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Identifying Mechanisms of Lung Tissue-Resident Memory CD8 T cell Decay

By

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Identifying Mechanisms of Lung Tissue-Resident Memory CD8 T cell Decay

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B.S., Wake Forest University, 2014

Advisor: Jacob E. Kohlmeier, Ph.D.

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Abstract

Identifying Mechanisms of Lung Tissue-Resident Memory CD8 T cell Decay

By Sarah Louise Hayward

Tissue-resident memory (T_{RM}) CD8 T cells are critical for cellular protection against pathogens at barrier tissues, where they can rapidly recognize and respond to invading pathogens due to their localization at the site of pathogen entry. In the lung, we find that protection against heterologous influenza virus infection wanes over time. This waning suggests a reduction of memory CD8 T cells. T_{RM} are known to be long-lived within the tissue, but we find this not the case in the lung. We find a loss of airway and interstitium T_{RM}, two distinct lung resident subsets, occurs gradually over 6-8 months. This loss is not due to loss of specific phenotypic subsets, nor due to established T_{RM} leaving the lung tissue as assessed using parabiosis experiments. We observed that airway and interstitium T_{RM} are prone to apoptosis in the tissue. Using paired RNA and ATAC-seq, we find that flu-specific airway and interstitium T_{RM} have distinct transcriptional and epigenetic profiles. The profile of airway T_{RM} revealed activation of the amino acid stress response pathway. Restoration of nutrients to airway T_{RM} revealed this pathway is reversible. Thus, the amino acid stress pathway plays a role in altering airway T_{RM} survival, leading to gradual cell death under homeostatic conditions and possibly preventing subsequent damage that could occur through non-specific bystander activation.

Most tissue-resident T cell memory is examined in the context of a single infection and under homeostatic conditions. We examined how multiple infections impact pre-existing lung T_{RM}. We found that parainfluenza virus infection induces a loss of influenza-specific cell-mediated protection against heterologous influenza challenge by driving the loss of pre-existing T_{RM}. This loss of T_{RM} was driven by local infection, but not by localized sterile inflammation or by competition for lung niches. Local infection in the lung drove release of significant extracellular ATP (eATP), known to induce cell death through the purinergic receptor P2RX7. Quenching of eATP using apyrase, which hydrolyzes ATP, prevented loss of T_{RM} from the lung. Together, these data suggest that each subsequent lung infection reduces pre-existing cellular immunity by depleting lung T_{RM}. Both the loss of airway resident T_{RM} due to amino acid starvation, and the selective loss of non-specific T_{RM} due to local infection, help define unique mechanisms of loss in lung T_{RM} populations.

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Chapter I: Introduction

There is an urgent need to design vaccines that induce durable cross-protective immunity against influenza A viruses. Despite an annual vaccination program, up to 20% of the population in the United States becomes infected in some years, and influenza viral infection leads to 36,000 deaths and over 200,0000 hospitalizations annually[1, 2]. Recent data suggests that global deaths due to seasonal influenza infections are between 290,000 and 650,000 annually. These deaths include secondary complications, and demonstrate an enormous global burden of influenza disease and a need to address it.[3] Every year a seasonal vaccine is produced to combat the burden of disease, and these vaccines target B cell responses to the HA and NA proteins of the virus in order to generate neutralizing antibodies that prevent viral infection[4, 5]. However, these viral proteins are subject to high mutation rates, leading to the inability of the vaccine to properly protect as the seasonal strains change, thus allowing evasion of preexisting humoral immunity by the virus[6]. Furthermore, our ability to predict the predominant influenza strains is not always accurate, resulting in a vaccine that is poorly matched against one or more circulating strains[6, 7]. Finally, influenza reassortants that harbor novel HA and NA proteins can arise and enter circulation, resulting in potential pandemics due to a lack of preexisting antibody responses in the human population[8]. Thus, it is important to explore additional strategies that can limit influenza infection and transmission, thereby reducing influenza-associated morbidity and mortality.

Humoral and cellular immunity to respiratory pathogens:

Respiratory pathogens and influenza virus structure:

Respiratory infections such influenza and Sendai virus, a murine parainfluenza type 1 virus, are restricted in their sites of infection. They bind to cells which express trypsin-like enzymes to cleave surface proteins. In influenza's case hemagglutinin must be cleaved to cause a productive infection[9]. In Sendai virus, the fusion glycoprotein requires cleaving for infectivity, a process that depends on proteases which are only present in certain cell types primarily in the lungs of mice[10]. Tissue restriction is further determined by binding of influenza and parainfluenza viruses to specific receptors on permissive cell types. In the case of influenza A, sialyloligosaccharides are implicated as these receptors. In human strains of influenza A, viruses preferentially infection host cells that express receptors which have SAα2,6Gal sequences[11, 12]. These restrictions limit the infection of both influenza and Sendai virus to the respiratory tract[13]. Influenza virus is cleared within two weeks and is not considered to be a chronic infection[8]. Despite the fact that the infection is localized, a broad adaptive immune response is generated against the infection. Beyond the initial innate response to the virus, B and T cells are crucial to the ability of the adaptive response to control the infection and can form long-lived memory responses to aid against reinfection. B cells form antibody responses against the rapidly mutating outer proteins of the viral capsid. T cells respond to epitopes from both the outer proteins and the conserved internal proteins of the respiratory virus. These arms of the adaptive immune system work in combination to control viral replication, limit immunopathology and prevent reinfection.

Influenza virus is a single stranded negative sense- RNA virus which has eight segments encoding 16 proteins[9, 14]. Its outer capsid contains the haemagglutinin (HA) and the neuraminidase (NA) proteins, and these proteins are the primary targets for antibody production. The HA segment codes for the receptor binding glycoprotein HA. The HA of influenza virus binds to sialic acid on epithelial cells within the respiratory tract and mediates membrane fusion. The NA segment

encode the NA protein. The NA cleaves sialic acid residues on the surface of the epithelial cell allowing the budding of newly formed virions following replication. The nonstructural segment of the influenza virus contains the NS1 and NS2 segments. The NS1 segment is a host antiviral antagonist. NS2 encodes a nuclear export protein. The matrix segment encodes three proteins: M1 the matrix protein, the ion channel protein M2, and the protein M42[14]. The polymerase segment codes for 3 proteins, PB1, PB2, and polymerase A (PA). The nucleoprotein (NP) segment encodes the nucleoproteins which maintain the RNA genome structure as single stranded. The influenza virus structure and life cycle are complex; thus the immune system has developed a multiple step approach to respond to the virus[15].

