenza epidemics amounting to $87.1 billion annually (63, 64). Emerging strains, such as the highly virulent avian H5N1 influenza virus variant arising in Hong Kong in 1997, present a pandemic concern, especially in the context of

the potential to successfully adapt to human hosts and based on the rapid spread of the 2009 H1N1 swine-origin virus which infected 11-21% of the world population prior to vaccination (65-67). With increased globalization, the impact on a global scale could be catastrophic in both human life and economic stability. Thus, there is a critical need to understand how pulmonary immunity can promote broad and enhanced immunity to respiratory pathogens. Influenza virus provides a clinically important and well-modeled pathogen for investigation of respiratory immunity.

Two Peas in a Pod, Airway & Lung Parenchyma T_{RM} Cells:

Mouse model studies have shown that the absolute number of virus-specific memory CD8 T cells in the airways progressively declines until reaching a stabilization point several months after the acute infection, while the number of systemic virus-specific memory CD8 T cells remains constant (68-71). However, ample animal model evidence demonstrates that CD8 T_{RM} cells significantly reduce viral loads, conferring protective immunity to respiratory viruses while promoting faster viral clearance and limiting immunopathology, as would be expected based on their unique localization at the site of viral infection and replication (72, 73). Notably, the number of airway CD8 T_{RM} cells correlates with the efficacy of cellular immunity to influenza; a steady decline in cellular immunity efficacy corresponds to the kinetic decline in virus-specific airway and LP T_{RM} cell numbers (35, 74, 75). Given the protection conveyed by virus-specific respiratory T_{RM} cells upon heterologous challenge, it is essential to understand their establishment, maintenance, and function.

Phenotypic Differences, LP vs Airway & CD4 vs CD8:

Airway CD8 T_{RM} cells have a decreased expression of CD11a that is not reflected on LP CD8 or CD4 T_{RM} cells. However, decreased CD11a expression reduces overall complexing

with CD18 for LFA-1 formation, limiting the ability of airway CD8 T_{RM} population to form a cytolytic synapse, ultimately resulting in decreased cytolytic function (76, 77). This down-regulation is most likely a result of the nutrient poor environment, as proliferation capacity and expression of CD11a are regained upon isolation and *in vitro* culture or adoptive transfer, demonstrating their plasticity (78). Furthermore, CD11a expression inversely correlates with the amount of time that CD8 T cells have resided in the airways, denoting a one way trip (79). Despite CD11a down-regulation, CD103 and CD69 co-expression is maintained following extravasation into the lung airways.

LP CD4 T_{RM} cells are noted for their expression of CD11a and CD69, lacking expression of CD103 (80). In contrast, LP CD8 T_{RM} cells express CD11a, CD69, and CD103 (36). Both populations are protected within the tissue proper from intravenous labeling with fluorophore-conjugated antibodies, allowing for delineation of these populations from those within the systemic circulation and lung microvasculature, which stain positively.

Establishment of Airway & LP Respiratory CD8 T_{RM} Populations:

The direct juxtaposition of the LP and airway T_{RM} populations suggest a similar point of origin rather than two distinct populations forming independently of one another. But whether these populations are generated *de novo* within the tissue proper or the draining lymph node (mediastinal LN), remains to be seen. In fact, some of our preliminary data indicate that the LP CD8 T_{RM} population may actually give rise to and replenish the airway CD8 T_{RM} population.

Ultimately, the challenge lies in localizing competent protectors to likely sites of infection. To do this, experiments must first elucidate the sufficiency and necessity of certain stimuli to induce long-lived T_{RM} populations; a topic discussed in greater detail in Chapter 4.

The route of infection is important for the establishment of LP and airway T_{RM} populations. Despite a robust antigen-specific systemic response, an intraperitoneal (IP) influenza virus infection is insufficient to generate a LP and airway CD8 T_{RM} population (81, 82). However, a subsequent IN infection with the same influenza virus can generate *de novo* LP and airway T_{RM} populations, indicating that pre-existing systemically primed T cell responses, with respect to localization, do not suffer from a similar antigenic sin that antibody responses experience (81). Moreover, memory CD8 T cells derived from an IN infection preferentially localize to the LP and airways from the circulation compared to cells from an IP infection; these progenitor cells establish the LP and airway T_{RM} populations which remain within the tissue, permitting continual steady-state cell recruitment to the airways in an antigen-independent manner (70). Finally, murine and human studies demonstrate that respiratory virus-specific memory T cells are more prevalent in the lung than T cells specific for non-respiratory pathogens (83, 84).

During the establishment process, LP and airway CD8 T_{RM} cell progenitors interact with APCs expressing TGF-β inducing down-regulation of T-bet and concomitant up-regulation of CD103 independent of signaling through SMAD4 (Sma- and Mad-related protein 4) (85, 86). CCR7 and CD62L are also down-regulated while interaction with APC pMHC, laden with local antigen, induces up-regulation of CD69 which antagonizes and ultimately suppress S1P1 expression via KLF2 down-regulation (38, 85, 87, 88). Down-regulation of CCR7 and S1P1 inhibits cell egress from the tissue, thereby promoting retention within the tissue. Temporally, antigen-specific CD8 T cell replication occurs within the lung by day 6 post-IN influenza virus infection with up-regulation of both CD69 and CD103 being observed between day 7 and day 10 post-infection for the LP and airway CD8 T_{RM} populations (89).

Notably, interactions between CD4 and CD8 T cells are necessary for the formation

of an efficacious and protective CD8 T_{RM} population in the LP and airways (85). CD4 help promotes the antigen-specific CD8 T cells to closely localize to the lung airway epithelium, up-regulate CD103, and enhance mediation of heterosubtypic immunity. Furthermore, localization of both CD4 and CD8 LP T_{RM} populations preferentially cluster around the lung airways (86, 90).

Maintenance of Airway & LP Respiratory CD8 T_{RM} Populations:

Unlike most T_{RM} populations in other anatomical locations, the airway T_{RM} population is not directly maintained via cytokine-driven homeostatic proliferation. Instead, they have a half-life of seven to ten days after entering the airway environment; thus, to compensate for cell death in the airways, they require continual cell recruitment from a replicative reservoir for replenishment following acute respiratory infection (70, 79). The airway T_{RM} population is maintained for years through continual recruitment, long after residual antigen reservoirs have been depleted (70). Thus, there is increasing experimental support for a long-term airway T_{RM} cell recruitment model which embraces steady-state T cell recruitment to the lung airways via an antigen-independent process. While the proximity of the LP T_{RM} population to the airways could be posited as said replicative reservoir, the mechanisms underlying the long-term maintenance, including size of population, and the antigen-independent recruitment of the airway T_{RM} cell population remains poorly understood and are an active topic of investigation within our laboratory.

LP and airway CD8 T_{RM} populations are also maintained in an IL-15 independent manner with steady-state recruitment to the airway also capable in the absence of IL-15 (78, 91). CD127 expression is lower on LP and airway T_{RM} populations as compared to the T_{EM} and T_{CM} populations, potentially indicating a decreased role for IL-7 in the long-term

maintenance of these populations or perhaps suggesting a cause for waning cell numbers over time due to limited access to IL-7 within the tissue proper. Beyond resolution of the acute infection, maintained expression of IFITM3, through hypomethylation of its promotor, conveys resistance from viral infection to antigen-specific airway and LP T_{RM} cells during a secondary infection, thereby enhancing protection from viral infection (92). Similarly to other T_{RM} populations, the LP T_{RM} population is maintained independently of communication with lymphocyte reservoirs in secondary lymphoid organs (90).

Function & Mechanisms of Protection

Recent studies have demonstrated the impact of cellular immunity upon influenza virus challenge in both the presence and absence of neutralizing antibody responses. During annual epidemics, in which cross-protective neutralizing antibodies may still be present, pre-existing cellular immunity to the challenge influenza virus resulted in drastically reduced symptom loads, to the point of some cases being completely asymptomatic, despite molecular evidence of an active infection (93). In the rarer and more dangerous case of a global pandemic, cross-reactive CD8 T cell populations in patients have been correlated with decreased morbidity and mortality during the 2009 H1N1 pandemic (94). When examining emerging influenza virus strains which have zoonotically transitioned from an avian source to humans in the past, such as the 2013 H7N9 influenza virus which resulted in an outbreak in poultry workers from southeast Asia, we identified that naturally occurring cross-reactive CD4 and CD8 T cells are able to protect mice upon lethal H7N9 challenge, reducing both morbidity and mortality (95). Acquired human samples from patients previously exposed to circulating influenza virus were also found to cross-react when exposed to the H7N9 influenza virus, indicating that these CD4 and CD8 T cell populations could convey a similar level of protection as we observed in

the mouse model. These findings are further discussed in Chapter 2 of this dissertation.

During heterologous challenge, there are three distinct waves of the cellular immunity recall response. The first line of protection consists of the airway and LP T_{RM} population, directly present at the site of infection. The second is the circulating memory CD8 T cells recruited in response to lung inflammation at around day 3 of infection; the final tier are the secondary effector CD8 T cells recruited from local lymphoid structures beginning around day 5 of infection (70, 77, 96-98). Given that the lung is a relatively inflammation intolerant organ, it would be optimal to enhance the first line of protection to limit pathogen spread early during the infection time course and thereby also limit unnecessary immunopathology.

It is known that protection from respiratory pathogens, including influenza virus, paramyxoviruses, and respiratory *mycobacterium tuberculosis* infections, correlates with number of airway T_{RM} cells present, with both CD4 and CD8 airway T_{RM} populations being able to mediate protection independently of one another (36, 99-101). Up until recently, the explicit role and functional capacity of the airway CD8 T_{RM} population in protective immunity was poorly defined despite the assumption that they decrease viral loads. The use of intratracheal transfer experiments definitively demonstrated that airway CD8 T_{RM} cells significantly decrease viral loads in an antigen-specific manner. As discussed further in Chapter 3, this recent investigation demonstrates the capacity of airway T_{RM} cells to limit viral burden when present, thereby promoting a more rapid viral clearance and limiting immunopathology.

While airway CD8 T_{RM} cells are poorly cytotoxic, they are still mediate protection by acting as sentinels capable of quickly detecting cognate antigen to rapidly produce effector cytokines, of which IFN-γ is produced in the greatest amounts (42, 77, 102). IL-10 is also produced in appreciable amounts, supposedly to promote a tempered immune response upon

secondary infection (103). Cytokine production can help to establish an early antiviral state and recruit a measured number of immune effectors to further contain the infection. Likewise, the LP CD8 T_{RM} population remains robustly cytolytic, effectively acting in concert with the airway T_{RM} population to clear infected cells.

Tools for Studying Airway & LP T_{RM} Populations

Tools for the study of LP and airway T_{RM} populations have improved drastically within the last few years. *In vivo* models have distinct advantages for studying T_{RM} populations, as contextual cues from other cells and within the tissue affect the development and actions of these populations. Parabiosis experiments remain a gold standard for the field to identify the peripheral tissues containing T_{RM} populations and to annotate the phenotype of the T_{RM} populations; this has led to the conclusion that CD103 and CD69 co-expression are specific but not sensitive for defining which cells are truly resident memory within a tissue and that traditional methods of isolating T_{RM} cells from tissues underestimate the size of the T_{RM} population (40). The practice of intravenously (IV) injecting a fluorophore-conjugated antibody prior to animal euthanasia conveys similar technical identification of T_{RM} populations as the parabiosis model, delineating those cells which are within the tissue proper instead of the systemic circulation or lung microvasculature (36, 42). Utilization of bone marrow (BM) chimeras is a powerful tool that permits the head-to-head, internally controlled comparison of different hematopoietic lineages, study of chemokine receptor necessity in trafficking to a target tissue, and investigation of developmental interactions required for T_{RM} cell formation by using selected APC knockout strategies (77). Furthermore, adoptive transfer of transgenic cell lines or dual adoptive cell transfer remains a strong asset for studying antigen-specific responses (87, 104). Finally, a mouse model new to immunology, Brainbow mice, allows an

investigator to track T cell ancestry on a population basis (105). Under inducible control with specific induction in cells expressing Thy1, Brainbow mice could be used to elucidate T cell lineage, including the deterministic source of the airway T_{RM} population, whether from the systemic circulation or perhaps the LP T_{RM} population.

Advancements to microscopy imaging, including spectral analyzer and *in situ* imaging provide spatial and temporal context to phenotypic data provided by flow cytometry. A snapshot of the lung and airway environment, immunofluorescence conveys important contextual evidence of direct interactions between cell subsets; surface molecules expressed, such as TGF-β and chemokines; and proximity to gross anatomical landmarks, such as vasculature endothelium and airway epithelium for the lung (85, 106). Alternatively, progress in the field of intravital imaging, permits a window into the lung to show lymphocyte trafficking in real time (107, 108).

Ultimately, an overarching goal to studying the airway and LP T_{RM} populations is to identify and validate correlates of protection. New vaccination techniques such as one employing local "pull" of systemic effectors, discussed in Chapter 4, can be best tested using traditional *in vivo* challenge systems as readouts of efficacy, including viral titers (98), weight loss studies (95), and molecular detection of viral loads (109).

Given the unique physiological setting in which the airway T_{RM} population resides, further study of changes in molecular regulation and metabolism would be critical to understand the constraints placed upon this population. Metabolic strictures, including the employment of aerobic and anaerobic metabolism or catabolic autophagy energy acquisition, of the airway T_{RM} population could be studied further with the use of a Seahorse Extracellular Flux Analyzer, as has been used for studying T cells within the secondary lymphoid organs

and systemic circulation (110, 111). Finally, molecular characterization of the airway and LP T_{RM} populations can be used to determine a common lineage through principal component analysis of common transcript profiles or transcription factor expression (41).

Challenges, Known Unknowns & Direct Applications of Knowledge:

To further examine the molecular phenotype of the LP and airway T_{RM} populations, the ability to selectively isolate these populations from transiently circulating T_{EM} populations is necessary. In the absence of globally associated phenotypic markers of T_{RM} populations, the most feasible means of doing this is to identify the necessary chemokine receptor and adhesion molecule pairing, as it is commonly accepted that a unique chemokine receptor and adhesion molecule pair is necessary for homing of T cell subsets to specific peripheral tissues. While the adhesion molecule VLA-1 (integrin $\alpha_4\beta_1$, CD49a) has been shown to be necessary to convey airway T_{RM} cell protection in influenza infections, the unique chemokine receptor of both the LP and airway T_{RM} populations remains undefined (35).

Following influenza virus infection, CXCR3 is important in the acute accumulation of antigen-specific CD8 T cells in the lung and airways (98, 112, 113). CXCR3 could be required to allow effector CD8 T cells entry to the inflamed lung tissue, much as is required for the skin T_{RM} population, so that the establishment of a LP and airway T_{RM} population can actually occur, but the establishment and long-term maintenance of that population could be under control of another chemokine receptor (41, 114).

A second chemokine receptor shown to be expressed on antigen-specific cells in the lung is CXCR6. CXCR6 is largely uncharacterized with respect to its role in T cell trafficking to peripheral tissues; albeit, it has been affiliated as a co-receptor for HIV and is highly expressed by hepatic NK T cells (115-117). Furthermore, it has been shown that human lung tissue is

enriched for CXCR6$^+$ T cells compared to blood, and the ligand for CXCR6, CXCL16, is highly expressed in the lung tissue by epithelial cells and the airways by alveolar macrophages (118, 119). Neither the environment which promotes CXCR6 expression nor the molecular cues that regulate CXCR6 expression on antigen-specific airway and LP T_{RM} cells are understood.

Perhaps one of the largest challenges of direct application of this research and subsequent uptake of any resident memory targeted vaccine to respiratory pathogens would be achieving protective durability over the year to decade time frame. Currently, protection conveyed from an initial live viral infection begins to drastically decrease by 180 days (74). Despite antigen-specific cells still being detectable and sensitive to antigen beyond one year, their numbers may not be large enough to effectively contain a pathogenic challenge. Thus, any practical vaccination strategy would require enhanced durability of response. An additional challenge includes determining whether one or both of the LP and airway T_{RM} populations are protective. Previous studies, including the one in Chapter 3, have shown that CD4 and CD8 airway T_{RM} cells lend protection independent of LP T_{RM} cells, but the question of protection by the LP T_{RM} population remains unanswered.

Current vaccination strategies do not ensure that cells are actually localized to the site where protection is most needed from secondary infection, assuming that evidence of systemic response is equivalent to downstream protection in many cases. It is becoming readily apparent, especially with the expansion of the resident memory field, that both location and functionality are crucial for consideration when designing future T cell mediated vaccines. A number of approaches have tried to accomplish LP and airway T_{RM} cell protection without direct infection of the upper respiratory tract.

One study IV transferred *in vitro* primed transgenic effector T cells followed by targeted

delivery of antigen to lung APC subsets, including respiratory $CD11b^+$ ($F4/80^-/MHCII^+/CD11c^+/CD11b^+$) and $CD103^+$ ($F4/80^-/MHCII^+/CD11c^+/CD103^+$) APCs. With dosing a source of inflammation, such as LPS, peptide linked α-Clec12A or α-DEC205 antibodies promoted selective uptake of the peptide with α-Clec12A targeting both $CD11b^+$ and $CD103^+$ APCs, while α-DEC205 specifically targets $CD103^+$ APCs, generating a memory population in the lungs derived from the IV transferred effector T cells (87). Other strategies of localizing antigen to the airways could have similar results, such as our vaccination strategy in Chapter 4 which merely uses peptide dosing of antigen in accompaniment with a TLR (Toll-like receptor) agonist, resulting in robust LP and airway T_{RM} populations from systemically primed effectors. One item to note, while systemic effector generation appears to be critical in establishing a LP and airway T_{RM} population when not directly infecting the upper respiratory tract, for the sake of developing a vaccine with practical clinical applications, focus should rely on establishing systemic effectors *in vivo*.

While the majority of resident memory studies have been completed in murine models, the phenotype of human T_{RM} populations has largely been found to be similar to their equivalent murine populations, including the predominance of respiratory pathogen-specific $CD103^+$ CD8 T cells in the LP in contrast to matched PBMC samples (120); these $CD103^+$ populations contain more robust cytokine production potential and less proliferative capacity than those which are $CD103^-$. Specifically, the human lung T_{RM} population contains an abundant number of influenza-specific T cells which are not found in matched T_{RM} populations of other tissues, and conversely, CD8 T cells specific for systemic pathogens, such as EBV (Epstein–Barr virus) and CMV (Cytomegalovirus), are not found in human lung tissue, let alone expressing CD103 (121).

However, it is largely implied that regulation of LP and airway T_{RM} populations would be considerably different in humans. The frequency of respiratory infections over the life of an individual would undoubtedly result in temporal fluctuations in the frequency of T_{RM} cells specific for any given antigen. While further insight into this question could be achieved through serial biopsies of the tissue, this is not feasibly accomplished in a healthy population; instead, secondary analysis of tissues from diagnostic biopsies could be used as a snapshot of T_{RM} population status. Another potential avenue of investigation could use longitudinal biopsies from donated organ specimens, prior to and post-transplantation, that would normally be used to evaluate any evidence of graft rejection but could secondarily be used to track changes in T_{RM} populations over time.

Due to differential tissue bioavailability and radiation shielding, T_{RM} cells tend to be more resistant to eradication by normal chemotherapeutics and radiation intended to target circulating lymphocytes. Depending on the scenario, this could be either harmful or beneficial.

Deleteriously, these populations could pose a problem whereby autoimmune reservoirs in peripheral tissues could complicate successful solid organ or bone marrow transplantation. For example, only CD8 T cells, expressing TGF-β-induced CD103, targeted host peripheral tissues during acute graft versus host disease; this selective destruction was ameliorated with the blocking of CD103 expression on graft CD8 T cells (122). Conversely, a similar phenomenon is observed in instances of host versus graft disease in a renal allograft model (123). Also, the resistance of T_{RM} cells to eradication presents distinct challenges when they are involved in disease, as is the case with a malignant skin T_{RM} population in cutaneous T cell lymphoma patients (124, 125).