B cell responses and influenza reassortment:

B cells form antibodies to the HA and NA proteins of the virus. These proteins are highly variable between seasonal influenza stains, as both antigenic drift and reassortments can lead to nonsynonymous mutations in these structures rendering previously formed antibodies of little use. Antigenic drift occurs when mutations that accumulate during viral replication due to the error-prone nature of the polymerase are selected based on an increase in viral fitness. Commonly drift mutations are made within the head region of the HA. These mutations can change the binding site recognized by HA antibodies created during a previous infection with strains from a prior influenza season[16]. This changing in epitopes recognized anti-HA antibodies over time, due to multiple mutations, eventually prevents the activity of antibodies and renders them unable to neutralize the virus. This is a means of escape and allows the individual to be re-infected in successive influenza seasons.

Antigenic reassortment or shift occurs when two different influenza viruses infect the same host cell and there is a mixing of the viral genomes. When this occurs, it can lead to creation of new influenza virus strain, with HA and NA combinations different from previously circulating strains. While antigenic reassortment is less common than drift, the sudden change can lead to pandemic strains, which cannot be controlled by previously formed antibodies. In addition, influenza vaccines currently are aimed at primarily generating antibody responses, thus when an antigenic shift occurs this leaves a large percentage of the population unprotected from the pandemic influenza strain[17, 18, 19].

2009, a new pandemic:

In 2009, a new pandemic H1N1 arose from porcine hosts. This was not a pure shift, however. The pandemic 2009 H1N1 (pH1N1) arose out of H1N1 viruses which were independently circulating in North American (a swine-lineage strain) and Eurasian (swine-origin) swine strains[20]. These viruses were combined in a host and adapted to infect and transmit between humans, forming a pandemic strain due to genetic drift occurring within the swine hosts. The H1N1 influenza virus had been previously seen in 1918 with the Spanish influenza. The North American swine lineage of the pH1N1 descended from 1918 H1N1 which began circulating in swine following the 1918-1919 influenza season. The Eurasian swine strain derives from an avian lineage of influenza which went directly into swine. Through multiple reassortments, the 2009 pandemic influenza is a hybrid of viruses with the HA and NA originally derived from1918 except they are dramatically different due to drift in the sequences and resulting proteins[21]. any antibodies formed to the previous 1918 were rendered ineffective due to the drifting and reassorting of the pH1N1[22]as despite the “similar” HA and NA epitopes are no longer shared.

Overall:

There is a complex adaptive immune response to respiratory pathogens, specifically in the case of influenza virus. Respiratory viruses are limited in their host range and tissue tropism due to the limited number of cell types that can support productive infection. B and T cells respond to different aspects of the physical nature of the virus. The influenza's complex structure means that antibody responses are formed to HA and NA extruding from the surface of the capsid and T cell responses are primarily directed against the NP, PA, M1, and PB1 proteins found within the capsid. There is very little evidence for selective pressure on influenza viruses due to T cells, resulting in conserved T cell epitopes across different influenza strains, enabling virus-specific T cells to provide cross-protection against many strains of influenza virus[23]. The cellular immune response to influenza and mediate pathogen clearance while limiting immunopathology is complex and centers on an intricate balance between co-stimulatory and co-inhibitory signals. This complicated relationship indicates the importance of understanding T cell-mediated control of viral infections.

T cell responses to influenza:

Experimental model:

In the event of a pandemic strain event, CD8+ and CD4+ T cells are critical mediators of the immune response to influenza and other respiratory pathogens. Immunodominant CD8+ and CD4+ T cell responses are directed against the NP, PA, PB1 and M proteins of the influenza virus in mice and humans[23, 24, 25]. These proteins are typically more conserved than HA and NA, as there is less selective pressure due to their location inside the virion. Due to the conserved nature of these proteins, T cells offer the opportunity to provide cross-protection between strains of

influenza. To examine cross protection in the laboratory setting, two viruses are typically used, Influenza A/PR8 and Influenza A/HKx31. PR8 is a mouse adapted H1N1 strain, and x31 is a lab adapted H3N2 virus[26]. Since as discussed before, the HA and NA protein are different, but the internal gene segments are identical between these viruses. There is no protection mediated by antibodies when the viruses are used in succession. A typical examination of a memory T cell response will involve infecting mice, C57Bl/6, with a sublethal dose of virus, typically x31. Then, once T cell memory has developed after 35 or more days, the mouse will be challenged with a lethal dose of PR8. Due to the protection provided by cross-reactive memory T cells generated during the initial x31 infection, the mice survive the lethal PR8 challenge.

Control of T cell activation during influenza infection:

Control of T cell activation during a primary influenza infection is a complex process involving the lung mucosa, infected epithelial cells and the recruitment of innate and adaptive immune cells to the site of infection. The lung mucosa is made up of a variable mixture of non-hematopoietic structural cells and hematopoietic immune cells. This milieu of cells includes both non-ciliated and ciliated lining cells, such as cuboidal and columnar epithelium, and alveolar epithelial cells, both type I and type II[27, 28]. These cells are targeted by influenza virus due to the sialic acid residues on their surface, which are bound by the HA of the virus and used to mediate fusion with the host cell[9]. The infection of these cells leads to production of type I interferons, and many inflammatory cytokines and chemokines, due to innate recognition of the invading pathogen by signaling pathways such as toll-like receptor pathways, Nod-like receptors and RIG signaling pathways. This production of inflammatory mediators induces the recruitment of the innate and

adaptive immune cells to the infected tissues. In addition, these cytokines and chemokine are capable of modulating the function and activation status of responding immune cells[27, 28, 29, 30].

The respiratory tract, where the infected epithelial cells are supporting productive viral replication, is where the initiation of the adaptive T cell responses begins. Viral antigen is captured by resident respiratory dendritic cells (DCs) that reside in close proximity to the airway epithelium, and within the parenchyma (interstitium) and the pleura of viscra[31]. This capture of antigen and viral particles by DCs leads to their activation, which in turn leads to their up regulation of CCR7 allowing them to respond to the gradient of CCL21 and migrate into the lymphatic system[32, 33]. Respiratory DCs migrate into the draining lymph node (mediastinal)[34, 35]. Respiratory DCs in mice can be divided into distinct subsets which vary in phenotype, distribution within the lung compartment, and function. Conventional DCs can be categorized into 3 different subsets found in the respiratory tract; CD103+CD11b+ DCs, CD103-CD11b^{hi} DCs and monocyte derived DCs. CD103+ DCs are typically found within the intraepithelial layer in the respiratory tract and the CD11b^{hi} DCs are found within the submucosa interstitial area of the lung. These distinct locations allow monitoring of the differential local environments[32, 36]. Another subset of DCs which make up respiratory DCs are the plasmacytoid DCs (pDCs). This subset is key for producing large quantities of type I interferons and inducing a highly inflammatory environment following their recognition of influenza due to infection via pathogen-associated molecular patterns (PAMPS)[37, 38]. Most antigen captured by DCs involved in the response to influenza is likely due to the direct infection of DCs by the virus, but DCs can also acquire influenza antigens by the phagocytosis of virions and dying infected cells within the respiratory tract[38].