In contrast, T_{RM} populations within the LP could also certainly serve as a protective

reservoir from opportunistic respiratory pathogens that could otherwise plague a patient with a compromised adaptive immune system.

Some of these challenges may be mitigated through improved understanding of the establishment and maintenance of the LP and airway T_{RM} populations, including the regulatory machinery required to initially establish a robust population. Only then can we optimize a system for a desired effect.

Summary:

Cellular immunity to influenza viruses holds great promise as a means to generate a protective immune response that is capable of recognizing and limiting infections from serologically distinct influenza strains. Studies in animal models and humans have shown that virus-specific memory T cells can protect against heterologous influenza challenge, but the durability of this protection and the relative contribution of different memory T cell subsets to heterosubtypic influenza immunity are not well understood (75, 94, 95). T_{RM} cells, a relatively new subset of T lymphocytes noted for their permanent positioning within peripheral tissues, are likely to be key mediators of cellular immunity against influenza viruses due to their localization in the lung (42). The total lung-resident T_{RM} population is composed of two distinct T_{RM} populations, the LP and the lung airway T_{RM} population, which reside in very different local microenvironments despite being in direct juxtaposition to one another. Notably, the effects of an environment with high oxygen tension and poor nutrient availability present unique challenges to the long-term survival and maintenance of the airway T_{RM} population (51, 52). While there is some understanding on the function of lung parenchymal and lung airway T_{RM} population in heterosubtypic influenza immunity, there exists a dearth of information with respect to how these two populations are established during a localized acute infection,

how they are maintained into immunological memory, and their individual roles in protective immunity. Work contained in this dissertation aims to synthesize the current understanding of the airway and LP T_{RM} cell populations for protection against influenza infection, enhancing current theories regarding the establishment and long-term maintenance of these populations, their antiviral mechanisms, and their importance for cellular immunity in the lung.

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Chapter 2

Memory T cells generated by prior exposure to influenza cross react with the novel H7N9 influenza virus and confer protective heterosubtypic immunity

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Abstract

Influenza virus is a source of significant health and economic burden from yearly epidemics and sporadic pandemics. Given the potential for the emerging H7N9 influenza virus to cause severe respiratory infections and the lack of exposure to H7 and N9 influenza viruses in the human population, we aimed to quantify the H7N9 cross-reactive memory T cell reservoir in humans and mice previously exposed to common circulating influenza viruses. We identified significant cross-reactive T cell populations in humans and mice; we also found that cross-reactive memory T cells afforded heterosubtypic protection by reducing morbidity and mortality upon lethal H7N9 challenge. In context with our observation that PR8-primed mice have limited humoral cross-reactivity with H7N9, our data suggest protection from H7N9 challenge is indeed mediated by cross-reactive T cell populations established upon previous priming with another influenza virus. Thus, pre-existing cross-reactive memory T cells may limit disease severity in the event of an H7N9 influenza virus pandemic.

Introduction

Influenza viruses are a primary cause of severe respiratory tract infections worldwide. In addition to the health risks from circulating seasonal strains of influenza, the potential for antigenic shift and emergence of zoonotic pandemic strains present significant risks for increased morbidity and mortality. In February of 2013, an H7N9 influenza A virus (H7N9) of avian origin was laboratory confirmed in four human cases, resulting in three deaths (1). Clinical symptoms in these cases included fever and intractable pneumonia unresponsive to antibiotics, which progressively extended to more severe systemic complications (2). The majority of human cases have been associated with either direct contact with avian sources or poultry markets, and transmission studies on H7N9 in guinea pigs and ferrets have demonstrated a limited ability to effectively transmit via respiratory droplets, suggesting that H7N9 has not achieved sustained human-to-human transmission (3-6). However, recombination with other influenza viruses with greater propensity to bind human respiratory epithelium would be a cause for concern (7), and a recent surveillance study showed evidence for increased pandemic potential of H7N9 following an outbreak of 127 confirmed cases during January 2014, ten cases fewer than all of the 2013 season (1, 8).

Antibodies generated against circulating influenza viruses following infection or vaccination do not convey neutralizing protection against the novel H7N9 virus (9). In contrast, two initial studies showed that PBMCs from healthy donors expanded *in vitro* contained T cells that were cross-reactive for H7N9-derived target peptides or infected cells (10, 11). This is important, as animal models have shown that influenza-specific memory T cells can confer protection against a lethal challenge from an unrelated influenza virus in the absence of neutralizing antibody (12). Furthermore, a recent study demonstrated an important

role for CD8 T cell (CTL) heterosubtypic immunity in decreasing clinical complications in humans during the 2009 H1N1 influenza pandemic (13). Thus, it is conceivable that cross-reactive influenza-specific memory T cell responses could similarly convey a milder clinical course and reduced morbidity during H7N9 infection.

The potential of pre-existing cellular immunity to influenza virus to provide some degree of protection against H7N9 influenza infection is currently unknown because we do not know the level of memory T cell cross-reactivity between common circulating strains and H7N9, nor have animal models addressed the potential for heterosubtypic immunity to protect against a lethal H7N9 challenge. In the current study, we investigated the native frequency of H7N9 cross-reactive memory T cells in mice following infection with common laboratory influenza strains and in human PBMCs from healthy donors. Our data show that there is a significant percentage of memory T cells that recognize H7N9 influenza in mice following infection with several different common influenza strains and also in human PBMCs from healthy donors. We have expanded previous studies investigating human CD8 T cell cross-reactivity to H7N9 influenza virus (10, 11) by demonstrating that there is also significant human CD4 T cell cross-reactivity and by demonstrating that cross-reactivity to H7N9 in humans is sufficiently robust to be identified by using *ex vivo* analyses instead of skewing populations through *in vitro* expansion of target populations. Furthermore, cross-reactive memory T cells in mice were able to confer protection from a lethal H7N9 challenge and led to more rapid viral clearance. Thus, our data suggest that cross-reactive memory T cell responses may play an important role in limiting the severity of H7N9 infection in humans.

Materials & Methods

Mice. C57BL/6J mice from The Jackson Laboratory were housed under specific ABSL2

conditions at Emory University. For H7N9 challenge studies at the University of Georgia, mice were housed under ABSL2 conditions for immunization and then transferred to ABSL3 facilities for H7N9 challenge.

Ethics Statement. All experiments in this study were approved and completed in accordance with the Institutional Animal Care and Use Committee guidelines of Emory University (Protocol Number: DAR-2001547-071315GN) and the University of Georgia (Protocol Number: A2014 04-025-Y1-A0). The above named Institutional Animal Care and Use Committees specifically approved this study. All efforts were made to minimize suffering.

Influenza infections. Intranasal infections with influenza A/HKx31 (H3N2), A/PR8 (H1N1) and pandemic A/California/09 (H1N1) were used to generate influenza virus-specific memory T cells in mice as previously described (14). Influenza A/PR8 (H1N1) was used to establish immunological memory prior to secondary challenge with either 0.5 mLD_{50} (mouse LD_{50}) or 5 mLD_{50} influenza A/Anhui/1/2013 (H7N9). After H7N9 challenge, mice were monitored for weight loss and clinical symptoms every other day. Animals reaching defined endpoints of less than 65% original weight were humanely euthanized by Tribromoethanol (Avertin) overdose (600mg/kg) followed by brachial exsanguination. No other analgesics or anesthetics were administered during the time course. Subsets of mice were humanely euthanized on days three and six post-H7N9 challenge for analysis of lung virus titers. A/Anhui/1/2013 (H7N9) was provided by Richard Webby (St. Jude Children's Research Hospital, Memphis, TN) through the WHO Global Influenza Surveillance and Response System (GISRS) and propagated in embryonated chicken eggs as previously described (15). All work with H7N9 influenza was conducted in BSL3 or ABSL3 facilities following protocols approved by the University of Georgia Institutional Biosafety Committee.

Viral antibody cross-reactivity. Bronchoalveolar lavage supernatants and serum were individually collected from PR8 memory mice, and anti-H7N9, anti-PR8, anti-X31 specific IgG antibody levels were determined quantitatively by enzyme-linked immunosorbent assay (ELISA) as previously described (16), using whole inactivated virus. Purified mouse IgG and goat anti-mouse-HRP for ELISA were purchased from Southern Biotechnology Associates. Optical density was read at 450nm.

Human PBMCs. Eleven de-identified human PBMCs purchased from Cellular Technology Limited were chosen on the basis of positive reactivity (as measured by IFN-γ ELISpot performed by Cellular Technology Limited) to peptides of common influenza virus T cell epitopes, described in Table 1. Patient demographic data delineated in Table 2. The country of origin of the human PBMC samples was the United States of America with ten of the eleven samples being collected between May 2006 and September 2012; a single patient in our study donated PBMCs early in February 2013.

CTL Peptide Number	**Virus, Protein$_{region}$**	**HLA-Allele**	**Peptide sequence**
CEF-1	Influenza A, PB-1$_{591-599}$	HLA-A1	VSDGGPNLY
CEF-2	Influenza A, NP$_{44-52}$	HLA-A1	CTELKLSDY
CEF-3	Influenza A, M1$_{58-66}$	HLA-A2	GILGFVFTL
CEF-4	Influenza A, PA$_{29-37}$	HLA-A2	FMYSDFHFI
CEF-8	Influenza A, NP$_{91-99}$	HLA-A68	KTGGPIYKR
CEF-9	Influenza A, NP$_{342-351}$	HLA-A3	RVLSFIKGTK
CEF-10	Influenza A, NP$_{265-274}$	HLA-A3	ILRGSVAHK
CEF-13	Influenza A, M1$_{13-21}$	HLA-A3/A11/A6	SIIPSGPLK
CEF-18	Influenza A, NP$_{418-426}$	HLA-B7	LPFDKTTVM
CEF-20	Influenza A, NP$_{380-388}$	HLA-B8	ELRSRYWAI
CEF-25	Influenza A, NP$_{383-391}$	HLA-B27	SRYWAIRTR
CEF-26	Influenza A, M1$_{4-11}$	HLA-B27	ASCMGLIY

Table 1. MHC Class I peptides from CTL to screen for previous exposure to influenza virus of human PBMC samples denoted in Figure 4.

Patient	Age	Gender	Ethnicity
1	36	Male	Caucasian
2	39	Male	Caucasian
3	49	Male	African American
4	36	Male	Caucasian
5	40	Male	Caucasian
6	27	Male	Caucasian
7	36	Female	Hispanic
8	35	Male	Hispanic
9	25	Female	Hispanic
10	33	Male	Hispanic
11	26	Male	Filipino

Table 2. Human patient demographic data for samples denoted in Figure 4.

Cellular stimulation & intracellular cytokine staining (ICS). Whole virus was heat-inactivated {Sendai virus, influenza A/HKx31 (H3N2), A/PR8 (H1N1) and pandemic H1N1} or β-propiolactone inactivated {H7N9} (15) and used separately to stimulate human PBMCs or mouse lung-derived lymphocytes for 18 hours. Brefeldin A was added during the last four hours of murine cell stimulation; Monensin was added with Brefeldin A to the human PBMCs for this time period. Following stimulation, human PBMCs were then stained with Zombie NIR (BioLegend) to exclude dead cells. Staining for intracellular cytokines was performed as previously described (14).

Flow cytometry. Monoclonal antibodies used to stain human PBMCs were from BioLegend CD27 [O323], CD8α [RPA-T8], CD3 [OKT3], IFN-γ [4S.B3], TNFα [MAb11], and BD Biosciences CD4 [RPA-T4]. Monoclonal antibodies used to stain lung-derived murine lymphocytes were BioLegend CD3 [17A2], CD8α [53-6.7], CD4 [RM4-5]; eBioscience CD11b [M1/70], CD44 [IM7]; and BD Biosciences IFN-γ [XMG1.2]. Samples were run on a BD Biosciences LSRII flow cytometer and analyzed with FlowJo software.

Viral titers. Tissue titers were determined as previously described (15, 17). Briefly, lungs were homogenized in 1mL PBS and cleared by centrifugation. Supernatants were titrated on MDCK cells in MEM containing 1μg/mL TPCK-trypsin (Worthington) and cultured for 72 hours. Supernatants were assayed for presence of influenza virus by hemagglutination using

0.5% chicken RBCs.

Statistical analysis. All analysis was performed in GraphPad Prism 6. One-tailed paired t tests were used to test significance of human and mouse ICS data when comparing H7N9 stimulated conditions to either the paired unstimulated or Sendai virus stimulated control. One-way ANOVA was used to analyze ELISA data with corrected multiple comparisons being evaluated for significance by Tukey's test. Two-tailed t tests were used to evaluate significance of viral titer data and mouse weight loss after Holm-Sidak correction for multiple comparisons. Differences in survival over the 14 day challenge period were evaluated for significance using the Logrank Mantel-Cox test.

Results

Cross-reactive CD4 and CD8 responses to H7N9 influenza virus in mice. We sought to quantify the frequency of H7N9 cross-reactive T cells under controlled influenza virus exposure. Thus, we infected mice with influenza A/HKx31 (X31), A/PR8 (PR8), or pandemic A/California/09 H1N1 (pH1N1) to evaluate the individual predisposition of each infection to generate H7N9 cross-reactive T cells. We harvested lymphocytes from the lungs of mice 35 days post-infection and stimulated cells with whole inactivated virus (X31, PR8, H7N9, pH1N1, or Sendai virus) to evaluate T cell reactivity via intracellular cytokine staining. CD8 and CD4 T cells were examined independently for the production of IFN-γ in response to virus stimulation (Figure 1A). IFN-γ responses to inactivated H7N9 influenza virus stimulations were directly compared to paired samples that were either left unstimulated or were stimulated with inactivated Sendai virus as a negative control, as Sendai virus does not share any cross-reactive T cell epitopes with influenza virus. For mice infected with each of the three priming conditions (PR8, X31, pH1N1), there was a significant percentage of CD8 (Figure 1B, top) and CD4

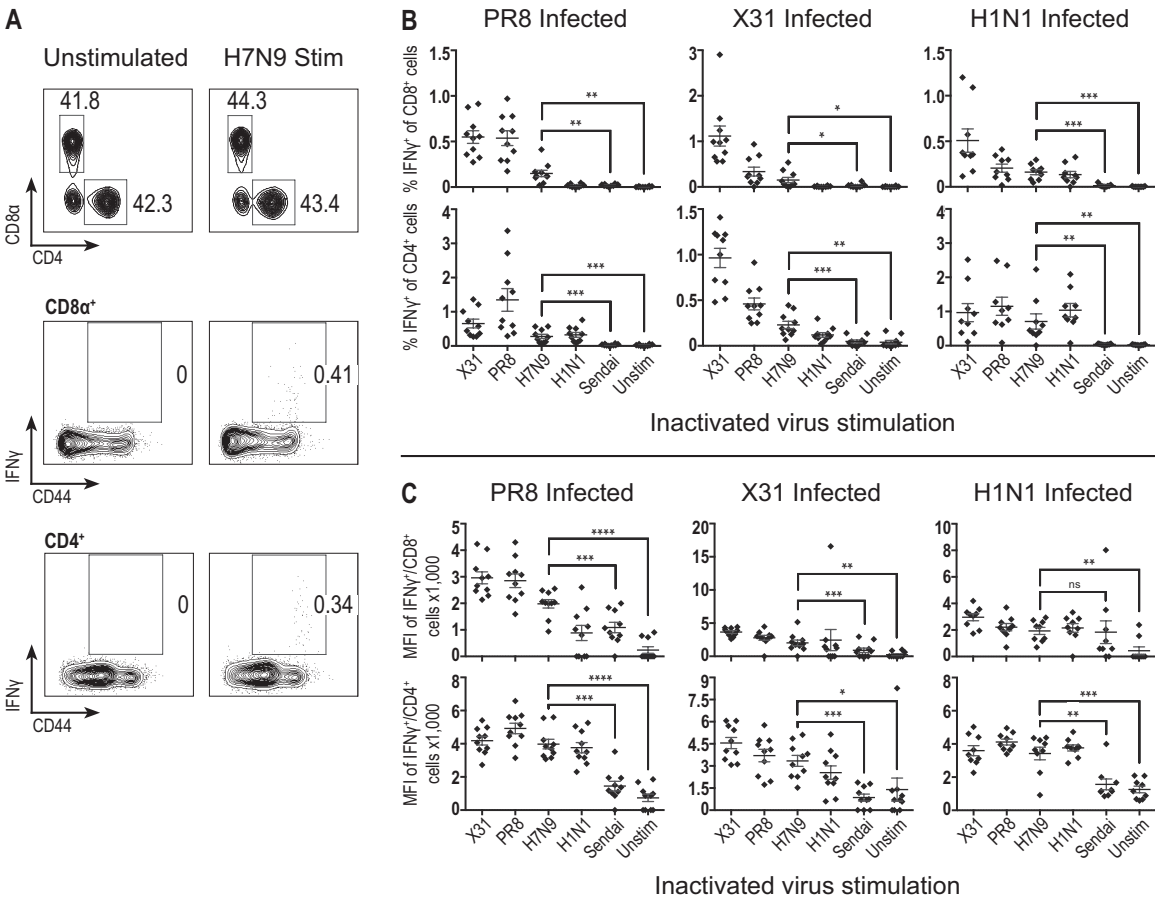

Figure 1. *H7N9 is recognized by memory CD4 and CD8 T cells derived from prior influenza virus exposure in mice.* A, Representative IFN-γ expression frequency in mouse $CD8^+$ and $CD4^+$ cells either unstimulated or stimulated for 18 hours with whole inactivated H7N9 influenza virus. $CD8^+$ and $CD4^+$ cells gated on a $CD11b^-/CD3\varepsilon^+$ population and derived from mouse lungs infected with 600 EID_{50} PR8 and rested 35 days. B, IFN-γ frequency in mouse $CD8^+$ [top] and $CD4^+$ [bottom] cells following 18 hours of stimulation with either whole inactivated influenza virus [X31, PR8, H7N9, pH1N1], whole inactivated Sendai virus, or left unstimulated. Cells for intracellular cytokine staining (ICS) were derived from mouse lungs 35 days post-infection with 600 EID_{50} PR8, 30,000 EID_{50} X31, or 1326 EID_{50} pH1N1 influenza virus. N=19-20 mice pooled from two experimental replicates; mean & SEM. Representative of seven experiments at memory and acute time points. C, Mean fluorescence intensity of IFN-γ^+/$CD8^+$ [top] or IFN-γ^+/$CD4^+$ [bottom] cells whose frequencies were displayed in *B*. Statistics: One-tailed paired t tests used in *1B* & *C* to compare H7N9 stimulated samples to either unstimulated or Sendai stimulated matched samples; p-values [* <0.05; ** <0.01; *** <0.001; **** <0.0001].

(Figure 1B, bottom) T cells cross-reactive to H7N9 as compared to matched unstimulated or Sendai virus stimulated samples. As expected, for all three priming conditions, the CD4 and CD8 T cell cross-reactivity to H7N9 was less than that observed following stimulation with homologous inactivated virus or heterologous inactivated virus with identical internal proteins (PR8 and X31). Moreover, for all three priming conditions, examination of the geometric

Mean Fluorescence Intensity (MFI) for IFN-γ following H7N9 stimulation was found to be significant compared to the matched unstimulated samples of both CD8 (Figure 1C, top) and CD4 (Figure 1C, bottom) T cells. When comparing IFN-γ MFI of H7N9 stimulated to Sendai virus stimulated samples, only the CD8 T cell population of PR8- and X31-primed mice stimulated with H7N9 were found to be significantly higher than the Sendai virus stimulation (Figure 1C, top); looking at CD4 IFN-γ MFI, all three priming conditions were found to produce a CD4 T cell population where the H7N9 stimulated samples have a significantly greater IFN-γ MFI as compared to those stimulated with Sendai virus (Figure 1C, bottom).

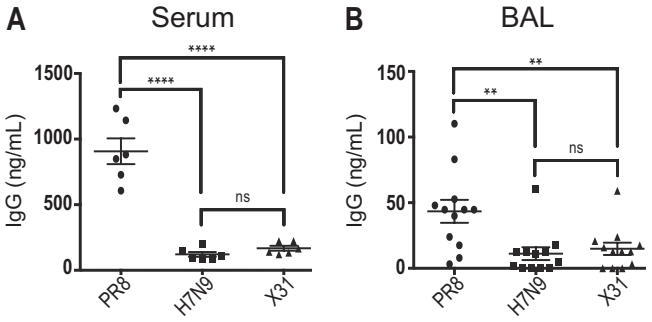

Figure 2. *Limited antibody cross-reactivity between PR8 and H7N9 influenza viruses.* A, Concentration of anti-PR8, anti-H7N9 and anti-X31 specific IgG antibody levels in the serum of mice infected with PR8 and rested to immunological memory. N=6; mean & SEM. B, Concentration of anti-PR8, anti-H7N9, and anti-X31 specific IgG antibody levels in the supernatant of a bronchoalveolar lavage (BAL) from mice infected with PR8 and rested to immunological memory. N=12; mean & SEM. Statistics: One-way ANOVA used to compare concentrations of cross-reactive IgG, multiple comparisons, with correction, were evaluated for significance by Tukey's test; p-values [** <0.01; **** <0.0001].

Limited anti-H7N9 cross-reactive antibody in PR8 memory mice. To confirm that priming with PR8, an H1N1 virus, did not generate any antibodies that recognized H7N9, we performed an ELISA on serum from PR8 memory mice. As expected in PR8-primed mice, we detected a significantly lower concentration of H7N9-reactive IgG antibody in the serum as compared to the concentration of PR8-reactive IgG antibody; in fact, the level of H7N9-reactive IgG was statistically similar to that of X31-reactive IgG (Figure 2A), which is known to be serologically distinct from PR8 (18). Furthermore, this observation held when we looked at the cross-reactive antibody concentrations in the lung airways, as determined by bronchoalveolar lavage (BAL), of PR8-primed mice. Anti-H7N9 cross-reactive IgG antibody

levels in PR8-primed lung airways were statistically similar to levels of anti-X31 cross-reactive IgG levels, both of which were significantly lower than the levels of PR8-reactive IgG antibody (Figure 2B).

Memory T cells from previous influenza virus exposure protect against H7N9 challenge. Given that the native frequency of H7N9 cross-reactive T cells was substantial in mice previously exposed to influenza virus, we wanted to know whether these memory T cells were able to convey protection upon challenge with H7N9. We tested this question by using mice mock-infected or primed with PR8 and rested to immunological memory before challenging with H7N9. We evaluated the infectious burden of the H7N9 0.5 mouse LD_{50} (mLD_{50}) challenge in PR8-primed and mock-primed mice by measuring viral titers at days three and six post-secondary challenge. While viral titers were not significantly different at day three post-H7N9 challenge between the mock-primed and PR8-primed mice, we did find a significant difference in titers at day six post-challenge (Figure 3A). This could infer that the cross-reactive memory T cells in the PR8-primed mice enabled a much more rapid clearance of H7N9 virus, despite the original infectious burden at day three remaining relatively the same between the two groups.

We also assessed clinical manifestations of the disease course by measuring weight loss and survival for 14 days following infection with 5 mLD_{50} or 0.5 mLD_{50} H7N9. We found large and significant divergences between the PR8-primed and the mock-primed groups four days following H7N9 challenge (Figure 3B); this divergence continued to grow until about day ten post-H7N9 challenge for the 0.5 mLD_{50} dose or until all mock-primed mice of the 5 mLD_{50} dose were dead by day eight post-H7N9 challenge (†). All PR8-primed mice survived challenge with either 0.5 mLD_{50} or 5 mLD_{50} H7N9, whereas all mock-primed mice from the

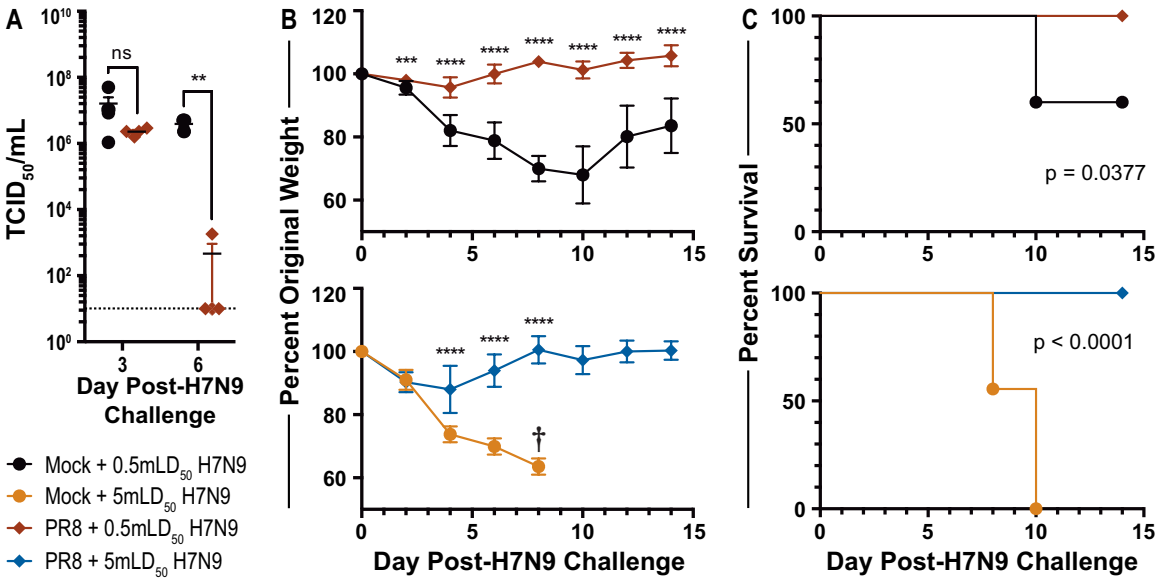

Figure 3. *Memory T cell responses generated following PR8 infection protect against lethal H7N9 challenge.* A, H7N9 influenza viral titers on day three or day six following challenge with 0.5 mouse LD_{50} (mLD_{50}) H7N9. Mice were mock-primed or PR8-primed (600 EID_{50}) and rested for 42 days prior to H7N9 challenge. N=4-5 mice/day/group; mean & SEM; representative of one experiment. Dotted line denotes ten as the level of detection. B, Weight loss over 14 days of mice challenged with 0.5 mLD_{50} [top] or 5 mLD_{50} [bottom] H7N9. Mice were primed and rested per A. † denotes all mice from 5 mLD_{50} H7N9 challenge group were dead or euthanized by day eight post-H7N9 challenge. N=9-10 mice per group; mean & SEM; representative of one experiment. C, Survival curve of mice challenged with 0.5 mLD_{50} [top] or 5 mLD_{50} [bottom] H7N9 over 14 days; same mice for which weight loss was measured in *B*. Mice were primed and rested per *A*. N=9-10 mice/group; representative of one experiment. Statistics: Two-tailed t tests used in *3A* & *B* to compare mock-primed and PR8-primed groups following H7N9 challenge; *3B* t tests corrected for multiple comparisons using Holm-Sidak correction; survival differences in *3C* evaluated with the Logrank Mantel-Cox test; p-values [** <0.01; *** <0.001; **** <0.0001].

5 mLD_{50} group and 40% from the 0.5 LD_{50} group died or were euthanized by day ten (Figure 3C). For the 0.5 mLD_{50} and 5 mLD_{50} challenge, the results between the PR8-primed and mock-primed groups were found to be statistically significant with p-values of 0.0377 and <0.0001, respectively.

CD4 and CD8 T cells derived from humans with previous exposure to influenza virus exhibit cross-reactivity to H7N9. Our findings on the level of H7N9 cross-reactivity in mice suggested that we might be able to detect the native frequency of H7N9 cross-reactive T cells in human samples. Thus, we procured human PBMCs from healthy donors with previous exposure to influenza virus; demographic data is provided in Table 2. Following stimulation with inactivated H7N9 virus, there was a significant increase in IFN-γ production compared

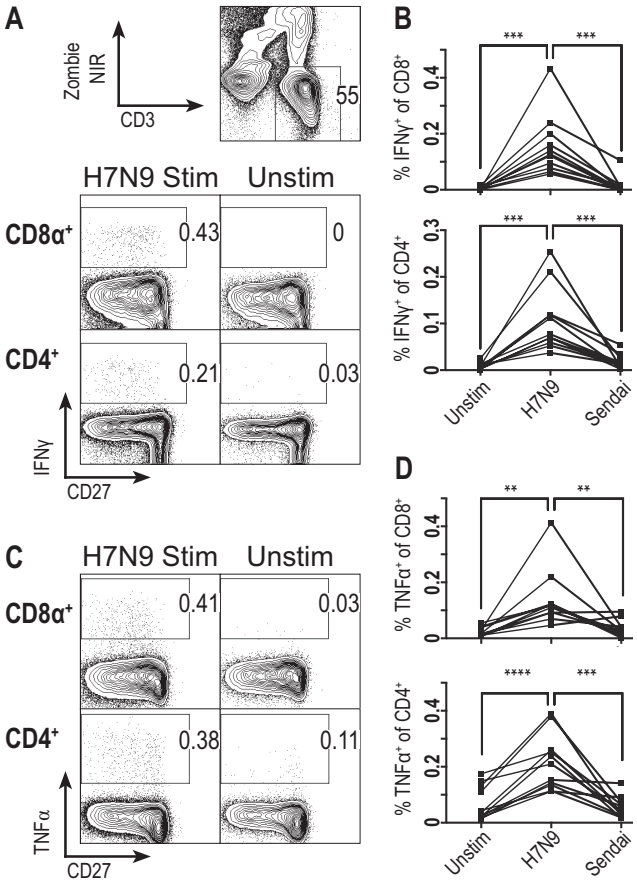

Figure 4. *H7N9 is recognized by memory CD4 and CD8 T cells derived from humans with prior influenza virus exposure.* A, Representative IFN-γ expression frequency in human $CD8^+$ and $CD4^+$ cells either unstimulated or stimulated for 18 hours with whole inactivated H7N9 influenza virus. $CD8^+$ and $CD4^+$ cells gated on a $ZombieNIR^-/CD3^+$ population and derived from human PBMC samples purchased from Cellular Technology Limited. B, IFN-γ frequency in live human $CD8^+$ [top] and $CD4^+$ [bottom] cells following 18 hours of stimulation with either whole inactivated H7N9 influenza virus, whole inactivated Sendai virus, or left unstimulated. N=11; stimulations for individual patients are interconnected with lines. C, Representative TNF-α expression frequency in human $CD8^+$ and $CD4^+$ cells either unstimulated or stimulated for 18 hours with whole inactivated H7N9 influenza virus, gated as described in *A*. D, TNF-α frequency in live human $CD8^+$ [top] and $CD4^+$ [bottom] cells described in *C*. Statistics: One-tailed paired t tests used in *4B & D* to compare H7N9 stimulated samples to either unstimulated or Sendai stimulated samples; p-values [** <0.01; *** <0.001; **** <0.0001].

to paired samples left unstimulated or stimulated with inactivated Sendai virus in both CD8 and CD4 T cell populations, when gating on live, $CD3^+$ cells (Figure 4A & 4B). Moreover, this significance also held when looking at TNF-α production in both CD8 and CD4 T cell populations when comparing paired samples stimulated with inactivated H7N9 virus to either unstimulated or inactivated Sendai virus stimulated samples (Figure 4C & 4D). The presence of cross-reactive T cells to H7N9 in humans could portend similar protection to what we observed with our murine H7N9 challenge.

Discussion

While nearly all of the identified cases of human H7N9 infection have occurred as a result of direct or indirect interaction with avian sources, there is a risk that the virus could gain the capacity to effectively transmit between humans. Given the absence of widespread human

exposure to either H7 or N9 influenza viruses, any type of pre-existing immunological protection would likely be derived from cross-reactive cellular immunity against other influenza virus strains. Because of this, our findings have broad implications in regard to previous influenza A virus exposure and development of cross-reactive T cells as a protective correlate to the emerging H7N9 influenza virus. We demonstrate that there exists a cross-reactive CD4 and CD8 memory T cell population found in both humans and mice able to recognize and produce antiviral cytokines in response to H7N9 exposure. Moreover, our H7N9 challenge study provides direct evidence that the existence of cross-reactive memory T cells from previous exposure to influenza A viruses, in our case PR8, correlates with reduced morbidity and mortality in the murine model. Furthermore, our results of limited antibody cross-reactivity between PR8 and H7N9 suggest that neutralizing antibodies are most likely not the source of the observed protection, affording additional credence that the protection from H7N9 challenge is mediated by pre-existing H7N9 cross-reactive T cells established from previous influenza virus exposure.

One could posit that such a correlation would also hold true for humans, as cross-reactive memory T cells could convey protection to H7N9 infection by limiting viral replication during the early stages of the immune response and thus limit the clinical manifestations of the infection. This is important since cross-reactive neutralizing antibodies generated by exposure to other circulating influenza viruses and capable of effectively targeting H7N9 have not been identified in the general human population, nor in poultry workers in China (19, 20); furthermore, H7 influenza A viruses have been characterized as being poorly immunogenic, including the emerging H7N9 virus where poor antibody generation and helper T cell function is predicted in humans (21). Therefore, in juxtaposition with the finding that CTL responses

aided in reducing morbidity during the 2009 H1N1 influenza virus pandemic (13), perhaps CTL responses could once again aid in limiting both morbidity and mortality in the event of an H7N9 influenza virus pandemic.

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Chapter 3

Airway-Resident Memory CD8 T cells Provide Antigen-Specific Protection against Respiratory Virus Challenge through Rapid IFN-γ Production

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Abstract

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

Introduction

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

Memory CD8 T cells canonically aid in controlling and clearing a pathogen through targeted lysis of infected cells (4) and modulation of the innate immune response at the site of infection through the local production of cytokines (5). There is ample evidence from animal models that memory CD8 T cells confer protective immunity to respiratory viruses by significantly decreasing viral loads, leading to faster clearance and decreased immunopathology (6-8). Recent studies in humans showed that increased numbers of circulating cross-reactive memory CD8 T cells correlated with significant decreases in viral loads and lower disease burden following heterosubtypic influenza virus challenge (9). Notably, studies in animal models that allow sampling of peripheral tissues have shown the number of memory CD8 T

cells in the lung correlates with the efficacy of cellular immunity to respiratory virus challenge, and a similar phenomenon has been observed in models of *M. tuberculosis* immunity (10, 11). Furthermore, the protective efficacy of cellular immunity to influenza virus slowly declines over several months post-infection with kinetics identical to the decline in the number of airway CD8 T_{RM} cells (12). Previous studies have shown that airway CD4 T_{RM} cells could mediate protection in mice lacking CD8 T cells (13), but despite the potential correlation between airway CD8 T_{RM} cells and protective cellular immunity in the lung, there is currently no direct evidence that demonstrates the protective efficacy or protective mechanism of these cells.

T_{RM} cells are generated in response to regional infections and have been documented in the lungs, skin, gut, and reproductive tract where they would have the ability to provide an initial line of defense against invading pathogens (14-19). T_{RM} populations consist of non-circulating cells characterized by permanent residence in peripheral tissues; expression of the tissue retention molecules CD69 and CD103; down-regulated expression of CD62L, CCR7, and sphingosine-1-phosphate receptor 1 (S1PR1); and a transcription program distinct from their circulating T_{EM} cell counterparts (20, 21). Despite sharing these hallmarks with T_{RM} populations in other tissues, lung airway T_{RM} cells have a distinct phenotype and are short-lived, likely due to the harsh airway microenvironment. Key features of this distinct phenotype are the down-regulation of the integrin CD11a and poor *in vivo* cytolytic capacity, which call into question the ability of these cells to participate in protective immunity (22, 23) Nevertheless, airway CD8 T_{RM} cells are in prime position to respond to a challenge from pathogens that infect the respiratory epithelium (24). Therefore, it is important to know whether these cells are sufficient to protect against secondary challenge and if so, how they mediate said protection.

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

Materials & Methods

Mice & infections. C57BL/6J (WT), B6.PL-Thy1^{a}/CyJ (CD90.1), B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ (CD45.1) and B6.129S7-Ifng^{tm1Ts}/J (IFN-γ KO) mice from The Jackson Laboratory were housed under specific ABSL2 conditions at Emory University and Trudeau Institute. Intranasal infection with influenza A/HKx31 (H3N2) at 30,000 50% egg infectious doses (EID_{50}) and Sendai virus at 282 EID_{50} established virus-specific T cells in mice as previously described (25). Influenza A/PR8 (H1N1) at 6,000 EID_{50} was used for challenge of transfer recipient mice. All experiments were completed in accordance with the Institutional Animal Care and Use Committee guidelines of Emory University and Trudeau Institute.

Cellular isolation, intratracheal transfer, intravital labeling & flow cytometry. Memory CD8 T cells, harvested from mice 35-45 days post-infection, were negatively selected from

bronchoalveolar lavage (BAL) using Miltenyi CD8α T Cell Isolation Kit II. Influenza $NP_{366-374}/D^{b+}$ tetramer quantification allowed for equal numbers of antigen-specific cells to be i.t. transferred from donor mice to naïve recipient mice. No more than $1.5x10^5$ antigen-specific airway CD8 T_{RM} cells were transferred per recipient to approximate physiological numbers of airway T_{RM} cells. Antibodies used for flow cytometry and cell sorting were BioLegend CD62L [MEL-14], CD8α [53-6.7], CXCR3 [CXCR-173]; eBioscience CD11a [M17/4], CD44 [IM7]; and BD Biosciences CD3ε [145-2C11], CD45.2 [104], CD90.2 [53-2.1], IFN-γ [XMG1.2]. Intravital staining was performed immediately before mouse euthanasia and tissue harvest as previously described (15). Briefly, to identify T cells resident in various tissues, including the lung parenchyma, 1.5μg of fluorophore-conjugated α-CD3ε antibody in 200λ 1x PBS was intravenously injected into the tail vein of mice; five minutes post-injection, mice were euthanized with Avertin (2,2,2-Tribromoethanol - Sigma) and exsanguinated prior to harvest of BAL and other tissues. Staining for intracellular cytokines was performed as previously described following stimulation in the presence of Brefeldin A for the indicated periods of time (25). To study cell proliferation, mice were given an intraperitoneal bolus of BrdU (0.8mg) at the time of infection and maintained on BrdU drinking water (0.8mg/mL) until harvest. BrdU incorporation was measured using the BrdU Flow kit (BD Biosciences) following tetramer and antibody staining. Samples were run on a BD Biosciences Canto II or LSR II flow cytometer and analyzed with FlowJo software. Sorting was performed on an Influx or Aria II cell sorter (BD Biosciences).