Following the migration of DCs to the draining lymph node, DCs present antigen to naive influenza-specific CD8+ T cells. This is primarily carried out by CD103+ DCs due to their

enhanced ability to migrate from the tissue to the lymph node. This was shown by the depletion of langerin-expressing CD103+ DCs before an influenza infection. This depletion of CD103+ DCs resulted in a decreased cytotoxic T lymphocyte (CD8 T cell) response, which in turn reduced viral clearance[39]. After this initial wave of CD8 T cell activation by the migrant CD103+DCs, influenza antigen is continuously presented by CD11b^{hi} DCs in the draining lymph node throughout the remaining course of infection. Additionally, pDCs have been implicated in capturing and presenting antigen to CD8 T cells, but it was shown in a sublethal influenza infection model that pDCs are unable to induce CD8 T cell responses by themselves[40].

Other cell types regulating T cell activation:

In addition to dendritic cells, many other cell types play a role in regulating T cell responses during influenza infection. CD45+ myeloid cells play a crucial role in this response. Myeloid cells include inflammatory granulocytes and mononuclear cells which infiltrate the lung tissue and airways in response to localized inflammation during infection[41, 42]. Neutrophils are one of the primary cell types infiltrating the lung following severe influenza infection. Neutrophils, while crucial to clearing infected cells and debris, can induce lung injury through production of reactive oxygen species (ROS) species and pro-inflammatory cytokines[43]. In these contexts, their involvement in influenza infection can be both helpful and harmful. In the context of a severe influenza infection, neutrophils are shown to be highly beneficial, but in the context of a mild infection neutrophils are shown to induce unnecessary tissue damage[44]. Inflammatory monocytes are another myeloid subset which produce ROS, TRAIL (TNF-related apoptosis-inducing ligand), iNOS (inducible nitric oxide synthase) and also pro-inflammatory cytokines. Inflammatory CCR2+ mononuclear cells have been shown to play a role in regulation of effector T cells. They

have been shown to promote growth and survival of virus specific CD8+ T cells and to modulate the cytokine production by these CD8 T cells in acutely infected lungs[42, 45, 46, 47].

CD8+ T cell effector mechanisms:

CD8+ effector T cells have various ways to control influenza infection. Conventionally, CD8 T cells kill influenza infected cells via Fas ligand/Fas mediated apoptosis or by perforin/granzyme-dependent granule exocytosis[48]. These methods destroy the infected host cell, and in the process can release additional DAMPs and PAMPs that further activate the innate immune system. In addition, effectors CD8 T cell produce the pro-inflammatory cytokines of interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). These cytokines further contribute to the recruitment and activation of innate inflammatory cells, including inflammatory DCs and pDCs. This activation by T effectors contributes to the ever-increasing storm of immune activation in the lung. As the infection progresses, effector CD8 T cells continue to interact with respiratory DCs, expanding and differentiating the virus-specific effector CD8 T cell pool to enable optimal antiviral effector activity and promote viral clearance. If they only interact with respiratory epithelial cells they are only able to lyse the infected target cell[45]. This is an interesting mechanism of control that serves to prevent excessive release of potentially destructive pro-inflammatory cytokines, thus limiting damage to the surrounding tissue and preventing deleterious immunopathology. An additional method of control with regard to the release of pro-inflammatory cytokines are the interactions of co-stimulatory receptors and their ligands. CD28 on effector CD8 T cells interacts with CD80 and CD86 which are displayed on inflammatory $CD11c^{hi}$ DCs[45]. There are also various mechanisms that regulate the cytolytic capabilities of CD8 T cells which together prevent unnecessary immunopathology.

Immunopathology as a result of immune activation:

While effector CD8 T cells are important in clearing influenza infection, an unintended consequence of eliminating infected host cells is the resulting lung injury, which in itself causes a significant health risk by increasing the susceptibility to secondary bacterial and viral pneumonias. The classic methods of cell lysis by CD8 T cell through FasL/Fas and granzyme/perforin mechanisms leads to massive cell death during infection[48]. Effector T cells, through their interactions with antigen-bearing cells inflammatory DCs in the lung, contribute huge quantities of pro-inflammatory chemokine and cytokines. These include IFN-γ, TNF-α, IL-17, and macrophage inflammatory protein-1α. These contribute to the damage and inflammation which is incurred during an acute influenza infection. While necessary for inducing an localized antiviral state and promoting apoptosis of infected cells, the problem with pro-inflammatory cytokines is that they are indiscriminate[49]. They target many cells, whether infected or not, and can induce non-specific damage to the respiratory tract[50]. In addition, T effector cells induce lung injury through the release of cytolytic granules and pro-inflammatory cytokines. The potential contribution of this damage on tissue resident memory T cells will be further explored below. Further studies, as will be discussed later in relation to tissue resident memory, suggest that this balance between immunopathology and cell-mediated protection may in part be tightly regulated by the activation of danger receptors on CD8 T cells, and the interactions between danger receptor signaling and recognition of specific antigen by CD8 T cells in the lung plays an important role in maintaining this balance.

Limiting immunopathology:

Effector T cells are also capable of limiting immunopathology. Effector T cells in the lung environment express several co-inhibitory molecules known to limit cellular proliferation and cytokine production. Effector CD8 T cells which infiltrate into the lung highly express an inhibitory receptor called NKG2A, which can bind to a on classical MHC class 1b molecule. Engagement of this pathways reduces TNF-alpha dependent inflammation, and blocking NKG2A interactions increases inflammation [51]. Additionally, classic inhibitory molecules such as Lag-3, PD-1, and Tim-3 are expressed on effector T cells during acute influenza infection and on memory T cells established following viral clearance[52].

The production of IL-10 by effector CD8 T cells is another major mechanism of immune suppression designed to limit immune pathology. IL-10 inhibits pulmonary inflammation during influenza infection and thusly controls the amount of effector T cell infiltration into the lung. Without IL-10 production by CD8 T cells, influenza infection results in lethal pulmonary inflammation and injury. However, mice which have the *Il10* gene deleted are more resistant to influenza infection as a result of elevated Th17 and antibody responses[53, 54, 55]. It has also been shown that Il-10 production by effector T cells is restricted to areas of the lungs which are actively infected with influenza. This suggests tightly regulated control over *Il10* transcription in T cells that limits IL-10 production to an inflamed environment where influenza antigens are present[56].