CTL assay. Donor airway CD8 T_{RM} cells were harvested from the airways of PBS control or PR8 challenged mice and sorted based on CD90.2 expression. Congenic ($CD45.1^+$) targets were pulsed with $FluNP_{366-374}$ peptide (specific targets) or a non-specific peptide (αHV $p79_{524-}$

$_{531}$ or SendaiNP$_{324–332}$) for two to four hours at 37°C with non-specific targets being labeled with 2.5μM CFSE; non-specific and FluNP targets were then mixed at a 1:1 ratio. Sorted airway CD8 T_{RM}, lung parenchymal CD8 T_{RM}, or splenic CD8 T_{EM} cells were incubated with targets at an effector to target (E:T) ratio of 4:1 or 1:1 for six, 14 or 24 hours. The ratio of live specific targets to live non-specific targets was determined by gating on propidium iodide$^-$/CD45.1$^+$/CFSE$^{+/-}$ cells following flow cytometry. Specific lysis was calculated by the formula: [1-(Ratio of targets only)/((Ratio of targets following incubation with effector cells))]×100.

Peptide stimulation and Luminex assay. Mice who received PBS, Sendai-specific airway CD8 T_{RM} cells, or influenza-specific airway CD8 T_{RM} cells followed by intranasal influenza virus challenge the following day had BAL isolated three days post-challenge. The supernatant from the single BAL pull was separated from cells via centrifugation prior to cytokine and chemokine analysis by Luminex. Alternatively, BAL and spleens were harvested from Sendai memory mice, sorted to isolate CD44^{hi}/CD62L$^-$/CD8$^+$ cells, and were stimulated 6 hours using irradiated congenic APCs pulsed with 1μg/mL SendaiNP$_{324–332}$ (FAPGNYPAL) or 1μg/mL FluNP$_{366-374}$ (ASNENMETM) prior to cytokine and chemokine analysis by Luminex.

Measurement of viral load. Sendai and influenza virus PFU titers were completed as previously described (7) following day three post-challenge with Sendai or x31 influenza virus, respectively. Quantitative PCR on influenza virus polymerase gene (PA) was completed as described (26), using High Capacity cDNA Reverse Transcription Kit (Life technologies) generated cDNA from 2μg RNA isolated from lung homogenates by TRIzol and RiboPure RNA Purification Kit (Ambion).

Results

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

Given their proximity to the respiratory epithelium, airway T_{RM} cells are ideally located to rapidly recognize and respond to respiratory viral infections. However, prior *ex vivo* studies have shown airway CD8 T_{RM} cells have a unique phenotype and effector function when compared to their systemic counterparts. Because of these differences, it is unclear if, and in what capacity, these cells contribute to protective immunity. To specifically test the protective capacity of airway CD8 T_{RM} cells in the absence of parenchymal T_{RM} and circulating T_{EM} cells, we intratracheally (i.t.) transferred Sendai or influenza virus-specific airway CD8 T_{RM} cells from the airways of immune mice directly into the airways of naïve recipient mice (Figure 1A). The transferred airway CD8 T_{RM} population expressed high levels of CXCR3 (Figure 2), which has been shown to be up-regulated on CD8 T cells in the airways during an acute infection and continues to be expressed into immunological memory (27). These cells also remain in the airways following i.t. transfer and do not egress from the airways to the lung parenchyma or mediastinal lymph node (MLN) (Figure 3). Recipient mice were challenged with influenza or Sendai virus one day after transfer and viral titers were measured three days

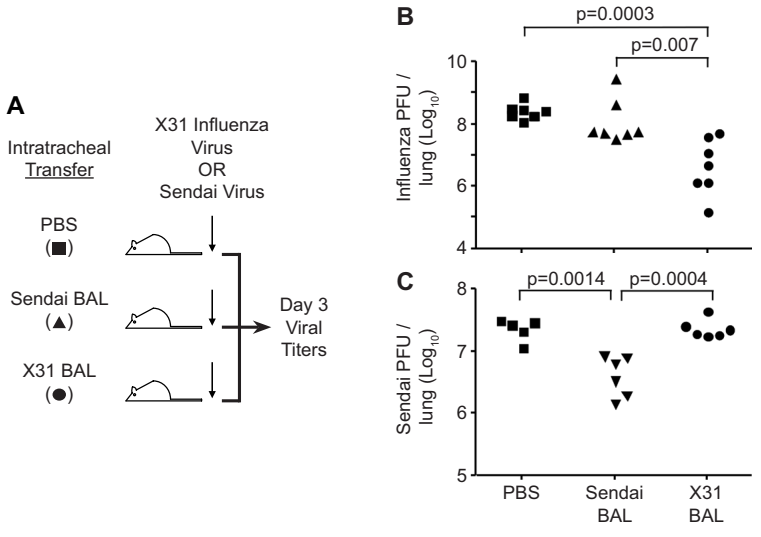

Figure 1: *Airway CD8 T_{RM} cells significantly decrease viral replication in an antigen-specific manner.* A, Intratracheal (i.t.) transfer experimental model where PBS or airway CD8 T_{RM} cells from Sendai virus or influenza virus (x31) memory mice were harvested from bronchoalveolar lavage (BAL)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

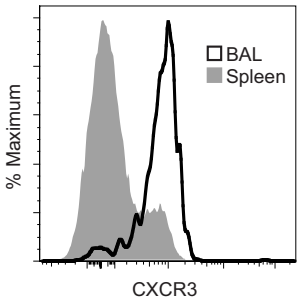

Figure 2: *Airway T_{RM} cells are CXCR3^{hi}.* CXCR3 extracellular staining on memory CD8 T cells from the spleen and airways at day 45 PI; representative of 6 independent experiments.

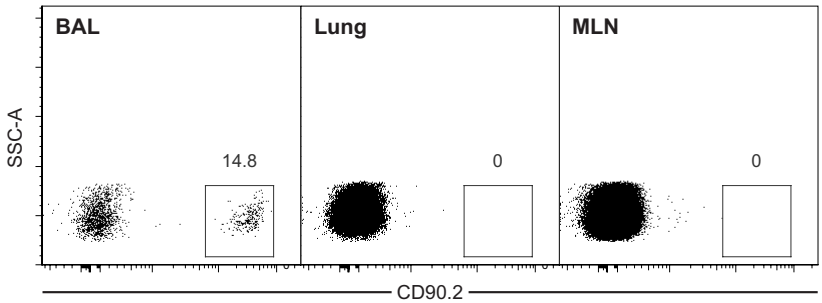

Figure 3: *Intratracheally transferred CD8 T cells do not leave the airways to enter the lung nor draining lymph node.* Sorted CD44^{hi}/CD62L^{lo} airway CD8 T_{RM} cells from x31 WT memory mice (CD90.2^{+}) were transferred i.t. into congenic recipients (CD90.1^{+}/CD90.2^{-}). Mice were sacrificed two days post-i.t. transfer and CD8 T cells were stained for congenic markers to determine if the i.t. transferred cells remained in the airways or migrated into the lungs or to the mediastinal lymph node (MLN); representative of 3 independent experiments.

after infection. As shown in Figures 1B and 1C, mice receiving airway CD8 T_{RM} cells specific to the challenge virus had a significant decrease in viral titers. In contrast, airway T_{RM} cells specific for a different virus showed no difference in titers compared to PBS controls. Thus, airway CD8 T_{RM} cells are sufficient to limit early viral replication through a mechanism that requires cognate antigen recognition.

Antigen-specific airway CD8 T_{RM} cells result in decreased expression of pro-inflammatory cytokines and chemokines within the lung airways upon challenge with cognate antigen.

Three days after intranasal challenge, the BAL supernatant was harvested from naïve mice who received an i.t. transfer of PBS, Sendai-specific airway CD8 T_{RM} cells, or influenza-specific airway CD8 T_{RM} cells one day before challenge (Figure 4A). Despite having the greatest reduction in viral titers upon challenge, mice receiving influenza-specific airway CD8 T_{RM} cells i.t. produced significantly lower levels of CXCL-1, CCL-2, IL-6 and TNF-α when compared to mice receiving PBS or Sendai-specific airway CD8 T_{RM} cells (Figure 4B). In contrast, the airways of naïve mice receiving Sendai-specific airway CD8 T_{RM} cells i.t. had higher levels of all four inflammatory cytokines than even the PBS controls as a result of the non-antigen-specific

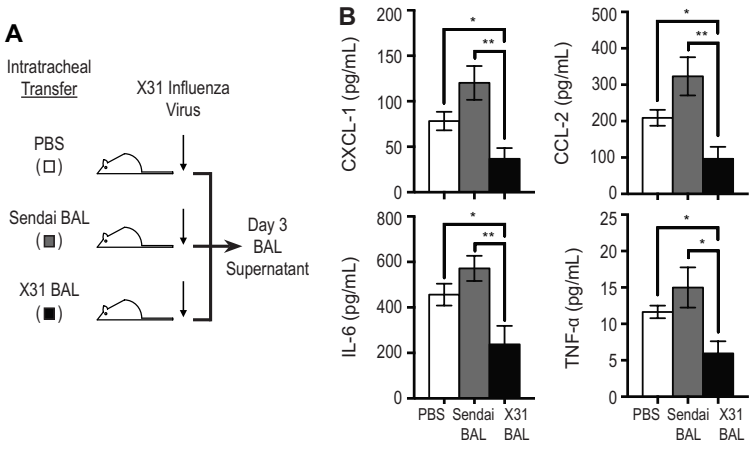

Figure 4: *Antigen-specific airway CD8 T_{RM} cells result in lower production of inflammatory cytokines within the lung airways upon challenge with cognate antigen.* A, PBS or airway CD8 T_{RM} cells from Sendai virus or influenza virus (x31) memory mice were harvested from BAL and i.t. transferred into naïve WT mice, which were i.n. challenged the following day and BAL supernatant was harvested 3 days later. B, BAL supernatant from each of the three groups receiving PBS, Sendai-specific airway CD8 T_{RM} cells or x31-specific airway CD8 T_{RM} cells i.t. as noted in *A* were isolated and Luminex assay performed to quantify the amount of CXCL-1 (KC), CCL-2 (MCP-1), IL-6, and TNF-α produced in the airways of the recipient mice. N=6-7 mice/group; unpaired two-tailed t tests; representative of 3 independent experiments; p-values [*<0.05; **<0.01].

influenza virus challenge. Therefore, upon exposure to cognate antigen, the ability of airway CD8 T_{RM} cells to rapidly decrease viral loads aids in restraining the local pro-inflammatory immune response and limiting unnecessary damage to the lungs.

Airway CD8 T_{RM} cells fail to gain rapid cytolytic function in vivo, even in the presence of cognate antigen. To understand the mechanism by which airway CD8 T_{RM} cells mediate protection, we examined the capacity of these cells to induce target cell death *in vitro* and their ability to proliferate upon secondary infection. In Figure 5A, we isolated and sorted airway CD8 T_{RM} and splenic CD8 T_{EM} cells from x31 influenza memory mice to compare their respective cytolytic capabilities. The specific lysis of airway CD8 T_{RM} cells was relatively negligible, remaining at ~10%, for E:T (effector to target) ratios ranging from 1:1 to 4:1, while the specific lysis of splenic CD8 T_{EM} cells from the same mice increase as the E:T ratio increases (Figure 5A). For all three E:T ratios, the specific lysis of the splenic CD8 T_{EM} cells was significantly higher than that of the airway CD8 T_{RM} cells. To directly compare the CTL activity of the airway (BAL) and lung parenchymal (LP) CD8 T_{RM} cells 35 days post-x31 influenza virus infection, we sorted cells from the airways and lung tissue which were protected

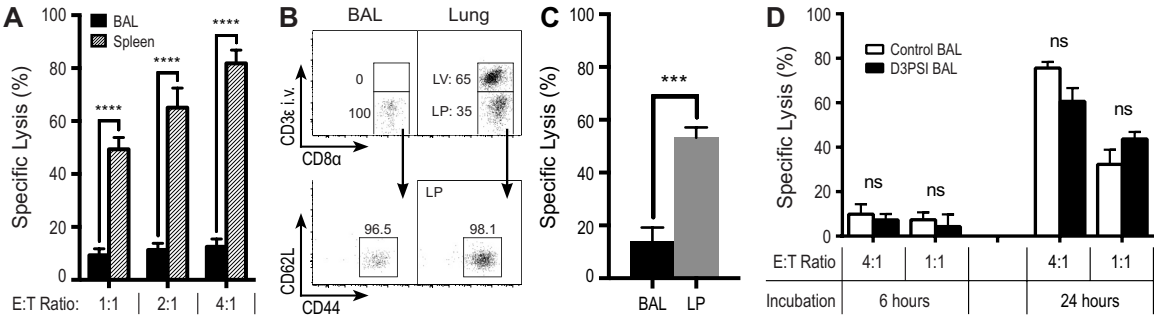

Figure 5: *Airway CD8 T_{RM} cells are slow to gain cytolytic function following secondary infection.* A, Sorted CD44^{hi}/CD62L^{lo} CD8 T cells from the airways and spleen of x31 memory mice and incubated with targets at an E:T ratio of 1:1, 2:1 or 4:1 for six hours. N=5 replicates per E:T ratio; representative of 4 independent experiments; mean & SEM; two-way ANOVA with Sidak test corrected multiple comparisons. B, CD44^{hi}/CD62L^{lo} CD8 T cells were sorted from the airways and lung parenchyma (LP) of x31 memory mice. Cells resident in the lung parenchyma were identified by intravital labeling using a fluorophore conjugated α-CD3ε antibody administered immediately prior to mouse euthanasia; cells within the LP are protected from being labeled by the intravenously administered antibody, while those within the lung vasculature (LV) are labeled. C, Cells sorted in *B* were incubated with targets at an E:T ratio of 1:1 for 14 hours to determine cytolytic capacity. N=4-5 replicates; representative of 2 independent experiments; mean & SEM; unpaired two-tailed t test. D, Sorted CD11a^{lo} airway CD8 T_{RM} cells from x31 memory mice were transferred i.t. into naïve congenic recipients challenged with PBS (Control) or PR8 for three days (D3PSI). Donor cells were isolated from the airways on day three and incubated with targets at an E:T ratio of 4:1 or 1:1 for six or 24 hours. N=4 replicates per time point per E:T ratio; representative of three independent experiments; mean and SEM; two-way ANOVA with Sidak test corrected multiple comparisons; p-values [***<0.001, ****<0.0001, ns=not significant].

from an intravital staining antibody, providing a CD44^{hi}/CD62L^{lo} CD8 T_{RM} population from each resident compartment (Figure 5B). Figure 5C shows that, at a 1:1 E:T ratio, the LP CD8 T_{RM} cells have significantly higher CTL activity than the airway CD8 T_{RM} cells, even after incubating with targets for 14 hours. Finally, to understand if the airway CD8 T_{RM} population gains CTL function by encountering cognate antigen, we transferred airway CD8 T_{RM} cells from x31 influenza-primed mice i.t. into congenic naive mice and challenged those mice with PBS (control) or PR8 influenza (PR8). On day three post-challenge, the transferred airway CD8 T_{RM} cells were isolated by cell sorting and assessed for cytolytic function. Even in the presence of cognate antigen stimulation *in vivo*, airway CD8 T_{RM} cells remained poorly cytolytic in a short-term CTL assay irrespective of the E:T ratio and did not display robust cytolytic function until 24 hours of target incubation (Figure 5D). Therefore, the airway CD8 T_{RM} population, once established, is poorly cytolytic and remains poorly cytolytic even during

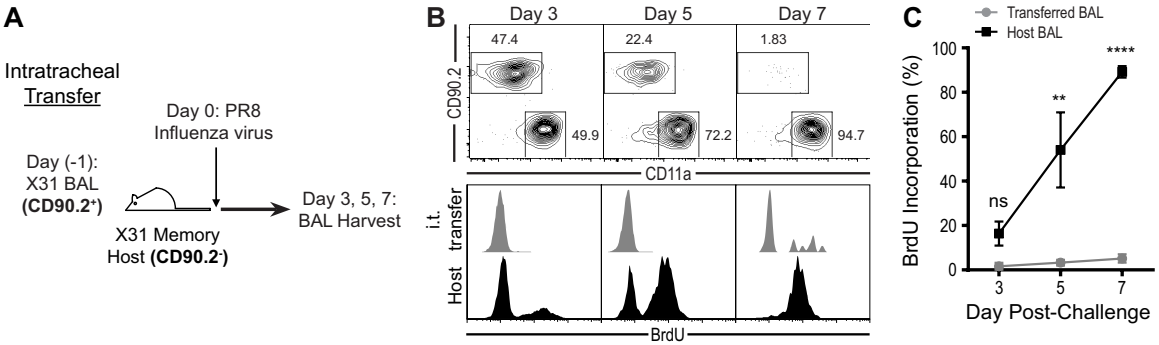

Figure 6: *Airway CD8 T_{RM} cells do not proliferate in vivo following secondary infection.* A, Schematic of the experimental design used in panels *B* and *C*. B, CD90.2$^+$ airway CD8 T_{RM} cells were i.t. transferred into x31 memory CD90.1$^+$/CD90.2$^-$ mice one day prior to PR8 i.n. challenge; host mice were maintained on BrdU following cell transfer through day seven of viral challenge. BAL was isolated at days three, five, and seven following challenge to evaluate donor airway CD8 T_{RM} expansion, CD11a expression [top row] and BrdU incorporation [bottom row]. Plots shown are gated of FluNP-specific cells. N=3 mice per time point per group; representative of three independent experiments; C, BrdU incorporation for donor and recipient airway CD8 T_{RM} cells from the plots in B is graphed as the mean & SEM; two-way ANOVA with Sidak test corrected multiple comparisons; p-values [**<0.01; ****<0.0001].

a secondary infection, while the lung parenchymal CD8 T_{RM} population retains their cytolytic capacity.

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

To investigate whether the rapid proliferation and expansion of airway CD8 T_{RM} cells may be important for their protective function, we transferred airway CD8 T_{RM} cells (CD90.2$^+$) from x31 influenza-primed mice i.t. into congenic (CD90.1$^+$) x31 influenza-primed mice, challenged with PR8 the following day, and maintained the mice on BrdU water for seven days (Figure 6A). The i.t. transferred population maintained their CD11a^{lo} status, did not incorporate BrdU, and failed to expand throughout the secondary response (Figure 6B and 6C). Notably, the only flu-specific CD8 T cells in the airways to incorporate BrdU were host cells that recently migrated to the airways, as noted by their CD11a^{hi} status; these host cells eventually dominate the secondary response. Together, these data infer that the airway CD8 T_{RM} cells do not need to proliferate within the airways or gain rapid cytolytic function to mediate protection to a secondary challenge.