Another crucial cytokine in the anti-inflammatory milieu is transforming growth factor (TGF)-β. Blockade of this cytokine results in lethality, lung destruction, and excessive inflammation in the context of influenza infection[57]. Alternatively, TGF- β1 is also known to be a pro-viral factor and acts to suppress the immune system long enough for viral replication[58]. It is not known exactly which cells produce TGF-β during an influenza infection but it is thought that T regulatory cells (T_{reg}) and pulmonary epithelial cells are important sources [59, 60]. T_{reg} are an

important mediator of immune suppression and healing. T_{reg}, T regulatory cells are a subset of CD4+ T cells that mediates and limits immune responses due to self-antigen and infection through a variety of mechanisms, including TGF-β [61]. Depletion of T_{reg} induced increased CD8+ T cell responses during influenza infection and required a transfer of exogenous T_{reg} to limit immune pathology and suppress excess immune activation[62, 63]. The production of TGF-β has also been shown to be important in the formation and maintenance of memory CD8 T cells following infection, and this aspect will be discussed further below. Another mediator of immune suppression and bringing the immune response within the lung environment back to homeostasis following influenza infection, is a molecule expressed by the respiratory epithelium itself. CD200 is expressed by epithelial cells in the reparatory tract and engages CD200R on lung macrophages. This interaction suppresses macrophage injury potential, specifically inhibiting the ability of macrophages to induce tissue injury through the release of reactive oxygen species intermediates, nitric oxide, proinflammatory cytokines, and hydrolytic enzymes[64]. In addition, CD200 is also expressed by effector T cells, providing another method for T cells to directly control immunopathology through their interaction with myeloid lineage cells[65, 66].

Therapies limiting the pathology of influenza:

Many therapies are focused on targeting the release of pro inflammatory cytokines by effector T cells as strategies to limit immune pathology while maintaining viral clearance. Two pathways that are currently the focus of research are OX-40 and ICOS. OX-40 is a co-stimulatory receptor which is found T cells and has been shown to promote T cell survival and proliferation. In influenza infection, a blockade of OX-40 is able reduce T cell infiltration, proliferation and the associated pro-inflammatory cytokine production. This leads to reduced weight loss and

pulmonary injury but still allows for efficient viral clearance[61, 67]. In contrast, blockade of the ICOS co-stimulatory receptor on T cells reduces T cell proliferation, infiltration and cytokine production but does not lessen pulmonary inflammation[61]. This is due to the fact that ICOS blockade seems to significantly enhance viral persistence within the lung tissue, leading to prolonged pro-inflammatory [61, 68]. While these studies hold great promise for lessening lung pathology by limiting infiltration of influenza-specific T cells in the lung, the effect of these approaches on the development of memory CD8 T cells is unknown. How resident memory CD8 T cells are formed within the lung is still unclear, and the impact of these therapies on the development of T cell memory must be further explored.

Formation of memory CD8+ T cells:

Following resolution of acute infection, a population of influenza-specific T cells forms the memory response. This memory response is critical to providing cell-mediated immunity. During an acute infection, virus-specific T cells can expand 10^4- to 10^5-fold, and they can divide every 6 to 8 hours[69, 70]. Following the peak of CD8 T cell expansion and the resolution of infection, the majority of virus-specific CD8 T cells, about 90-95%, die by apoptosis within three weeks[70, 71, 72]. The remaining cells will undergo transcriptional changes to transition to long-lived memory CD8 T cells. [73, 74, 75]. It is a gradual developmental program that occurs as a CD8 T cell transitions from an effector T cell to a memory CD8+ T cells. There are three main changes that occur. First the ability to produce the cytokine IL-2 increases. Effector T cells produce only modest amounts of IL-2, and one of the key steps in the differentiation process is the ability to increase IL-2 production[76, 77]. The second change which occurs is an increase in the proliferative potential of the cell in response to antigen or homeostatic signaling. While terminally differentiated effector T

cells are unable to proliferate, it is key for a memory T cell to be able to replicate in the event it reencounters its specific antigen. It is also important that memory CD8 T cells be able to undergo homeostatic turnover in response to both IL-7 and IL-15, and that these abilities can only be acquired by fully differentiated memory CD8 T cells[75, 77]. Additionally, some effector CD8 T cells fail to reacquire the ability to home to the lymph nodes via high endothelial venules, and preferentially locate to non-lymphoid tissues as they remain CD62^{Lo} and CCR7^{Lo}. Eventually very long-lived memory CD8 T cells can convert and reacquire CD62L and CCR7. In murine models of influenza infection, this process can take between 90-360 days[77]. One of the most important features of memory CD8+ T cell is that they maintain the ability to rapidly reactivate antiviral effector functions such as cytokine production following stimulation with their cognate antigen[76]. Thus, memory CD8 T cells are one of the first lines of defense against reinfection.

Importance of T cell mediated Cross protection:

Recently the importance of T cell mediated protection was tested due to the 2009 pandemic H1N1, previously discussed within this chapter. In a study examining the role for T cell protection during influenza infection, mice were infected with viruses distantly related to the pH1N1 Ca 2009 influenza strain, including PR8 (H1N1), x31(H3N2), and New Caledonia (H1N1). These viruses were distant enough that sera isolated from mice infected with these viruses provided no protection when a mouse was infected with the 2009 pH1N1. This meant that antibody responses alone would not protect a mouse from the 2009 pH1N1 strain. In order to examine the T cell response, mice were initially infected with one of the three previously stated viruses. The mice were then rested to allow formation of influenza specific memory CD8+ T cells. Then, mice were challenged with the 2009 pH1N1strain and weight loss, death, and viral titers were measured in the subjects. All

mice which were previously infected with one of the distantly related virus strains had reduced viral titers, weight loss, and mortality compared with unprimed mice[78]. Given that sera containing antibodies to these viruses was of no protection against the 2009 pH1N1 strain, it is clear that T cell-mediated immunity was critical to cross-protection against seasonal strains, and also very important in protection against possible pandemic strains. These experiments were a valuable addition to the initial experiments from Walter Gerhard's laboratory examining the heterologous influenza protection provided by effector CD8 T cells, and the subsequent decline in heterologous immunity over time, which is the focus of this dissertation[79].

Resident memory:

Subtypes of memory T cells:

Memory T cells have traditionally been divided into central memory (T_{CM}) T cells that reside in lymphoid tissues and effector memory (T_{EM}) T cells that recirculate through peripheral tissues[80]. T_{CM} cells express CCR7 and CD62L in addition to CD127 and CD44. T_{CM} remaining in the lymphoid tissue are capable of proliferating extensively in response to antigen, in contrast to the T_{EM} subset which are less proliferative and produce interferon (IFN)-γ in contrast to interleukin(IL)-2[81]. T_{EM} subsets are more heterogeneous than T_{CM} cells. Recently, a population of non-circulating memory T cells localized permanently to the peripheral tissues, termed resident memory (T_{RM}) T cells, has been identified at sites such as the gut, skin, lung, reproductive tract, and brain[82, 83]. These cells are shown to express CD44, are CD62L and CCR7 low (like T_{EM}), CD11a high, and can express both CD69 and CD103[84].

Transcriptional profile of tissue resident CD8+ T cells:

T_{RM} cells have been shown to have a unique transcriptional and phenotypic profile. They express various unique retention markers depending on the tissue in which they reside and their spatial arrangement within the tissue. CD69 and CD103 expression prevent egress from the tissue. CD69 is an antagonist of S1P1R, which mediates migration from the tissue in response to S1P gradients in the blood. CD103, whose gene name is ITGAE, is an integrin which binds to E-cadherin, CDH1, expressed on the surface of epithelial cells. This combination of CD69, preventing tissue egress, and CD103, binding these cells to the lung epithelium, provides the mechanisms to enable these memory CD8 T cells to remain within the tissue[85]. The expression of these proteins is governed by overarching transcriptional networks. Examining the network governing CD69 expression: the transcriptional regulation of *klf2* and *s1pr1*, which encodes S1p1, have been implicated in the establishment and maintenance of tissue resident CD8 T cells. Specifically, KLF2 is a transcription factor that promotes expression of S1p1, and *klf2* and *s1pr1* genes are found to be down regulated in CD8 T_{RM} cells[86]. TGF-β is a cytokine that has been implicated in the establishment of T_{RM} via the induction of CD103 expression and down regulation of KLF2. In addition, continued TGF-β signaling may be important for the long-term maintenance of CD8 T_{RM}[59, 87]. Finally, it has recently been shown that *zfp683*, which encodes the transcription factor Hobit, is a master regulator of the T_{RM} transcriptional program[88].

Another set of transcription factors crucial to understanding the transcriptional regulation of tissue resident CD8 T cells are the T-box transcription factors. These transcription factors are Eomesodermin (Eomes) and a related homolog called T-bet. These two transcription factors combine to control the formation of CD8+ CD103+ tissue resident memory T cells. There is a coordinated down-regulation of Eomes in conjunction with TGF-β cytokine signaling. This TGF-

β signaling resulted in reciprocal down-regulation of the T-box transcription factors. There are subtle differences in the regulation of these two transcription factors during T_{RM} development, as while the complete loss of Eomes signaling was necessary for CD8+ CD103+ tissue resident memory T cell development, T-bet was required to be maintained at a low level in order to allow for the cell surface expression of interleukin-15 receptor beta chain to remain expressed. Thus, modest expression of T-bet was necessary for tissue resident T cells to maintain responsiveness to homeostatic cytokines. The reciprocal nature of the relationship between T-box transcription factors and the control of T_{RM} development by the TGF-β and IL-15 cytokines demonstrates the complexity of understanding the formation and maintenance of tissue resident memory T cells[89].

Tissue Resident T cells as sentinels of the immune system:

Tissue resident CD8 T cells have been shown to play a critical role in protective immunity as sentinels of the immune system at sites of pathogen entry[90, 91, 92]. For example, in the mouse female reproductive tract, tissue-resident CD8 T cells rapidly induce strong local chemokine expression when they interact with their cognate antigen, leading to the rapid accumulation of circulating immune cells in the infected tissue[93]. Within the lung these populations have been shown to be highly protective against influenza virus and other pulmonary virus infections. They are critical to providing cross-protection against heterologous influenza infections via their CTL activity[94]. Additionally, within the lung they have been shown to be heterogeneous in phenotype, proliferative, and multifunctional producing several different cytokines[95].

Within the small intestine, extensive work has been done to parse out tissue resident memory T cells and their role in protection. This work has led to the idea that within the small intestine there are two populations of resident T cells which are distinct and play different roles.

In a model of *Yersinia pseudotuberculosis* (Yptb)*,* work from Michael Bevan's group has shown that the inflammatory microenvironment of the gut leads to both CD103- and CD103+ T_{RM} which are developmentally and spatially distinct. Mice were initially infected using a Yptb expressing both OVA and YopE, a specific antigen in Yptb infection. They found that CD8 T cells in the intestine underwent a typical phenotypic transition into memory cells expressing CD69, CD103 and granzyme B expression. In the intestinal epithelium compartment the CD103+ cells quickly became stable, but in the lamina propria a heterogeneous population of cells was observed. These were both CD103 positive and negative. They found the CD103+CD8+ tissue resident cells to be TGF-beta dependent and scattered throughout the lamina propria. In contrast, they found the CD103- tissue resident cells to be independent of TGF-beta, clustered around CD4+ T cells and CX3CR1+ macrophage and/or DCs. The close proximity to APCs suggested to them that these CD103- tissue resident cells served more of an alarm function[91]. While this study was interesting and does suggest that tissue resident CD8 T cells could function as sentinels in different ways. As will be discussed later, the problem with using a depleting antibody to define tissue residency is that peripheral tissues, and thus the T cells within them, by definition have little access to the circulation. This is a problem because at any one time the T cell population within a tissue is comprised of both permanently-lodged T_{RM} and transiting T_{EM} that are surveilling the tissue. How is it known if the CD103- population is not this transient T_{EM} population? There are still many questions regarding what is a true resident CD8 T cell.

Sterilizing immunity:

Most importantly, T_{RM} in peripheral tissues have been shown to provide nearly sterilizing immunity when present in sufficient numbers, likely through their ability to rapidly respond to the

invading pathogen before it can replicate to sufficient numbers[83, 90, 91, 93, 94]. In a parabiosis system in which a vaccinia-immune mouse is sewn to a naïve partner mouse, circulating T cells are free to move between the mice. In this case, it was shown that truly resident T cells are formed in the skin of the previously infected partner and are unable to move into the skin of the naïve partner. These vaccinia-specific tissue resident T cells are shown to be potent effector T cells capable of producing high levels of IFN-γ upon antigen re-encounter. It is also possible to continually boost this population of vaccinia-specific tissue resident T cells by repeated infections, and these T_{RM} are highly protective when the mice are challenged[83].