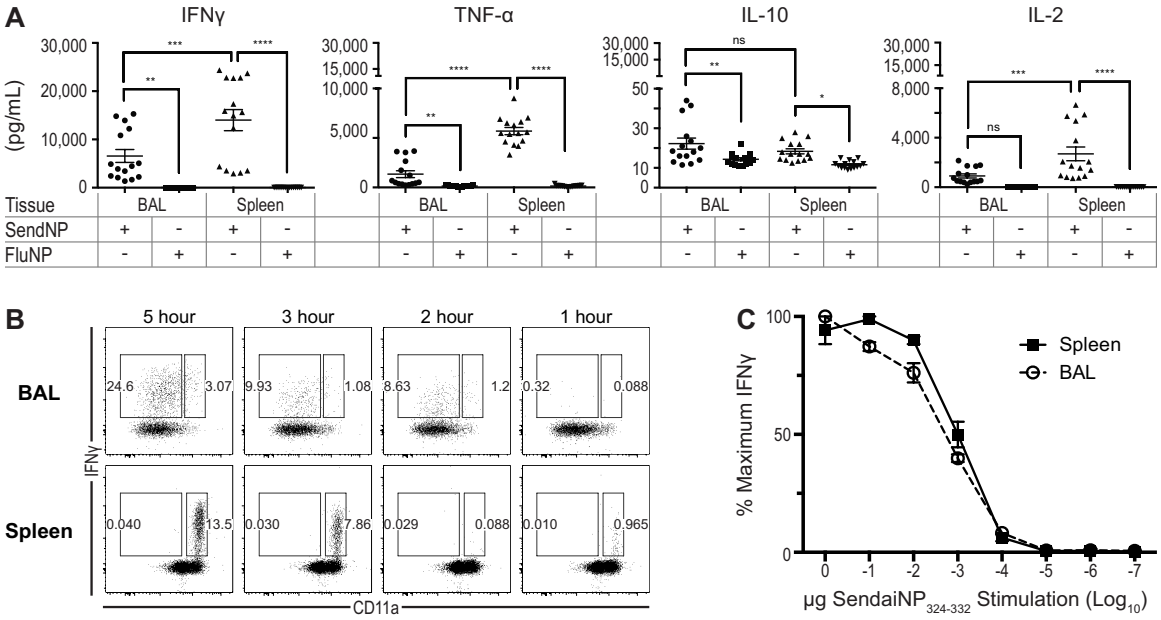

Figure 7: *Airway CD8 T_{RM} cells are capable of quickly producing cytokines, especially IFN-γ, upon recognition of cognate antigen.* A, Mice i.n. infected with Sendai virus and rested to immunological memory were sacrificed on day 45. Sorted CD62L^{-}/CD8^{+} cells from BAL and spleen were stimulated with SendaiNP$_{324–332}$ (SendNP) or FluNP$_{366-374}$ peptide for six hours before Luminex assay; N=15 mice/group; compiled from three independent experiments; mean & SEM; one-way ANOVA with Tukey's test corrected multiple comparisons; p-values [*<0.05; **<0.01; ***<0.001; ****<0.0001]. B, Airway CD8 T_{RM} cells and splenic-derived CD8 T_{EM} cells were stimulated with 1 µg/mL SendNP for one to five hours prior to intracellular staining (ICS) for IFN-γ production. N=4 replicates per time point; representative of three independent experiments. C, Percentage maximum of IFN-γ production of airway CD8 T_{RM} cells (BAL) or splenic-derived CD8 T_{EM} cells (Spleen) following stimulation with serial dilutions of SendNP for five hours before ICS for IFN-γ production. N=2 replicates per group per time point; representative of four independent experiments; mean & SEM. *B* & *C* results gated on CD8^{+}/CD45.2^{+} cells.

Airway CD8 T_{RM} cells are capable of rapidly producing antiviral cytokines. Given their suboptimal cytolytic activity, we hypothesized that airway CD8 T_{RM} cells may provide protection by rapidly detecting cognate antigen and secreting antiviral cytokines in response to secondary challenge. To test this, we examined the cytokine profile of airway CD8 T_{RM} cells and splenic-derived CD8 T_{EM} cells from Sendai-immune mice in response to their cognate antigen (SendNP) or an unrelated peptide (FluNP). As shown in Figure 7A, after six hours of stimulation with cognate antigen, airway CD8 T_{RM} cells produced significant amounts of IFN-γ, TNF-α, and IL-10; splenic CD8 T_{EM} cells also produced significant amounts of these cytokines plus IL-2. Notably, out of the cytokines produced by airway CD8 T_{RM} cells, IFN-γ

was most impressive with respect to magnitude and merited further investigation.

We suspected that the rate at which the airway CD8 T_{RM} population senses its cognate antigen could be another difference between the two populations, as rapid cytokine production is a hallmark of T_{RM}-mediated protection in other peripheral sites (18). This would corroborate the idea that the airway CD8 T_{RM} population acts as an early warning sensor to mediate protection in an antigen-specific manner. Thus, when we compared the airway CD8 T_{RM} and splenic T_{EM} cell IFN-**γ** production at early times after cognate peptide stimulation (Figure 7B), we observed that the airway CD8 T_{RM} population reacted faster (within two hours) than the splenic CD8 T_{EM} population. Furthermore, it was the $CD11a^{lo}$ airway CD8 T_{RM} population, which has resided in the airway the longest, that had the fastest rate of IFN-**γ** production. One explanation for the quicker IFN-**γ** response by the airway CD8 T_{RM} cells is that they are more sensitive to antigen than splenic CD8 T_{EM} cells. However, there was no difference in peptide affinity between airway CD8 T_{RM} and splenic CD8 T_{EM} cells (Figure 7C). This lack of difference is especially true at lower concentrations where a divergence would be expected if the airway CD8 T_{RM} cells had greater functional avidity to their cognate antigen than the splenic CD8 T_{EM} cells. Together, these data demonstrate that airway CD8 T_{RM} are able to rapidly produce antiviral cytokines upon antigen recognition and suggest that airway CD8 T_{RM} cell-derived IFN-**γ** may be a crucial mediator of protection against respiratory virus challenge.

IFN-γ-deficient airway CD8 T_{RM} cells show a significant defect in protective immunity. To test if IFN-**γ** was important for airway CD8 T_{RM} cell-mediated protection during an influenza virus infection, we i.t. transferred equal numbers of FluNP-specific airway CD8 T_{RM} cells from either WT or IFN-**γ**-deficient mice into naïve recipients, followed by PR8 influenza challenge one day later (Figure 8A). We found that mice receiving influenza-specific WT

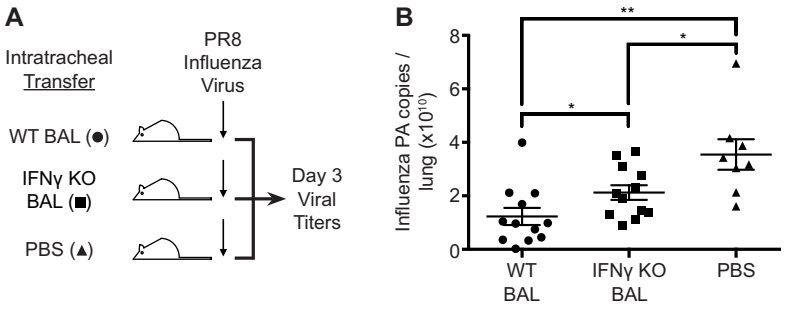

Figure 8: *IFN-γ-deficient airway CD8 T_{RM} cells are less effective at conveying heterologous protection.* A, Experimental i.t. transfer model where PBS or influenza-specific airway CD8 T_{RM} cells from WT or IFN-γ KO mice were i.t. transferred into naïve WT mice, which were i.n. challenged with PR8 influenza virus one day after transfer and viral titers were measured three days later. B, Influenza titers from each of the three groups receiving PBS, IFN-γ KO airway CD8 T_{RM} cells or WT airway CD8 T_{RM} cells as noted in *A*. N=8-12 mice/group; compiled from 2 independent experiments; mean & SEM; unpaired two-tailed t tests; p-values [*<0.05; **<0.01].

airway CD8 T_{RM} cells have significantly lower viral copies than those mice receiving airway CD8 T_{RM} cells from IFN-γ-deficient mice following PR8 challenge (Figure 8B). Moreover, mice receiving IFN-γ-deficient airway CD8 T_{RM} cells still showed a significant decrease in virus copies compared to PBS control mice, suggesting that other antiviral mechanisms are likely involved, such as production of TNF-α by airway CD8 T_{RM} cells observed in Figure 7A. Nevertheless, while it has been shown that IFN-γ is not necessary to survive a lethal primary influenza virus infection (28), these data show that IFN-γ produced by airway CD8 T_{RM} cells plays an important role in limiting viral loads following secondary challenge, which can be important in limiting immunopathology during an infection (29).

Discussion

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

of their cognate antigen, suggesting that the ability to rapidly produce cytokines is critical for their protective efficacy. In support of this, airway CD8 T_{RM} cells lacking IFN-**γ** had a significant defect in protective immunity compared to wild-type controls. Together, these data demonstrate that the airway CD8 T_{RM} population plays an important role in secondary cellular immunity against respiratory viruses by providing a rapid, local source of cytokines to promote an early anti-viral state.

Many studies have observed a correlation between the steady decline in numbers of airway CD8 T_{RM} cells in the months after primary infection and the steady decline in heterosubtypic immunity against influenza virus challenge. However, demonstrating that the decline in protective immunity is a direct consequence of a decline in airway CD8 T_{RM} population has been difficult because delineating the individual contributions of airway T_{RM}, lung parenchymal T_{RM}, and circulating T_{EM} populations are not possible through traditional antibody depletion approaches. The importance of analyzing the role of airway CD8 T_{RM} cells independently of these other subsets was further highlighted in a recent study that observed that lung parenchymal T_{RM} cell numbers also decline in the months post-infection, and the decline in protection may have been solely attributable to this phenomenon (10). Our data do not preclude a role for parenchymal T_{RM} cells in heterologous immunity, but rather suggest that these populations may act in concert to limit early viral replication. Unlike the airway T_{RM} population, T_{RM} populations within other tissues display strong cytolytic activity, and the lung parenchymal T_{RM} population maintains expression of CD11a, enabling their cytolytic activity (Figure 5C). It has been shown that infected lung epithelial cells can present antigen to T cells on the apical surface lining the airways in addition to the basolateral surface; so, it is possible that an infected epithelial cell would be presenting antigen to both the airway

and lung parenchymal T_{RM} subsets (30). Thus, there may be a division of labor between these populations where airway T_{RM} cells serve more of a sentinel function through the rapid production of cytokines to condition the local microenvironment and lung parenchymal T_{RM} cells mediate direct killing of infected cells.

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

Although IFN-γ-deficient mice show no defect in antiviral immunity following a primary influenza infection (28), its impact on protective cellular immunity to heterologous influenza challenge is less clear, with several conflicting reports regarding the protective role of IFN-γ during secondary challenge (35-38). Our data show that the inability of airway CD8 T_{RM} cells to produce IFN-γ resulted in a significant increase in viral titers compared to wild-type airway CD8 T_{RM} cells; it may be that the impact, positive or negative, of IFN-γ on protective immunity during influenza challenge depends on the timing of IFN-γ production. For example, it has been shown that IFN-γ production at the later stages of the acute response

can lead to enhanced pathology (39), whereas our data suggest that early production of IFN-γ by airway CD8 T_{RM} cells results in decreased levels of pro-inflammatory cytokines, likely due to decreased viral replication. It should also be noted that airway CD8 T_{RM} cells also produced TNF-α and IL-10 and that these cytokines may account for the limited protective effect observed when IFN-γ-deficient airway CD8 T_{RM} cells were transferred into the airways of naïve mice compared to PBS controls. In particular, the low levels of IL-10 produced may also limit early pro-inflammatory cytokine production and decrease pathology (40).

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

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Chapter 4

CXCR6 and CD49a co-expression promote airway & lung parenchymal resident memory CD8 T cell establishment following local antigen exposure

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Abstract

Resident memory CD8 T (T_{RM}) cells in the lung parenchyma (LP) and airways provide heterologous protection against influenza virus challenge. However, scant knowledge exists regarding factors necessary to establish and maintain airway and LP CD8 T_{RM} cells. Here we demonstrate that, in contrast to mechanisms described for other tissues, lung CD8 T_{RM} cell establishment require cognate antigen recognition following systemic effector cell recruitment to the lung by local inflammation. This "pulled" population forms long-lasting LP and airway CD8 T_{RM} populations, conveying protection equal to a native intranasal infection upon heterologous challenge. Finally, these populations co-express a unique chemokine receptor and adhesion molecule pairing, CXCR6 and CD49a, respectively, which we postulate as unique identifiers of the LP and airway CD8 T_{RM} populations.

Introduction

Viral clearance following acute infection results in the establishment of three T cell memory reservoirs: central memory T (T_{CM}) cells, largely found in secondary lymphoid organs; effector memory T (T_{EM}) cells, which systemically circulate, transiently entering peripheral tissues; and resident memory T (T_{RM}) cells, a non-circulating, self-renewing population found in peripheral tissues exposed to the acute pathogenic assault (1-3). T_{RM} cells are protected from intravital labeling by a fluorophore-conjugated antibody intravenously injected prior to animal euthanasia, and many, but not all, of these populations co-express the activation marker CD69 and integrin molecule CD103 (4, 5).

Acting as sentinels capable of quickly organizing a secondary immune response upon pathogen challenge, CD8 T_{RM} populations are initially primed by microenvironment cues and APC licensing following acute infection of peripheral tissue. Studies have shown that cellular immunity is in part responsible for mitigating morbidity and mortality during exposure to pandemic influenza virus strains with protection directly correlated with the number of detectable cross-reactive T cells (6, 7). In fact, we have shown that the airway CD8 T_{RM} population is sufficient to mediate protection from heterologous challenge, working in concert with the LP CD8 T_{RM} population to rapidly produce cytokines and kill infected cells (4).

Given the protective benefits that are gleaned from these populations, investigators are seeking out how these populations actually arise, noting the stimuli necessary for the establishment of a robust and protective T_{RM} population. For example, studies on the skin and female reproductive tract (FRT) T_{RM} populations demonstrate that local inflammatory signals alone are capable of establishing long-lived and protective T_{RM} populations from primed systemic effectors (8, 9). However, despite their capacity for heterologous protection

against respiratory infections, including influenza virus, paramyxoviruses, and respiratory *mycobacterium tuberculosis* infections, scant knowledge exists as to factors necessary to establish and maintain airway and LP CD8 T_{RM} populations (10-12).

T_{RM} populations of different peripheral tissues are typified by the expression of a unique chemokine receptor and adhesion molecule pairing acquired during licensing of population precursors (13). Two notable and well-studied T_{RM} populations are found in the skin and gut; gut T_{RM} cells express the chemokine receptor CCR9 and adhesion molecule integrin $\alpha_4\beta_7$; skin T_{RM} cells express the chemokine receptor CCR10 and adhesion molecule cutaneous lymphocyte-associated antigen (CLA), conveying local protection to pathogens such as herpes simplex virus (14, 15). However, the chemokine receptor characteristic of and necessary for establishing and maintaining the airway and LP CD8 T_{RM} cells remains elusive.

In the presented study, we identify that local antigen is required to generate airway and LP CD8 T_{RM} populations. In doing so, we employ a new vaccination method to establish protective antigen-specific airway and LP CD8 T_{RM} populations on par with immune responses generated by a native intranasal infection; local intranasal (IN) inflammation and antigen draw in systemic effectors, established through intramuscular (IM) priming, to effectively create airway and LP CD8 T_{RM} cells specific for the dosed antigen. Furthermore, we identify that CXCR6 and CD49a represent a unique chemokine receptor and adhesion molecule pairing which promotes the establishment and maintenance of airway and LP CD8 T_{RM} cells in both our new vaccination model and native IN infections.

Materials & Methods

Mice. C57BL/6J (WT), B6.PL-Thy1^{a}/CyJ (CD90.1), B6.SJL-Ptprca Pepc^{b}/BoyJ (CD45.1) and C.129P2-Cxcr6^{tm1Litt}/J (CXCR6 KO) mice from The Jackson Laboratory were housed

under specific ABSL2 conditions at Emory University. Mixed bone marrow (BM) chimeras were generated following two 475 rad irradiation doses of recipient mice followed by intravenous transfer of at least $1x10^7$ donor BM cells from each genotype (WT and CXCR6 KO) in a 1:1 ratio; chimeras were maintained on a solid food diet with 1.2% Sulfamethoxazole and 0.2% Trimethoprim (TestDiet 5TYG) for four weeks and rested for an additional two weeks prior to use, allowing for immune reconstitution. All experiments were completed in accordance with the Institutional Animal Care and Use Committee guidelines of Emory University.

Infections & "pull" vaccination. Intranasal infections used influenza A/HKx31 (H3N2) at 30,000 50% egg infectious doses (EID_{50}) or Sendai virus at 282 EID_{50}; intramuscular infections used influenza A/HKx31 (H3N2) at $1x10^6$ EID_{50} (16). Influenza A/PR8 (H1N1) at 6,000 EID_{50} was used for heterologous challenge of vaccinated mice. Peptides used for local antigen dosing, $InfluenzaNP_{311-325}$ (QVYSLIRPNENPAHK) and $InfluenzaNP_{366-374}$ (ASNENMETM), were dosed at 5μg. Dosed at 5μg, InvivoGen manufactured ODN 1826 (CpG) was used as the mediator of local inflammation.

Intravital cell labeling & cellular isolation. To delineate T cells resident in tissue and those in the vasculature, mice were intravenously injected with a fluorophore conjugated antibody (1.5μg of fluorophore-conjugated α-CD3ε antibody in 200λ 1x PBS) five minutes before euthanasia with Avertin (2,2,2-Tribromoethanol - Sigma) and exsanguination; after which point, tissues were harvested (4). Bronchoalveolar lavage was harvested directly from euthanized mice; mediastinal lymph nodes and spleen were mechanically dissociated into single cell suspensions; lungs were mechanically dissociated and enzymatically digested in Collagenase D (Sigma) and DNAse (Roche).

Flow cytometry. Antibodies and staining reagents used for flow cytometry and cell sorting

include Biolegend CD62L [MEL-14], CD8α [53-6.7], CXCR3 [173], CD90.1 [OX-7], CD90.2 [53-2.1], CD69 [H1.2F3], CD4 [RM4-5], CCR4 [2G12], CCR9 [CW-1.2], CCR6 [29-2L17], CCR5 [HM-CCR5]; eBioscience CD11a [M17/4], CD44 [IM7]; BD Biosciences CD3ε [145-2C11], CD45.2 [104], CD103 [M290], CD49a [Ha31/8]; Jackson ImmunoResearch Polyclonal goat α-human IgG FCγ specific $F(ab')_2$ Fragment; and a CXCL16-hFC fusion protein (17). Staining for intracellular and nuclear markers used BD cytofix/cytoperm kit and eBioscience transcription factor staining buffer set, respectively. Tetramers, provided by the NIH Tetramer Core Facility, used for detection of antigen-specific cells include H-2D^b Influenza A $NP_{366-374}$ (ASNENMETM), H-2D^b Sendai $NP_{324–332}$ (FAPGNYPAL), and H-2D^b Influenza A $PA_{224-233}$ (SSLENFRAYV). Samples were run on a BD Biosciences LSR II flow cytometer and sorted on an Aria II cell sorter (BD Biosciences) with analysis by FlowJo software.

Quantitative PCR (qPCR) & measurement of viral burden. Antigen-specific CD8 T cells at D12PI were sorted from the lung vasculature and lung parenchyma, RNA isolated using TRIzol and RiboPure RNA Purification Kit (Ambion) and cDNA generated using iScript cDNA Synthesis Kit (Biorad) was used to determine the expression of target genes. Primers for target genes include: *sell* [F: CTA ATT TCC CCT CGC TCA TTC AT & R: GCA TTA GCT TCT GTG CTG AAT TGA], *cd69* [F: TGG TCC TCA TCA CGT CCT TAA TAA & R: TCC AAC TTC TCG TAC AAG CCT G], *s1pr1* [F: GTG TAG ACC CAG AGT CCT GCG & R: AGC TTT TCC TTG GCT GGA GAG], *klf2* [F: ACC AAC TGC GGC AAG ACC TA & R: CAT CCT TCC CAG TTG CAA TGA], *itgae* [F: TGG CTC TCA ATT ATC CCA GAA & R: CAT GAC CAG GAC AGA AGC AA], *hprt* [F: CAT TAT GCC GAG GAT TTG GAA & R: CAC ACA GAG GGC CAC AAT GT] (18-20). Viral burden was measured

as previously described (4). Briefly, qPCR, using High Capacity cDNA Reverse Transcription Kit (Life Technologies), to detect levels of the influenza polymerase gene was run on cDNA generated from 2μg RNA isolated from lung homogenates through TRIzol and RiboPure RNA Purification Kit (Ambion).

Data Analysis & Statistics. All analysis was performed in GraphPad Prism 6. Figures represent mean and SEM. Specific statistical tests for each data set are noted in the respective figure legend. Relative recruitment of antigen-specific cells to target tissues (TT) in the BM chimeras were calculated using [({(# WT Ag-specific cells in TT)+1}/{(# WT Ag-specific cells in spleen)+1})/({(# CXCR6$^{-/-}$ Ag-specific cells in TT)+1}/{(# CXCR6$^{-/-}$Ag-specific cells in spleen)+1})], where target tissues include airways, LP, LV, MLN, and spleen.

Results

IM infected mice fail to generate a LP and airway CD8 T_{RM} population. It is known that the route of infection impacts generation of a T_{RM} population within the lung and airways (21). Given that the majority of vaccination methods employ an IM inoculation route, we employed an IM versus IN infection platform on which we could try to elicit a LP and airway CD8 T_{RM} population from an initial IM inoculation. As expected, IM infected mice fail to generate as robust of an influenza (flu)-specific and flu-specific CD103^{+}/CD69^{+} population in the LP and airways as compared to an IN infection; this was observed despite both infection routes generating an equally strong systemic flu-specific population, as denoted by the spleen (Figure 1A).

Acute IN dosing of local antigen and inflammation is sufficient to generate long-lived LP and airway CD8 T_{RM} populations. We permuted our system by IN dosing IM infected mice at D7PI (day 7 post-infection) (Figure 1B) to examine if local inflammation (CpG) alone or local

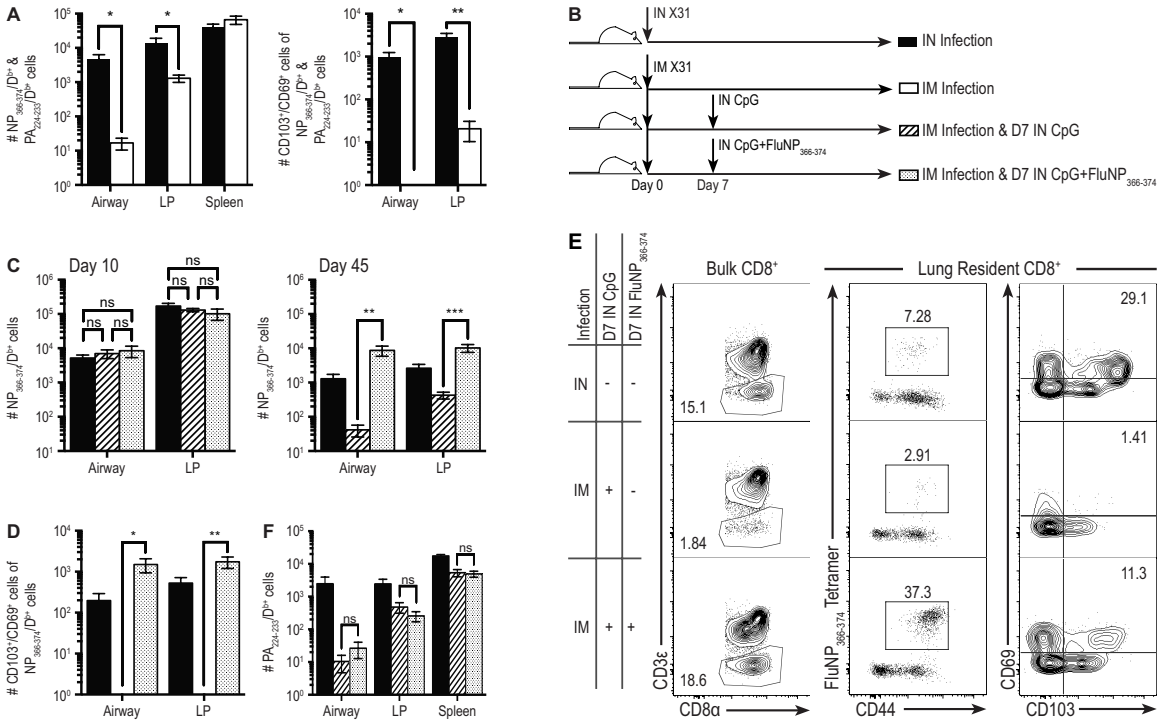

Figure 1. *Systemic effectors are "pulled" into and establish resident memory populations in the LP and airways following acute encounter with local antigen and inflammation.* A, Number of influenza-specific CD8 T cells ($NP_{366\text{-}374}/D^{b+}$ & $PA_{224\text{-}233}/D^{b+}$) and $CD103^+/CD69^+$ influenza-specific cells in the lung airway, lung parenchyma (LP), and spleen of mice at day 45 post-intranasal (IN) infection or intramuscular (IM) infection with x31 influenza virus. B, Experimental model denoting timing of infection relative to IN "pull" through the dosing of local inflammation (CpG) +/- local antigen ($FluNP_{366\text{-}374}$) at day seven post-IM infection. C, Number of $FluNP_{366\text{-}374}$-specific CD8 T cells at day ten and 45 post-infection (PI) within the airway and LP of mice treated as described in *B*. D, Number of $CD103^+/CD69^+$ cells of those noted in *C* at day 45PI. E, Representative gating of IN infection and two "pull" treatments at day 45PI, delineating systemic and resident populations through the use of CD3ε as an i.v. injected antibody prior to mouse sacrifice; representative frequencies of $FluNP_{366\text{-}374}$-specific cells and $CD103^+/CD69^+$ cells are shown for the bulk lung resident CD8 T cell population (i.v. injected $CD3\varepsilon^-$). F, Number of $FluPA_{224\text{-}233}/D^{b+}$-specific cells within the airway, LP, and spleen of mice treated as in b at day 45PI. *A, C, D, F* antigen-specific resident populations are gated on a $CD8^+$/i.v. injected $CD3\varepsilon^-$ population; *A, F:* spleen gated on bulk $CD8^+$ population. Mean & SEM; N=3-5 mice/group/time point; representative of 3-5 experiments; statistical analysis includes, *A:* unpaired t test with Holm-Sidak corrected multiple comparisons, *C, D, F:* two-way ANOVA with Tukey corrected multiple comparisons.

antigen and inflammation (CpG+Ag) were sufficient to "pull" systemic effectors to the LP and airway so as to seed a T_{RM} population. As our target antigen, we used the peptide $FluNP_{366\text{-}374}$ which is a known immunodominant CD8 T cell epitope within the influenza virus nuclear protein (FluNP) that we use experimentally. Both IN dosing schema are sufficient to draw flu-specific cells to the LP and airway acutely (D10PI), but only local antigen and inflammation were able to generate a robust flu-specific CD8 T_{RM} population in the LP and airways (Figure

1C). This finding at memory held true for the flu-specific/CD103+/CD69+ population as well when looking at either cell counts or population frequencies (Figure 1D & 1E). In fact, the FluNP-specific airway and LP CD8 T_{RM} population responses generated in mice IN dosed with CpG+Ag was slightly stronger than that observed in mice receiving a native IN influenza virus infection.

Of importance, the observed response was specific for the IN-dosed antigen, as demonstrated by the equally poor establishment of LP and airway CD8 T_{RM} populations specific for the influenza polymerase gene (FluPA) in both groups of mice IN dosed at D7 post-IM infection (Figure 1F). This is expected and serves as an internal control, as these animals are only exposed to FluPA during the initial IM infection.

Antigen is necessary in conjunction with local inflammation to generate a LP and airway CD8 T_{RM} population. Two potential explanations for the lack of LP and airway T_{RM} populations in mice receiving only local inflammation could be that the lack of local antigen 1) Results in defective T_{RM} population formation or 2) Generates a transient T_{RM} population which is only short-lived, resulting in a steady decline in the number and frequency into immunological memory. Performing a time course out to memory following the D7PI IN "pull" with CpG or CpG+Ag allowed us to answer this question. Representing a direct comparison between the IM and IN infected mice prior to any IN "pull," the D7PI time point shows a significant defect of FluNP-specific localization to the LP and airways of IM infected mice as compared to IN infected mice (Figure 2A). Three days following IN dosing with CpG or CpG+Ag of IM infected mice (D10PI), the number of FluNP-specific cells was in parity between both "pull" groups and those mice IN infected. However, over the next four days, a significant divergence became evident in the LP and airways between the CpG and CpG+Ag treated groups. From

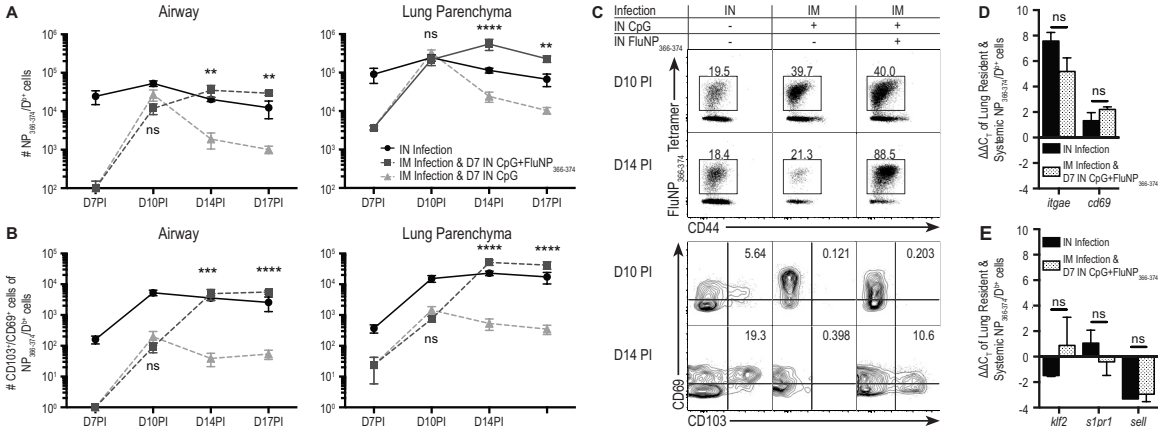

Figure 2. *"Pulling" systemic effectors with IN inflammation and antigen results in an IN infection-like immunological phenotype by day 14 post-infection.* A, Number of FluNP$_{366-374}$-specific CD8 T cells within the lung airways and LP at day 7, 10, 14 and 17PI by IN infection or IM infection followed by IN "pull" at day 7 post-IM infection. Gated on CD8⁺/i.v. injected CD3ε⁻ population. B, Number of CD103⁺/CD69⁺ cells of the FluNP$_{366-374}$-specific population noted through the ten day time course in the airways and LP. C, Representative gating at D10PI and D14PI of FluNP$_{366-374}$-specific cells [Top] within the LP, gated on CD8⁺/i.v. injected CD3ε⁻ population; [Bottom] representative frequencies and plots of CD103⁺/CD69⁺ on the FluNP$_{366-374}$-specific LP CD8 T cells noted on [Top]. D, Of genes typically up-regulated on T_{RM} populations, relative gene expression (ΔΔCT) between FluNP$_{366-374}$-specific cells of the LP and lung vasculature (LV) for mice at D12PI of mice receiving an IN infection or IM infection with CpG+Ag "pull." E, Same conditions as *D* but of genes normally down-regulated on T_{RM} populations. Mean & SEM; N=5 mice/group/time point; representative of 3 experiments; *A, B* significance noted between IM infection with CpG+Ag and IM infection with CpG groups at designated time points; statistical analysis includes *A & B:* two-way ANOVA with Tukey corrected multiple comparisons, *D & E:* unpaired t tests with Holm-Sidak corrected multiple comparisons.

D14PI (D7 post-IN "pull"), the FluNP-specific cell counts of the CpG+Ag group continued to resemble the findings of an IN infected mouse while the CpG "pull" group experienced further contraction from its initial peak in numbers at D10PI. This trend observed between the two "pull" groups and the IN infected group was also confirmed when looking at the FluNP-specific/CD103⁺/CD69⁺ population in the LP and airways (Figure 2B).

The CpG+Ag LP and airway CD8 T cell population undergoes a striking phenotypic transition between D10PI and D14PI, increasingly resembling that of the IN infected group, while in parallel, the CpG "pull" group adopts a phenotype more closely to that of an IM infected animal. Between D10PI and D14PI, the frequency of FluNP-specific cells decreased in the CpG "pull" group while it continued to expand in the CpG+Ag "pull" group (Figure

2C, Top). Furthermore, a population of the FluNP-specific cells in the LP and airways of the CpG+Ag "pull" groups adopted a $CD103^{+}/CD69^{+}$ phenotype by D14PI, while this is not evident in the CpG "pull" group (Figure 2C, Bottom). Based on this evidence, it appears as if the CpG "pull" group fails to generate a CD8 T_{RM} population, while the addition of local antigen (CpG+Ag) is sufficient to generate a long-lived T_{RM} population within the LP and airways.

By D12PI, the differences of expression between antigen-specific cells of the lung vasculature (LV) as compared to those of the LP denote a similar gene regulation between the IN infected mice and those mice receiving IM infection with a D7PI CpG+Ag "pull." The fold change for *itgae* (gene controlling CD103 expression) and *cd69* each show preferential upregulation on the FluNP-specific lung resident CD8 T cell population, found in the LP, as compared to those of the systemic circulation (Figure 2D). Furthermore, low expression of *klf2*, *s1pr1*, and *sell* (gene controlling CD62L expression) on the FluNP-specific lung resident CD8 T cell population (Figure 2E) are in line with previous findings characterizing early T_{RM} precursors of LCMV infected mice (18). Thus, by D12PI, the antigen-specific systemic effectors drawn in by the D7PI IN CpG+Ag dosing have adopted programming similar to that observed in natively IN infected mice.

Acute local antigen and inflammation "pull" is sufficient to protect from secondary heterologous challenge. Given that we were able to generate a targeted antigen-specific LP and airway CD8 T_{RM} population through CpG+Ag "pull" that equaled the magnitude of said population from an IN infection, we sought whether this population was protective when heterologously challenged. With inclusion of the peptide for the CD4 and CD8 epitope of FluNP when IN dosing, we observed statistically equal protection between immunological

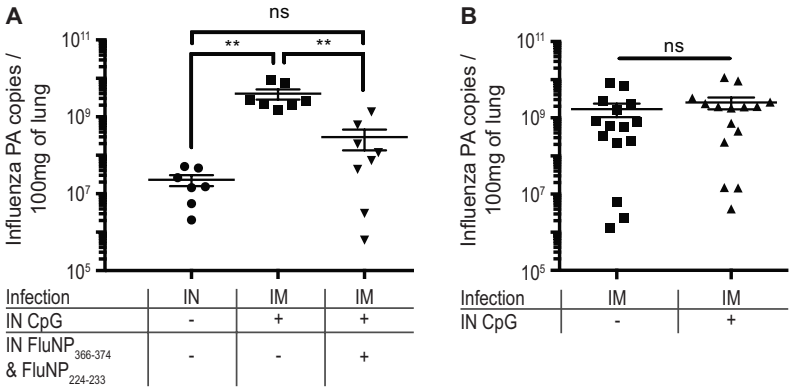

Figure 3. *T_{RM} population generated by antigen & inflammation "pull" provides heterologous protection.* A, Mice 46 days PI and 39 days post-IN "pull," if applicable, were heterosubtypically challenged IN with PR8 influenza virus. Lungs were harvested at day six post-secondary infection (D6PSI) to evaluate the viral load via FluPA RNA copies/100mg of lung. B, Mice 45 days PI and 38 days post-IN "pull," if applicable, were heterosubtypically challenged IN with PR8. Lungs were harvested at D6PSI to evaluate the viral load via FluPA RNA copies/100mg of lung, compiled from two independent experiments. Mean & SEM; N=7-8 mice/group/experiment; p-values [**<0.01]; representative of 2-3 independent experiments; statistical analysis includes *A*: one-way ANOVA with Tukey corrected multiple comparisons, *B*: unpaired t test.

memory mice that were either IN infection primed or IM primed and "pulled" at D7PI with CpG+Ag (Figure 3A). However, mice IM primed and "pulled" at D7PI with local inflammation alone did not realize any protection. In fact, these mice had viral loads similar to that of mice only receiving a primary IM infection with no subsequent IN "pull" (Figure 3B). Thus, "pulling" a primed systemic effector population with both local antigen and inflammation is able to establish a robust and protective CD8 T_{RM} population in the airway and LP that rivals a native primary IN infection.

CXCR6 and CD49a denote a paired signature for LP and airway CD8 T_{RM} populations. It is known that T_{RM} populations of different tissues can be characterized by the expression of a unique pairing of a chemokine receptor and an adhesion molecule, which arise through tissue imprinting during acute infection of a peripheral tissue. Additionally, it has been shown that CD49a expression on airway T_{RM} cells is necessary for protection during a secondary influenza virus challenge (22). Furthermore, comparison of the chemokine receptor expression on human $CD3^+$ cell populations of the lung with those in the blood, identified a considerable enrichment in the expression of CXCR6 within the lung, noting additional chemokine receptors such as

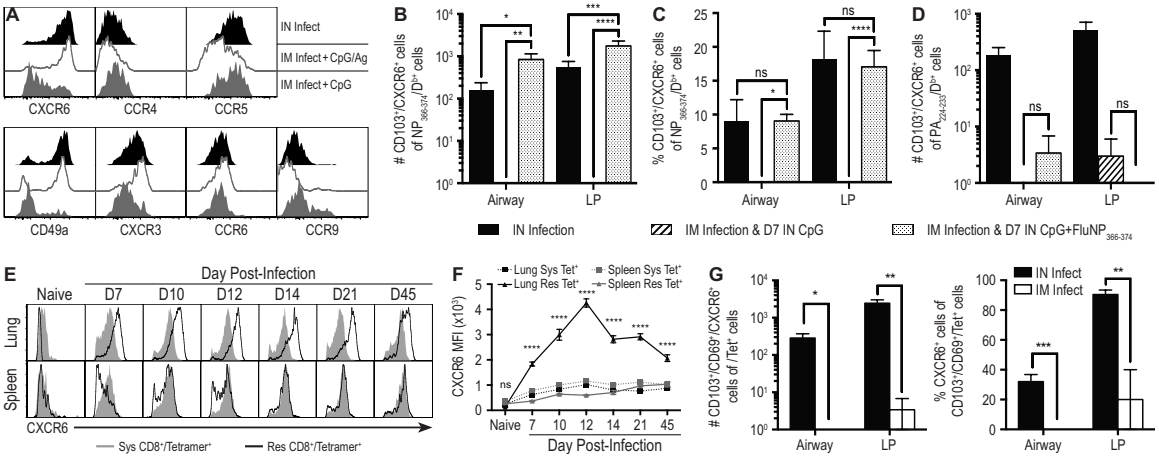

Figure 4. *CXCR6 and CD49a expression following IN inflammation and antigen "pull" closely resembles that of a native IN infection at memory, overcoming the deficits observed from an IM infection alone.* A, Representative staining for the noted chemokine receptors and CD49a of LP CD8 T_{RM} cells at day 45 post-IN infection [Black] or IM infection followed by IN "pull" with CpG+Ag [Grey outline] or CpG [Grey filled]. B, Number of CD103⁺/CXCR6⁺ cells of the $FluNP_{366-374}$-specific population at D45PI within the airway and LP. C, Frequency of CD103⁺/CXCR6⁺ cells of the $FluNP_{366-374}$-specific population at D45PI within the airway and LP. D, Number of CD103⁺/CXCR6⁺ cells of the $FluPA_{224-233}$-specific population at D45PI within the airway and LP. E, Representative histograms denoting CXCR6 expression on the systemic (i.v. injected CD3ε⁺) CD8⁺/$FluNP_{366-374}$- & $FluPA_{224-233}$-specific (Tetramer⁺) population and the resident (i.v. injected CD3ε⁻) CD8⁺/Tetramer⁺ population of the lung and spleen over a 45 day time course following IN infection with x31 influenza virus. F, CXCR6 geometric MFI of the two populations noted in e within the lung and spleen; gated on bulk CD8⁺ population, and i.v. injected CD3ε$^{+/-}$. G, Number and frequency of CXCR6⁺ cells of a CD103⁺/CD69⁺/influenza-specific ($FluNP_{366-374}$- & $FluPA_{224-233}$-specific) population at D45 post-IN or IM infection within the airway and LP. *A-D, G*: gated on CD8⁺/i.v. injected CD3ε⁻ population. Mean & SEM; N=5 mice/group/time point; representative of 2-3 experiments; *F*: significance noted between Lung Res Tet⁺ and Spleen Sys Tet⁺; statistical analysis includes *B-D*: two-way ANOVA with Sidak corrected multiple comparison, *F*: two-way ANOVA with Dunnett corrected multiple comparison, *G*: unpaired t test with Holm-Sidak corrected multiple comparisons.