In another model of skin infection using herpes simplex virus (HSV), it is found that HSV-specific tissue resident memory CD8 T cells preferentially form on the ipsilateral flank relative to the infection site. This accumulation on the same side as infection leads to 100-fold greater control of infection upon challenge on the ipsilateral flank versus the contralateral flank in B cell deficient mice. B cell deficient mice are used to control for antibody mediated protection, allowing for the investigation of protection on the basis of T cell responses[83].

In the case of the lung and respiratory infections, it has been shown that tissue resident memory CD8 T cells are critical to protection against heterologous influenza infections. Linda Cauley's group showed in a heterosubtypic reinfection model, using WSN-OVA and X31-OVA (H1N1 primary, challenged with an H3N2 virus), that viral clearance after reinfection is very closely linked to the number of antigen-specific tissue resident CD8 T cells in the lung. They also show that new effector T cells, developed as a result of the heterologous virus, play very little role in protection. This is shown through BrdU labeling of proliferating cells, as BrdU is not incorporated by the new effector T cells before virus is cleared[94]. Our laboratory has shown that the transfer of virus-specific CD8 T cell directly into the airways of naïve mice is sufficient to

confer protection from respiratory virus challenge in an antigen-dependent manner[96]. This manner of protection is dependent on the production of IFN-γ, as transfer IFN-γ deficient cells into the airways failed to control virus replication as efficiently as WT cells[96]. Interestingly, it was revealed recently that the expression of an interferon induced transmembrane protein, IFITM3, by lung T_{RM} cells aids in survival of T_{RM} following secondary or challenge infections. This protein expression, conferring broad resistance to many viral infections, was selective and promoted survival and cellular protection following influenza challenge[97].

Key methods:

One of the key developments in the study of tissue resident memory T cells was the development of intravascular staining. In many of the studies referenced in this dissertation, and in much of our own work, intravascular staining is the gold standard for defining tissue resident T cells. In the initial studies describing this approach from David Masopust's laboratory, mice are injected with 3ug of anti-CD8 in APC or PE[98]. This has been further developed to include the possibility of staining with CD3e or a pan-CD45 marker in many other fluorophores. After 3 to 5 minutes, blood was taken from the mice to confirm sufficient labeling and the mice are euthanized. Masopust's group went as far as to perfuse the mice with PBS following sacrifice to ensure that antibody was removed from the system. Tissue were processed and again rinsed free of antibody. Interestingly, donor naive T cells staining by the intravenous (i.v.) antibody varied according to the permissiveness of the distinct tissue, with this permissiveness being dictated by the vascularity of the tissue. In a successful intravital labeling experiment, all of the donor cells within the blood were stained with the i.v. antibody, some cells within the spleen with the antibody, and almost no

cells within the lymph node were stained. Within the spleen, labeled cells were seen in the red pulp, whereas the unlabeled cells were localized to the white pulp. Within the lungs, labeled CD8 T cells were found to be closely associated with pulmonary capillary beds. Using these methods Masopust was able to distinguish that some cells protected from i.v. labeling were in fact CD103 and CD69 positive, and that protected lung cells down regulated the integrin CD11a[98]. Some example staining is shown here in Figure 1 distinguishing resident from systemic T cells in the lung. The development of this technique transformed the field of tissue residency as it was an efficient approach for which cells were in the tissue versus the blood stream. However, this method does have its caveats. At any one time there are transient T_{EM} cells moving through a tissue. These cells will momentarily not have access to the vasculature, and thus could be falsely labeled as resident memory T cells. Other methods to determining tissue residency include using depletion antibodies, as a cell outside the vasculature should be protected from the depleting antibody. Unfortunately, this has some of the same flaws as i.v. labeling, plus depletion antibodies can vary in their efficacy depending on the tissue.

Key points regarding Tissue Resident memory T cells:

Tissue resident memory CD8 T cells are shown to be highly protective in the peripheral tissues of the intestinal tract, the female reproductive tract, the skin, and the lung. They have been shown to be sentinels using chemokine gradients and cytokine release to pull innate lymphocytes and leukocytes to the site of a reinfection. They have been shown to be protective against multiple strains of influenza. The genetic profile of tissue resident CD8+ T cells is still currently being parsed apart, and although the transcriptional regulation of T_{RM} development and maintenance is

a developing field, it is clear that T_{RM} are a distinct T cell subset with a unique genetic program. T_{RM} in different peripheral tissues have been shown to provide robust protection against local reinfection, and importantly in the context of influenza infection these cells are critical to providing cell-mediated protection in the event of a pandemic strain or influenza vaccine/seasonal strain mismatch.

Loss of cellular immunity over time:

A note on the unique formation of tissue resident memory in the lung:

Unlike in other peripheral tissues, we have found tissue resident memory in the lung requires that effector T cells encounter antigen at the site of infection within the lung to promote T_{RM} formation. Our laboratory and others have shown that intranasal delivery of peptide plus an inflammatory stimulus such as CpG is necessary and sufficient for forming protective T_{RM} populations.[96] This is in contrast to other tissues such as the skin and genital tract, where recruitment of effector T cells into the tissue is sufficient for the development of T_{RM}, even in the absence of local antigen.

Importance of tissue resident memory:

Following clearance of a primary influenza infection, memory T cells are found in secondary lymphoid organs, such as the spleen, mediastinal lymph node (MLN), and bone marrow, as well as peripheral tissues, such as the lung parenchyma (interstitium) and lung airways[99]. 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[94, 100, 101]. Also, recent studies in humans showed that increased numbers of circulating cross-reactive memory T cells correlated with significant decreases in viral loads and lower disease burden following heterosubtypic influenza challenge[102, 103, 104].

Waning of influenza specific memory over time:

However, the efficacy of heterologous cellular immunity against influenza viruses has been shown to wane over time. Studies in the mouse model have demonstrated that the number of virus-specific memory CD8 T cells present in the lung following respiratory virus clearance progressively declines over several months post-clearance, before stabilizing at a low number for the life of the animal[84, 105, 106, 107]. In contrast, the number of systemic virus-specific memory CD8 T cells remains relatively constant.