CCR4, CCR5, CXCR3, CCR6, and CCR9 which are found at various expression levels on T cells in the human lung (23).

When we examined the expression of these chemokine receptors on the LP CD8 T_{RM} population, we noticed a similar profile between the IN infected mice and CpG+Ag "pull" group which differed from the CpG "pull" group. Both CXCR6 and CD49a were highly expressed in IN infected and CpG+Ag "pull" groups, while both were poorly expressed in the CpG "pull" group (Figure 4A). In contrast, the expression of CCR4, CCR5, CXCR3, CCR6 and CCR9 were comparably expressed on IN infected, CpG+Ag "pull," and CpG "pull" treated mice. CXCR6 expression was found on greater numbers of FluNP-specific/

CD103⁺ CD8 T_{RM} cells in the airways and LP of mice which received antigen IN (IN infected or CpG+Ag "pull"), while the CpG "pull" group did not generate a detectable population at D45PI (Figure 4B). On a frequency basis, this correlation held, with co-expression of CXCR6 and CD103 on FluNP-specific CD8 T_{RM} cells in the airways and LP being equal between IN infected and CpG+Ag "pull" groups, while this population was non-existent in the CpG "pull" group (Figure 4C). Notably, this response corresponded only to the IN dosed antigen, as both "pull" groups generated equally poor numbers of CXCR6 expressing FluPA-specific/CD103⁺ CD8 T_{RM} cells in the airways and LP, as compared to IN infected mice (Figure 4D).

CXCR6 is rapidly upregulated and highly expressed on flu-specific CD8 T cells in the LP following IN infection. After observing the expression of CXCR6 on the LP and airway CD8 T_{RM} population, we were curious about the temporal and spatial dynamics of CXCR6 expression on antigen-specific and bulk CD8 T cells in the lung vasculature (LV - lung sys CD8⁺) and LP (lung res CD8⁺) as well as the spleen red pulp (spleen sys CD8⁺) and white pulp (spleen res CD8⁺). In Figure 5A, we show that CXCR6 is quickly up-regulated to high levels on CD8 T cells in the LP, and more specifically flu-specific cells (Tetramer⁺) within the LP, by day seven post-IN infection, maintaining a high expression into immunological memory (Figure 4E). In the LV, flu-specific cells also up-regulate CXCR6 but not to as high of an extent as those in the LP (Figure 4E). Furthermore, the bulk LV CD8 T cell population does not up-regulate CXCR6, unlike the bulk compartment of LP CD8 T cells (Figure 5A). Additionally, the flu-specific populations in the spleen normalize their CXCR6 levels by D14PI, achieving equivalent expression levels as the LV flu-specific cells (Figure 4E). Initial differences in CXCR6 expression between flu-specific cells of the red and white pulp in the spleen normalize by D14PI as the two populations, likely occurring as cells transition between compartments, as

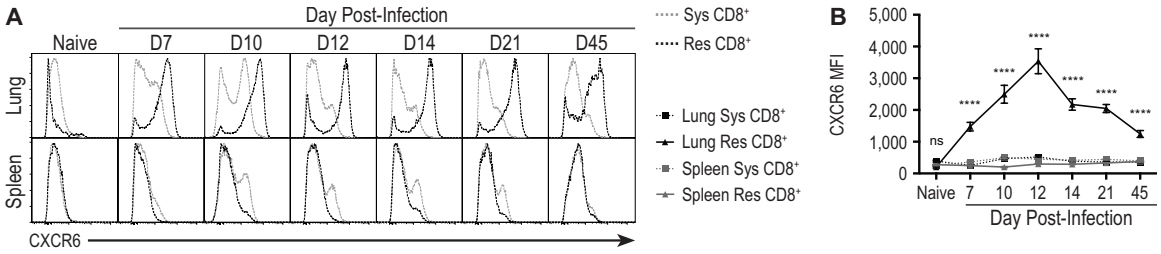

Figure 5. *Lung resident CD8 T cells highly express CXCR6 following IN infection.* A, Representative histograms denoting CXCR6 expression on the systemic CD8⁺ population and the resident CD8⁺ populations of the lung and spleen over a 45 day time course following IN x31 influenza virus infection. B, CXCR6 geometric MFI of the two populations noted in *A* within the lung and spleen; gated on bulk CD8⁺ population, and i.v. injected CD3ε$^{+/-}$. Mean & SEM; N=5 mice/group/time point; representative of 2-3 experiments; *B* significance noted between Lung Res CD8⁺ and Spleen Sys CD8⁺ by two-way ANOVA with Dunnett corrected multiple comparisons.

shown previously in parabiosis experiments (5). Importantly, the bulk CD8 T cell populations in the spleen white and red pulp express very low levels of CXCR6, indicating that CXCR6 expression is not globally induced on CD8 T cells following IN influenza virus infection (Figure 5A). Instead, it is sequestered to cells which reside at the site of the primary infection and/or have responded to the pathogenic insult either locally or in the regional lymphoid tissue.

Thus, in summary, CXCR6 is expressed at 1) High levels on LP flu-specific CD8 T cells and bulk LP CD8 T cells; 2) Intermediate levels on systemic flu-specific CD8 T cells found in the LV as well as the white and red pulp of the spleen; 3) Low levels on the bulk CD8 T cells of the LV and both compartments of the spleen, which include the majority of non-flu-specific CD8 T cells.

Moreover, when examining the geometric MFI (gMFI) of CXCR6 expression on the bulk and antigen-specific populations of the resident and systemic compartments of the spleen and lung, we found that CXCR6 expression is highest on CD8 T cells within the lung parenchyma with flu-specific CD8 T cells exhibiting an even higher level of CXCR6 expression (Figure 5B & Figure 4F). CXCR6 expression on these populations peaks by D12PI and then declines slightly as the acute CD8 T cell effector population contracts; it then levels off as the

CD8 T cell population of the LP transitions into memory, remaining highly expressed relative to its expression on memory CD8 T cells of the LV and spleen.

CXCR6 expression is limited to LP and airway CD8 T_{RM} cells exposed to local antigen during the acute immune response. Noting the differences in phenotype of CXCR6 expression between the two "pull" groups and the dynamic CXCR6 expression within the LP of IN infected animals, we returned to our initial model system of differential route of infection to see if CXCR6 failed to be expressed on flu-specific LP and airway CD8 T_{RM} cells of IM infected mice. From both a number [Left] and frequency [Right] perspective at D45PI, only IN infected mice expressed CXCR6 at appreciable levels on LP and airway flu-specific CD8 T_{RM} cells, while IM infected mice failed to co-express CXCR6, CD103, and CD69 on these populations (Figure 4G).

CXCR6 deficient cells fail to form LP and airway CD8 T_{RM} populations following acute respiratory viral infection. To understand if cells lacking CXCR6 were truly at a disadvantage at establishing LP and airway CD8 T_{RM} populations, we created mixed bone marrow (BM) chimeras, reconstituting the hematopoietic compartment of lethally irradiated hosts with a 1:1 ratio of WT:CXCR6 KO BM (Figure 6A). After immune reconstitution, we infected the mixed BM chimeras IN with x31 influenza virus to determine if cells of WT or CXCR6 KO ancestry preferentially mediated the acute and memory CD8 T cell response to the insult.

Following infection with influenza virus, relative localization in the LP of flu-specific cells (Figure 6B, Left) favors WT over CXCR6$^{-/-}$-derived cells at the peak of immune response (D10PI) and into memory (D45PI); this differential localization is significantly different from that observed in the LV, where the numbers of WT or CXCR6$^{-/-}$-derived cells are nearly equal across the three time points. Notably, this finding of preferential localization of WT over

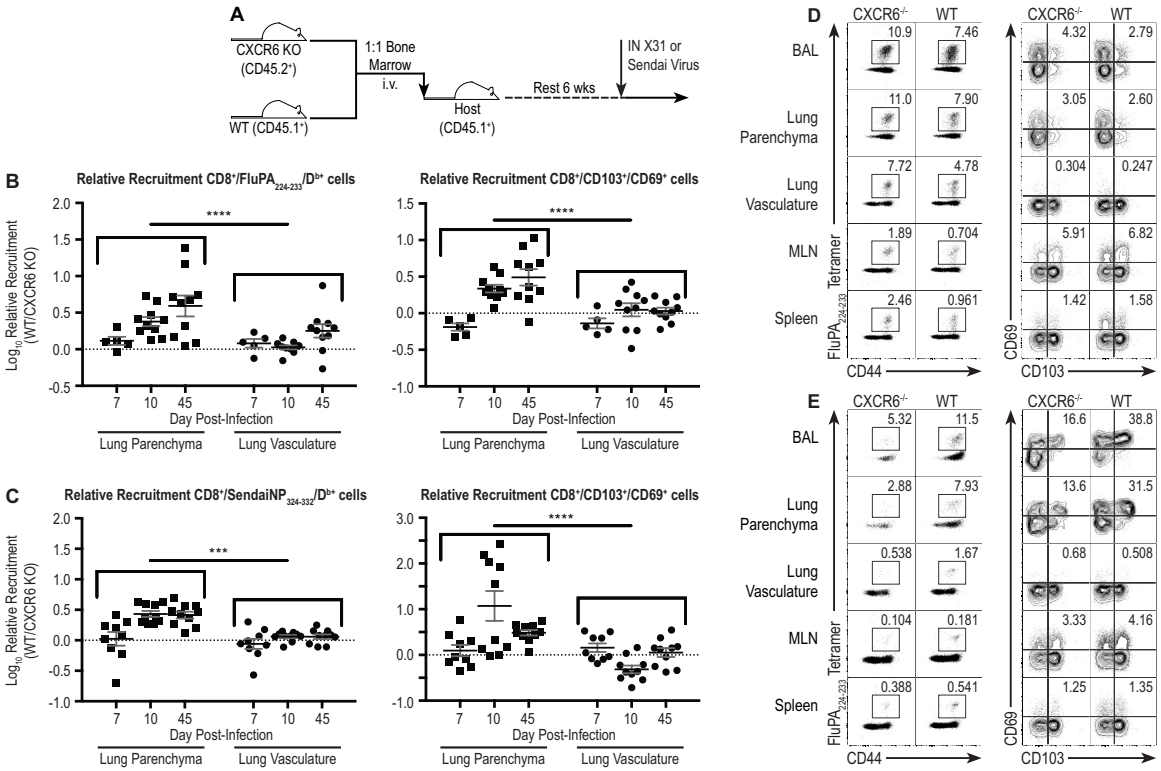

Figure 6. *WT cells, over CXCR6$^{-/-}$, preferentially form the antigen-specific & CD103⁺/CD69⁺ T_{RM} populations after viral respiratory infection of mixed BM chimeras.* A, Experimental outline of mixed bone marrow (BM) chimera creation with congenic WT and CXCR6$^{-/-}$ BM at 1:1 ratio followed by infection with Sendai or x31 influenza virus after immune reconstitution. B, Relative recruitment (RR) of WT:CXCR6$^{-/-}$ cells to the LP and LV of mixed BM chimeras post-IN influenza virus infection. [Left] RR lineage of CD8⁺/FluPA$_{224-233}$-specific cells and [Right] CD8⁺/CD103⁺/CD69⁺ cells at acute (D7PI & D10PI) and memory (D45PI) time points. C, RR of WT:CXCR6$^{-/-}$ cells to the LP and LV of mixed BM chimeras post-IN Sendai virus infection. [Left] RR lineage of CD8⁺/SendaiNP$_{324-332}$-specific cells and [Right] CD8⁺/CD103⁺/CD69⁺ cells at D7PI, D10PI, and D40PI. Values >0 indicate greater localization of WT-derived over CXCR6$^{-/-}$-derived cells to a tissue compartment. D, Representative staining of FluPA$_{224-233}$-specific cells [Left] and CD103⁺/CD69⁺ [Right] at D7PI of mixed BM chimeras. E, Representative staining of FluPA$_{224-233}$-specific cells [Left] and CD103⁺/CD69⁺ [Right] at D45PI of mixed BM chimeras. As also demonstrated in *B*, CXCR6$^{-/-}$-derived cells fail to form antigen-specific and CD103⁺/CD69⁺ populations in the LP. *B-E:* gated on CD8⁺ congenic cell populations, denoting marrow ancestry. Mean & SEM; N=5-10 mice/time point; p-values [***<0.001, ****<0.0001]; representative of 2-3 experiments; statistical analysis includes *B & C*: two-way ANOVA with Tukey corrected for main column effect multiple comparisons.

CXCR6$^{-/-}$-derived cells also held true and was statistically significant for the CD103⁺/CD69⁺ flu-specific populations of the LP but not the LV (Figure 6B, Right). Similarly, we observed the same trend in mixed BM chimeras infected with Sendai virus, a natural murine respiratory pathogen, where the Sendai-specific and CD103⁺/CD69⁺/Sendai-specific populations were predominately WT-derived in the LP, while equally WT and CXCR6$^{-/-}$-derived in the LV (Figure 6C). In support of these findings, we observed a similar defect in the localization of

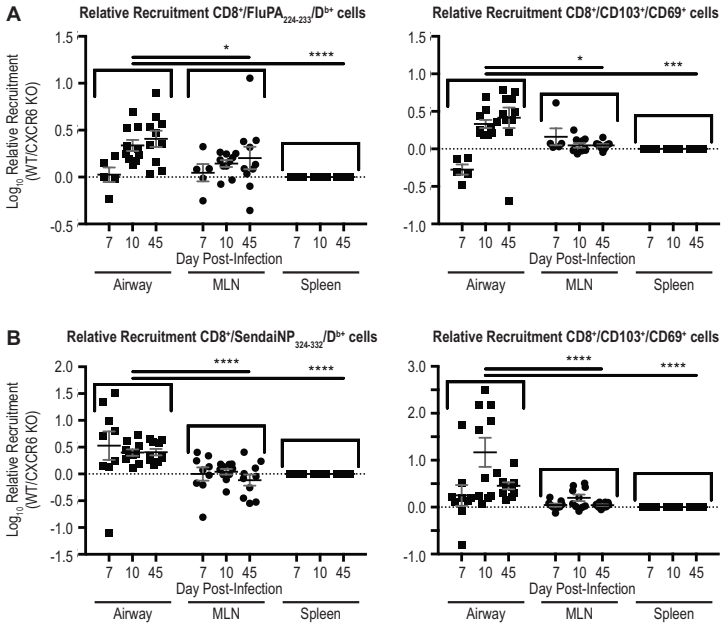

Figure 7. *WT cells, over CXCR6$^{-/-}$, preferentially form the airway antigen-specific & CD103⁺/CD69⁺ T_{RM} populations after viral respiratory infection of mixed BM chimeras.* A, Relative recruitment (RR) of WT:CXCR6$^{-/-}$ cells to the airways (BAL), mediastinal lymph node (MLN) and spleen of mixed BM chimeras post-IN influenza virus infection. [Left] RR lineage of CD8⁺/FluPA$_{224-233}$-specific cells and [Right] CD8⁺/CD103⁺/CD69⁺ cells at acute (D7PI & D10PI) and memory (D45PI) time points. B, RR of WT:CXCR6$^{-/-}$ cells to the BAL, MLN and spleen of mixed BM chimeras post-IN Sendai virus infection. [Left] RR lineage of CD8⁺/SendaiNP$_{324-332}$-specific cells and [Right] CD8⁺/CD103⁺/CD69⁺ cells at acute (D7PI & D10PI) and memory (D40PI) time points. Values >0 indicate greater localization of WT-derived over CXCR6$^{-/-}$-derived cells to a tissue compartment. *A & B* gated on CD8⁺ congenic cell populations, denoting marrow ancestry. Mean & SEM; N=5-10 mice/time point; p-values [*<0.05, ***<0.001, ****<0.0001]; representative of 2-3 experiments; statistical analysis includes *A & B*: two-way ANOVA with Tukey corrected for main column effect multiple comparisons.

CXCR6$^{-/-}$-derived cells in the lung airways at D10PI and D45PI, which was not observed in the mediastinal lymph node (MLN) nor spleen; this held true when examining an antigen-specific or CD103⁺/CD69⁺/antigen-specific population following influenza virus (Figure 7A) or Sendai virus (Figure 7B) infection. The nearly identical findings regarding LP and airway CD8 T_{RM} population establishment following both influenza and Sendai virus infection indicate the importance of CXCR6 expression by these two populations following respiratory virus infection, yet could also portend the importance of CXCR6 expression on similar populations following infection by other respiratory pathogens.

It is significant that, in both the influenza and Sendai virus infection systems, the D7PI time point results in near parity of WT and CXCR6$^{-/-}$-derived cells in all tissues examined. Only D10PI and beyond do we begin to see divergence in the ancestral composition of the LP and airway compartments from the LV, MLN and spleen. It is notable that this same timing

corresponds to the nascent co-expression of CD69 and CD103 by the antigen-specific LP and airway CD8 T cell populations (Figure 2C). This is best exemplified by juxtaposition of example flow plots at D7PI (Figure 6D) and D45PI (Figure 6E). At D7PI, the antigen-specific responses between the two genotypes appear relatively equal between all tissues, with a slight, but not statistically significant, skewing toward a CXCR6$^{-/-}$ predominant ancestry in all tissues (Figure 6D, Left); on the basis of CD69/CD103 co-expression, frequencies between the two genotypes are nearly equal (Figure 6D, Right). However, at D45PI, the antigen-specific responses in the airway and LP are skewed toward a WT ancestry, while WT and CXCR6$^{-/-}$ antigen-specific responses within the LV, MLN and spleen are nearly equal. This disparity in favor of a WT lineage is also evident in the CD69 and CD103 co-expressing population of the airway and LP, despite frequencies in the LV, MLN, and spleen existing in parity.

Based on this data, it is evident that CXCR6 expression is necessary for the establishment of robust airway and LP CD8 T_{RM} populations, supporting our theory that CXCR6 along with CD49a is the canonical chemokine receptor and adhesion molecule pairing specific to and characterizing of these airway and LP CD8 T_{RM} populations.

Discussion

In summary, we have shown that local inflammation and antigen is necessary to generate a robust antigen-specific LP and airway CD8 T_{RM} population from systemic effector CD8 T cells, while local inflammation is not sufficient despite drawing in systemic effectors acutely. The LP and airway CD8 T_{RM} population generated from this local antigen and inflammation "pull" provides heterologous protection upon secondary challenge that is not observed in mice receiving only IN "pull" with local inflammation. Antigen-specific lung and airway CD8 T_{RM} cells established from a native IN infection or IM infection followed by IN antigen and

inflammation "pull" express both CXCR6 and CD49a, denoting a unique chemokine and adhesion molecule pairing distinct to the airways and LP. Finally, we show that there exists a defect in relative localization to the LP and airways by antigen-specific CXCR6$^{-/-}$ cells, resulting in a diminished resident memory population following IN respiratory virus infection.

The two impactful findings contained in this work are: 1) The identification of the stimuli required to establish LP and airway CD8 T_{RM} populations, including the development and characterization of a novel vaccination system to establish said T_{RM} populations without direct infection of the respiratory system and 2) The suggestion that CXCR6 and CD49a denote the elusive chemokine receptor and adhesion molecule pairing required for the establishment and long-term maintenance of the airway and LP CD8 T_{RM} populations.

First, the ability to establish T_{RM} populations in a targeted manner which ensures cells are localized only to the tissues of greatest need for protection from an acute pathogen challenge is the acme of vaccination development, especially when it can be done without risking the accidental dissemination of an infectious vaccine vector. Our IM infection followed by local antigen and inflammation "pull" does just that; it yields T cell protection to the respiratory tract without requiring direct infection of the lungs. Influenza virus is uniquely suited to this strategy since IM infection with influenza virus results in a single round of replication, thereby generating a pool of systemic effectors, before resulting in an abortive infection due to incomplete virion maturation (24). Therefore, a live attenuated influenza virus could be administered IM with limited concern of virus mutation and undesired public transmission via the respiratory route. Direct application of this research which yields a direct proof of concept, advancing influenza virus T cell vaccine generation, could have tremendous impact on annual epidemics and periodic pandemics. Additionally, current research continues

to reveal the merits of naturally occurring cross-reactive T cells to influenza virus challenge, noting their capacity to reduce morbidity and mortality, even resulting in largely asymptomatic responses in some cases (6, 7, 25).

Aside from the potential downstream therapeutic applications, this new vaccination strategy explicitly aided in the delineation of stimuli necessary for the establishment of the LP and airway CD8 T_{RM} populations. In contrast to mechanisms described for the skin and FRT, where administration of local inflammatory signals was sufficient to establish a robust and protective T_{RM} population from a systemic effector population, both local antigen and inflammation are required for the establishment of robust and protective LP and airway T_{RM} populations (8, 9).

Additionally, the co-expression of CXCR6 and CD49a (Figure 4B) denotes the protective population (Figure 3A) of antigen-specific CD8 T cells in the LP and airways generated through either IN infection or IM infection with local antigen and inflammation "pull." This provides a correlate of protection and a more targeted means to further investigate the LP and airway CD8 T_{RM} populations.

Finally, based upon our findings, we propose the following model which incorporates this new knowledge with the body of work already published (Figure 8). In our "pull" vaccination system, an IM infection generates a population of systemic effector T cells. Expressing CXCR3, a chemokine receptor which is highly up-regulated on T cells in inflammatory environments, these systemically primed T cells traffic into the LP non-specifically upon encountering the IN dosed inflammatory stimulus (26). Endocytosing the IN dosed antigen, the local APCs process and present it upon MHC-I (Major Histocompatibility Complex-I) following activation by the IN dosed inflammatory stimulus. CXCL16, which is known to be highly expressed in the

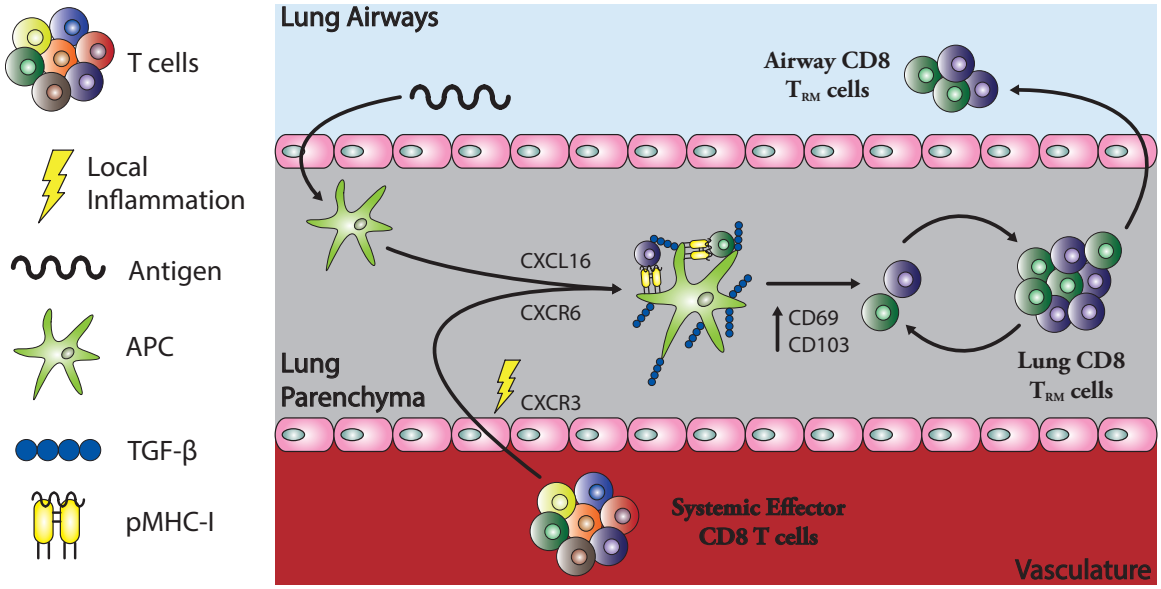

Figure 8. *Proposed model of airway and LP CD8 T_{RM} cell establishment following IN "pull."* An IM infection primes a systemic influenza-specific effector CD8 T cell response. Dosing with local inflammation induces T effector cells to traffic into the lung parenchyma via CXCR3 mediated translocation; in the absence of antigen, these cells either re-enter the systemic circulation or perish. If antigen is present, antigen-presenting cells (APCs) acquire the peptide, presenting it upon MHC-I molecules (pMHC-I). CD8 T cells in the LP, in response to inflammation, up-regulate CXCR6 and chemotax along a CXCL16 gradient to encounter cognate antigen and TGF-β on local APCs, promoting CD69 and CD103 expression, respectively. These licensed CD8 T cells locally proliferate to establish a LP CD8 T_{RM} population, which could act as a replicative reservoir for the airway CD8 T_{RM} population.

lung parenchyma and airways by resident APCs, is cleaved from the local APCs by ADAM10 (A Disintegrin and metalloproteinase domain-containing protein 10) (27-29). Chemotaxis along the local CXCL16 gradient by CXCR6 up-regulating systemically primed effectors in the LP facilitates direct interaction with activated local APCs. CD8 T cell interaction with peptide-MHC-I presentation and TGF-β on the local APC surface promotes CD69 and CD103 expression, respectively, resulting in the establishment of the LP CD8 T_{RM} cell population, which could give rise to and act as the replicative reservoir for the airway CD8 T_{RM} cell population. Further investigation is needed to confirm aspects of this model, but this study equips the field with a solid foundation and yields a powerful experimental system on which future protection studies could be employed.

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Chapter 5

Synthesis: Final Perspective, Extrapolative Implications & Future of Respiratory Pathogen Protection

Summary

Chapter 1 presented an overview of the adaptive immune system, delineating the role that resident memory T (T_{RM}) cells play in limiting pathogenic insults. It provides specific insight into the current state of respiratory resident memory T cell research. The successive chapters build on this foundation to provide a better understanding of the function of CD8 T_{RM} in the lung parenchyma and airways as well as the factors regulating the establishment and long-term maintenance of these populations.

As validated in a murine model, Chapter 2 demonstrated the capacity for T cells to protect and decrease severity of infection following encounter with a heterologous influenza virus such as H7N9. Human PBMC samples had a similar cross-reactive population which could also mediate protection, if challenged. This correlate of protection, which decreases morbidity and mortality in the host, is especially important in the absence of neutralizing antibodies against the virus. Such instances can occur when 1) vaccine coverage is mismatched with the actual circulating annual epidemic influenza virus strain and 2) influenza virus recombination events result in a pandemic. The absence of effective protection during these events results in considerable increases in morbidity and mortality with concomitant impact on economic productivity. Thus, being able to mediate protection in the absence of neutralizing antibody coverage would have a significant global impact given the ubiquity of influenza virus infections.

Chapter 3 provided the first evidence that airway CD8 T_{RM} cells are sufficient to convey protection from respiratory virus challenge and that this protection is mediated in part by their ability to produce IFN-γ. Notably, the data also suggests a potentially cooperative interaction between the airway and lung parenchymal CD8 T_{RM} populations, whereby the airway CD8 T_{RM} population acts as a highly sensitive and antigen-specific sentinel to signal in secondary immune mediators, while the lung parenchymal CD8 T_{RM} population retains its cytolytic function to aid in clearing infected cells, further expediting pathogen clearance.

In Chapter 4, presented data implicate CXCR6 and CD49a as the unique chemokine receptor and adhesion molecule pairing responsible for the establishment and maintenance of airway and lung parenchymal CD8 T_{RM} cells. Furthermore, the identification and testing of a new vaccination system, whereby systemic effector T cells are "pulled" to the airways and lung parenchyma to establish CD8 T_{RM} populations, successfully demonstrates a proof of concept that protective airway and lung parenchymal CD8 T_{RM} populations can be established in the absence of direct infection of respiratory tissues. This finding alone yields a tremendous experimental tool and potential future therapeutic application of work discussed in this dissertation, providing a potential solution to a public health problem that arises when a neutralizing antibody response generated by the current influenza virus vaccine strategy is insufficient to fully protect upon challenge.

Overall, these collective data advance the respiratory resident memory as well as the broader resident memory T cell field as a whole, yielding a number of future research avenues, discussed herein.

Improving the Global Definition of T_{RM} Cell Populations

A query presented in Chapter 1 of this dissertation was how to best define a resident

memory T cell population. The data exemplified in Chapters 1 and 2 demonstrate the proposed functionality definition, that T_{RM} cells should ultimately convey local protection upon acute challenge with a pathogen containing cognate antigen epitopes for which the cells were initially primed. Our characterization and identification of the unique chemokine receptor and adhesion molecule pairing of the airway and lung parenchymal T_{RM} populations denotes the local or tissue specific definition presented in Chapter 1.

However, this still begs the question of how best to globally define a resident memory population. Many caveats are introduced with this inquiry, as the experimental systems used to examine the T_{RM} populations across varied peripheral tissues are not standardized, albeit understandably so as pathogens have tropism for different tissues. Even the study of airway and lung parenchymal T_{RM} populations do not use a single infection model, varying between intranasal administration of vesicular stomatitis virus (VSV), Sendai virus and influenza virus (1, 2). While it is beneficial to understand if viral versus bacterial, systemic versus local, or even acute versus persistent or chronic infections vary in how T_{RM} populations are established, differences or artifacts that result based upon the experimental system used may confound insight to the broader resident memory T cell field. Therefore, a certain burden lies upon the investigator to test in good faith, through use of different experimental systems, the generalizability of their experimental findings to the field as a whole.

Using and comparing results from both Sendai virus, a natural murine respiratory virus, and influenza virus, a murine adapted respiratory pathogen, make us confident in our findings of the airway and lung parenchymal T_{RM} populations. The co-expression of CXCR6 and CD49a is unique to these two resident memory populations and is not found globally, per our testing with LCMV (Lymphocytic Choriomeningitis Virus) Armstrong. Furthermore,

comparison of inflammatory adjuvants such as Lipopolysaccharide (LPS) and CpG when testing our "pull" vaccination model indicate that any method of inducing properly timed local inflammation, in conjunction with dosing local antigen, is sufficient to generate airway and lung parenchymal T_{RM} populations from systemic effectors, albeit, certain adjuvants have greater potency at inducing inflammation than others.

Further studies employing parabiotic animals, the gold standard for resident memory experiments, could confirm the CXCR6 and CD49a co-expression findings. This would be significant, as parabiosis experiments using a local intranasal pathogen have yet to be executed, yielding a potentially large boon for the respiratory resident memory field. Furthermore, validation of the "pull" vaccination method could be done with a true internal control where both mice receive an intramuscular influenza virus priming, yet one receives only local intranasal inflammation and the other receives both local intranasal inflammation and antigen one week later. Furthermore, given that other protective T_{RM} populations, including that of the female reproductive tract (FRT) and the skin, can be established with local inflammatory signals alone (3, 4), it would be interesting to see if supplementing in local antigen in addition would enhance the protection that these populations are able to covey.

Finally, the identification of a new lymphoid T_{RM} population brings to question whether the T_{RM} population of the draining lymph node arise independently of the populations in which the acute infection occurs or whether the detected lymphoid T_{RM} population actually gives rise to the T_{RM} population of the acutely infected tissue (5). Our preliminary studies appear to suggest that, at least in the case of the lung parenchymal and airway T_{RM} populations, T_{RM} populations can develop even after communication between the lymphatic system and peripheral tissue is interrupted; we have tested this just prior to the peak of the immune response

and resolution of influenza virus infection, through intraperitoneal treatment of FTY720, a S1P receptor antagonist, from day 7 through day 21 post-infection. Additional experiments with FTY720 lead us to believe that the lung parenchymal T_{RM} population maintains the airway T_{RM} population, replenishing its homeostatic turn over every 10-14 days.

The origin and education of T_{RM} population precursors during an acute infection will most likely be one of the forthcoming unifying findings for the resident memory T cell field, yielding insight into the necessary tissue licensing and transcriptional programming which must occur for a protective and self-maintaining population to be generated.

Advancing the Future of T Cell Mediated Vaccines to Respiratory Insults

In Chapter 4, we demonstrate a novel method of establishing airway and lung parenchyma CD8 T_{RM} populations, which protect as effectively as the CD8 T_{RM} populations established through a normal intranasal infection. This vaccination strategy has the potential to provided targeted immunity in a potentially prophylactic and therapeutic manner. It is conceivable that protective immunodominant epitopes for a variety of pathogenic insults could be targeted by intranasal peptide dosing in conjunction with local inflammatory adjuvants. Such a vaccination model for influenza virus could be as simple as intramuscularly inoculating a patient with a live attenuated influenza virus followed by provision of an inhaler/nebulizer to be used at home one week after intramuscular priming. While this concept is still very far from uptake in the clinical environment, the data in this dissertation provide a solid proof of concept for further investigation and extrapolation to other infection models, including targeting T_{RM} cells against respiratory cancers expressing unique epitopes.

Pertinent unknowns, not previously discussed, would need to be investigated prior to considering this approach in a human population. It is not yet known whether the effectiveness

of the intranasally dosed peptide to establish an expanded antigen-specific T_{RM} population in the airway and lung parenchyma would remain if a cocktail of antigens were dosed. Expansion of said population may depend on the frequency of the primed systemic effector population. Additionally, the magnitude of the local inflammatory stimulus likely plays a role in the effectiveness of establishing airway and lung parenchymal T_{RM} populations, as we have found that certain adjuvants, such as CpG when compared to LPS, have a greater effect on the initial systemic effector recruitment and memory establishment when dosed with an equal amount of antigen. Finally, we know, albeit the data is not shown in Chapter 4, that dosing local inflammation with a CD8 peptide does not protect upon heterologous challenge, despite the formation of robust expansion and memory CD8 T cell formation specific for the dosed peptide; however, with the supplementation of the CD4 epitope of the same viral protein, we can generate a protective response. Therefore, it would be important to understand how to best modulate the CD4 T cell help to ensure proper CD8 T cell education and protective memory formation.

Extrapolating the vaccination method described in this dissertation, a future vaccine could employ an adjuvant, which promotes the co-expression of CXCR6 and CD49a and is co-administered with the initial intramuscular attenuated influenza virus injection. This method of simultaneously priming a systemic effector population and licensing them to express lung homing markers would have greater efficacy, on a population basis, by removing the issue of patient compliance (i.e. not relying on the patient to take their adjuvant inhaler seven days post-initial priming), reduce cost of implementation (not assuming for differences in research and development), and improve clinical outcomes for all patients. Evidence that such adjuvants exist was recently presented in a presentation at the American Association of

Immunologists 2015 Annual Meeting where the use of dmLT (double mutant *Escherichia coli* heat labile toxin, LT[R192G/L211A]) induced expression of integrin $\alpha_4\beta_7$, a known gut homing marker, to establish a gut resident memory population (6). Therefore, this approach could be employed to target antigen-specific T cell populations to other peripheral tissues once tissue licensing adjuvants have been identified for each target tissue. Ultimately, employing this strategy would require understanding of the antigen-presenting cell interactions necessary to establish T_{RM} populations.

Implications of T_{RM} Cells on Allogeneic Transplantation

Aside from the potential protection that T_{RM} populations can convey during peripheral tissue infection, perhaps the next most significant point of concern is their interaction during allogeneic transplantation. As more T_{RM} populations are identified, it is becoming readily apparent that these donor T_{RM} populations are being transplanted with the target organ. While the majority of systemic T cells and serum-based antibodies are perfused from the organ during preparation and preservation, T_{RM} populations would not as they reside within the tissue proper. We still do not know how these populations affect host circulating T cell populations or if they may play a role in stimulating a smoldering host response, resulting in chronic graft rejection.

Furthermore, the converse scenario of bone marrow transplantation could confer additional insight into interactions between lymphocyte populations from a new graft and those T_{RM} populations already established in the solid organs of the host.

It would be surprising if these T_{RM} populations were not affected by allogeneic transplantation. Greater understanding of any interactions may enable more efficacious and longer lasting grafts by promoting tolerogenesis and harnessing the protective benefits of the

radioresistant T_{RM} population upon pathogen re-exposure.

Final Perspective: Ten Year Projection

Through my graduate work I have gained both technical and scholarly insight to the greater field of immunology, including the intricacies and interdependencies of the individual mediators to effectively detect and manage pathogenic insults. As is the nature of scientific work, one often leaves with more questions than what one initially sought out to explore. Since its inception with the initial discovery and characterization of a T_{RM} cell subset in the gut (7), other investigators have sought to identify and specifically characterize T_{RM} cell subsets in other peripheral tissues. While there are still areas for initial characterization and observation, the next step for the field is to begin understanding the origins of the T_{RM} populations followed by embracement of this newly expanded knowledge to begin pioneering applications and next generation therapeutics. Below, I include a number of questions and hypothesis which I believe will need to be addressed for the continual success and growth of the T_{RM} cell field over the next ten years.

One of my running hypotheses is that T_{RM} populations can be established in two distinct ways: Acute priming of T_{RM} cell precursors, which can give rise to a proper T_{RM} population following resolution of the acute infection, can occur 1) directly within the tissue and is mediated by local antigen-presenting cells (APCs), quickly giving rise to a T_{RM} population and 2) through priming in the draining lymph node(s), as mediated by APCs draining from the infected tissue. While direct priming within the tissue could quickly give rise to a T_{RM} population due to proximity, the lymphoid generated population ultimately gives rise to the bulk of the T_{RM} population, which, if the generation through this route were to be fully blocked, would result in compromised protection upon challenge.

Perhaps the most significant contribution to the resident memory T cell field that could be made in the near future is to determine the antigen presenting cell subsets responsible for priming the resident memory T cell response. I would hypothesize that the APCs responsible for priming T_{RM} population precursors are not tissue specific but are derived from inflammatory monocytes, which traffic into the tissue, mature into dendritic cells and mediate local antigen presentation. These APCs can then drain to the lymph nodes after accumulating antigen to prime a second round of T_{RM} cell precursors, in accordance with the aforementioned hypothesis addressing the location of T_{RM} cell precursor priming.

Aside from identifying the correlates of protection, a known challenge for generating vaccines is the balance between crippling a pathogen to eliminate pathogenicity while trying to maintain its immunogenicity. The vaccination strategy suggested above and detailed in this dissertation could represent the best of both worlds, at least with respect to influenza virus, where a robust immune response is generated despite the intramuscular viral inoculation resulting in only a single round of infection. A similar strategy could be employed for other infections which result in an abortive viral intramuscular infection due to the cells being susceptible but not permissive to the viral infection.

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