Gerhard's group showed that over time heterologous immunity to influenza virus also waned. They showed this through a series of experiments in which they infected mice with PR8 and challenged these mice with x31 at various times after initial PR8 infection (days 0, 3, 7, 10, 14, 21, etc). Next, they determined influenza viral titers in both the lung and upper respiratory tract. They compared these viral titers to previously uninfected mice which were challenged with the heterologous x31 virus. They observed that as time progressed further away from the initial PR8 infection, the ability of the previously vaccinated mice to control viral loads decreased. Once sufficient time had elapsed, the previously vaccinated mice showed similar viral titers to the unvaccinated control mice. This suggested that T cell memory to the reparatory pathogen was lost over time[79]. These data correlated with data I have produced as part of my dissertation project. In Figure 2 (derived from data in chapter II), mice were previously infected with x31, an H3N2 influenza virus, and then rested for 1 month or 6 months prior to challenge with a $10LD_{50}$ PR8 (10

times the lethal dose that result in the death of 50% of the mice). There was a significant loss of life for mice that were 6 months from initial infection compared to mice 1 month from initial infection. Figure 1A shows dramatic weight loss for these late memory mice, and in fact all mice were sacrificed by day 7 due to falling below the weight loss limits mandated in our animal protocol. In contrast, the early memory (1 month post-infection) mice lost little weight and only one mouse from the study died. This experiment was a simple demonstration of the loss of cellular immune protection against influenza virus, which we will demonstrate in this dissertation correlates with the loss of the protective tissue resident CD8 T cells.

Two scenarios for examining the loss of T_{RM} from the lung:

Homeostatic loss due to lung microenvironment:

As mentioned before, cellular immunity to influenza gradually wanes as time from initial influenza priming increases[79, 84, 108]. As opposed to the stability of tissue resident memory in other tissues, we and many others suspected that the loss of heterologous cellular immunity was largely due to a loss of tissue resident memory T cells in the lung. It was recently reported that T_{RM} from the lung were rapidly lost under steady-state, homeostatic conditions, and that the lung T_{RM} population as a whole had to be continually replenished by circulating T_{EM} through an antigen-independent process[109]. However, this finding conflicted several reports that demonstrated antigen was required for the establishment of lung T_{RM}. Recently, however, it was shown that under specific inflammatory conditions using the glucan zymosan, tissue resident memory T cells can form within the lung in the absence of antigen encounter within the tissue[110]. This contradiction,

and its implications for lung T_{RM} maintenance and decline, will be further explored in this dissertation.

Loss of lung T_{RM} due to damage from unrelated infections:

As previously discussed, during the course of influenza infection DAMPs and PAMPs are released in response to innate recognition of the virus and tissue damage. One of these DAMPs is extracellular ATP (eATP). ATP release is tightly regulated in the lung and the dysregulation of ATP release during an infection further amplifies the pro-inflammatory program[111, 112]. The release of eATP drives systemic inflammation via the release pro-inflammatory cytokines such as TNF and Il-1β[113, 114]. This release of eATP also activates a family of receptors, called purinergic receptors, including the receptor P2RX7[114] . P2RX7 has recently been implicated as being very important in memory CD8 T cells. The activation of P2RX7 has been implicated in regulating mitochondrial homeostasis in memory CD8 T cells and was posited to be critical to tissue resident memory survival[115]. Van Lier and colleagues found that tissue resident memory CD8 T cells expressed P2RX7 at high levels, and activation of the receptor via a sterile inflammation model of liver damage was sufficient to induce rapid death of liver TRM[116]. For most studies, tissue resident memory has been studied in the context of a single infection, so the effect of subsequent, unrelated infections and the tissue damage they induce hasn't been thoroughly examined. The secondary effects of DAMPs and PAMPs on pre-existing T_{RM} need to be further considered with regards to the decline of cellular immunity in the lung.

Unanswered questions:

We now know that the number of memory CD8 T cells in the lung correlates with the efficacy of cellular immunity to influenza challenge[79, 117]. Thus, the waning of lung T_{RM} is likely responsible for the gradual loss of protective cellular immunity against secondary pathogen encounters in the lung. However, a number of unanswered questions regarding the gradual loss of lung CD8 T_{RM} remain, including: (a) whether the inability to maintain T_{RM} is unique to the lung environment or occurs distinct subsets of T_{RM} within the lung, (b) what are the mechanisms that regulate the slow decline of lung T_{RM} over time, and (c) how unrelated infections or localized inflammation affects pre-existing lung CD8 T_{RM}. Given the contribution of lung T_{RM} to the cellular immunity against respiratory pathogens, it is essential we understand the factors that govern their long-term maintenance.

Additional future studies outside the scope of this dissertation:

Prime boosting or use of adenoviral vectors? A way of ameliorating lung T_{RM} loss:

A series of prime-boosting regimens could be one way to prevent this loss of lung T_{RM} and cellular immunity. Prime-boost strategies have been shown to increase the number of antigen-specific memory CD8 T cells through repeated antigen exposure, and have been used to successfully improve the efficacy of cellular immunity against pathogen challenge[118, 119, 120, 121]. Furthermore, repeated boosting of pre-existing memory T cells can lead to improved memory T cell responses on a per cell basis, suggesting that secondary or tertiary memory lung T_{RM} may acquire intrinsic properties that enhance their longevity[122 123, 124, 125]. Another strategy to improve lung T_{RM} numbers that has recently been demonstrated is the use of adenoviral vectors in immunization strategies. These strategies have been used in the design of vaccines for avian

influenza and they are ideal vaccine candidates due to the diverse tissue tropism of the vector, large quantities of antigen produced following immunization, and the non-productive infection that prevents the adenoviral vector from inducing adverse pathology[126, 127, 128]. Adenoviral vectors are being further explored as methods for inducing large T cell responses and protective cellular immunity.

Is the immune compartment within the lung truly fixed in size?

Contrasting views exist as to whether the loss of memory CD8 T cells is environmental or structural. Can the lung memory T cell compartment expand indefinitely, or is it limited in its size and variation due limitations for space and resources or subsequent inflammatory events? This question, regarding whether the memory compartment can continue expanding, or whether the memory compartment is culled by subsequent infection through inflammation-dependent attrition was taken up by Masopust and Welsh[129, 130]. Masopust's group used a series of prime-boost strategies to increase the size of the memory CD8 compartment in the blood. They transferred LCMV specific P14 cells into mice and then used a series of heterologous prime-boosts to examine the expansion and contraction of this memory population. They observed that there was an overall increase in the memory compartment, and the increase in this compartment was almost entirely due to an increase in CD44 high CD8 T cells. These data suggested that the memory compartment does not undergo attrition due to inflammatory cytokines, at least in the blood. Further studies are needed to examine the expansive nature of the immune compartment of the lung and determine whether there are tissue-specific limitations on the size of the memory T cell compartment.

How does tissue remolding following infection modulate tissue resident memory within the lung environment?

Following primary infection and clearance of the virus, tissue remolding occurs during the healing process as a result of repairing the damage induced by the immune response to an active infection. As previously discussed, PAMPs and DAMPs are released as a result of influenza infection. These PAMPs and DAMPs activate various receptors, including TLRs and purinergic receptors, respectively[116, 131]. Additionally, it was shown that S100A9, a molecule expressed by influenza-infected epithelial cells, is a DAMP that is capable of inducing inflammation through the TLR4-MyD88 pathway[131]. The TLR4-TRIF pathway alternatively activates oxidative stress pathways which contribute to acute lung injury[132]. In response to these DAMPs and PAMPs, considerable damage occurs which must be healed, resulting in tissue repair and remodeling. ILC_S are critical to helping restore functionality through their expression of epidermal growth factors which help heal the damaged tissues[133]. These epidermal growth factors also remodel the airway following acute injury[134]. As a result of recurrent lung infections, sites of tissue repair, which are known to preferential niches for T_{RM} in the lung, are continuously being destroyed and remolded[135, 136]. Future studies should examine how T_{RM} within the lung adapt to their ever-changing environment following subsequent infections.

Figure 1

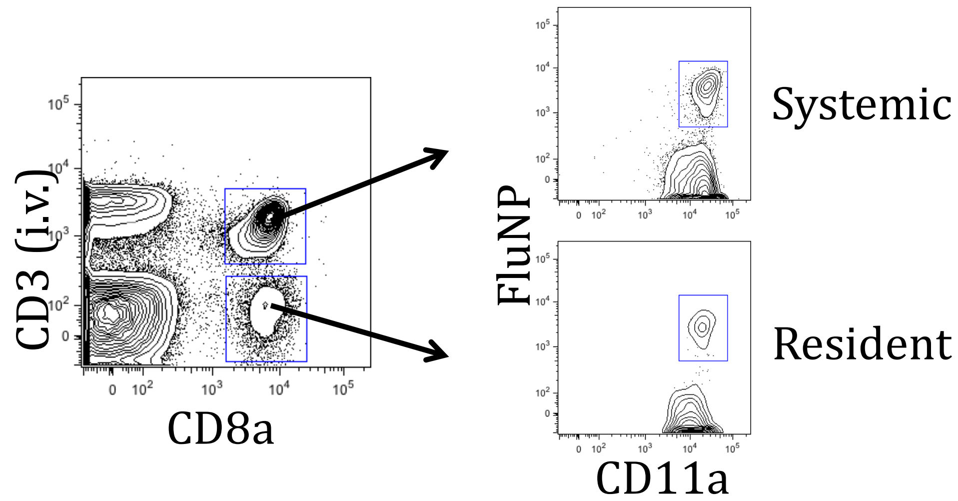

Figure 1. Example staining showing the use of intra-vital labeling to distinguish systemic from resident populations within tissues. Used to determine tissue resident lymphocytes in this case CD8a+ cells.

Figure 2:

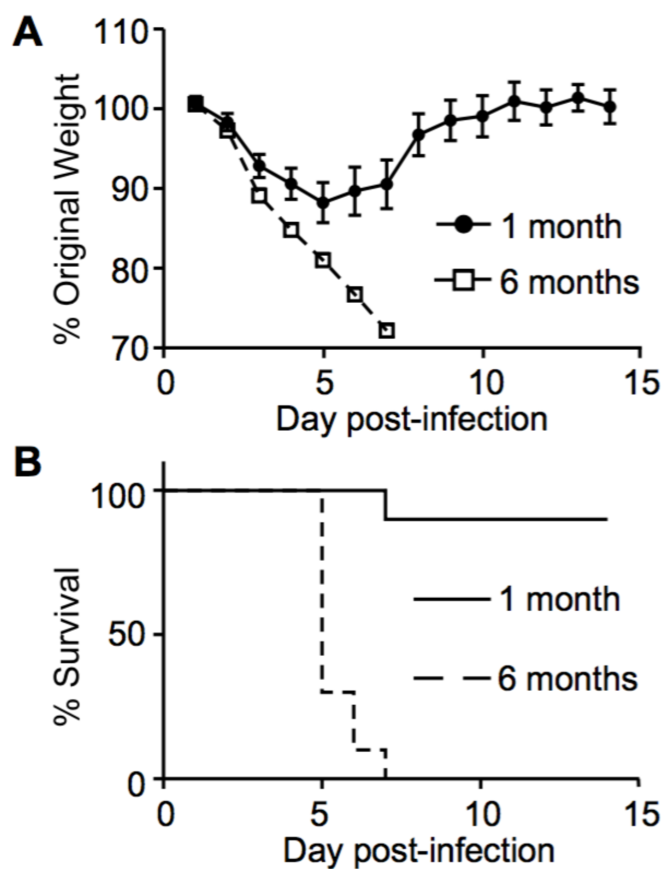

Figure 2. The efficacy of heterosubtypic immunity to influenza virus declines over time. Mice were infected with x31 influenza virus, challenged with 10 LD_{50} PR8 influenza virus 1 month or 6 months later, and monitored for (A) weight loss and (B) mortality.

References:

1. Thompson, W.W., Shay, D.K., Weintraub, E., Brammer, L., Cox, N., Anderson, L.J. & Fukuda, K. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* **289**, 179-186 (2003).

2. Glezen, W.P. Serious morbidity and mortality associated with influenza epidemics. *Epidemiol Rev* **4**, 25-44 (1982).

3. Iuliano, A.D., Roguski, K.M., Chang, H.H., Muscatello, D.J., Palekar, R., Tempia, S., Cohen, C., Gran, J.M., Schanzer, D., Cowling, B.J., Wu, P., Kyncl, J., Ang, L.W., Park, M., Redlberger-Fritz, M., Yu, H., Espenhain, L., Krishnan, A., Emukule, G., van Asten, L., Pereira da Silva, S., Aungkulanon, S., Buchholz, U., Widdowson, M.A., Bresee, J.S. & Global Seasonal Influenza-associated Mortality Collaborator, N. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. *Lancet* **391**, 1285-1300 (2018). PMC5935243

4. Fiore, A.E., Bridges, C.B. & Cox, N.J. Seasonal influenza vaccines. *Curr Top Microbiol Immunol* **333**, 43-82 (2009).

5. DeDiego, M.L., Anderson, C.S., Yang, H., Holden-Wiltse, J., Fitzgerald, T., Treanor, J.J. & Topham, D.J. Directed selection of influenza virus produces antigenic variants that match circulating human virus isolates and escape from vaccine-mediated immune protection. *Immunology* **148**, 160-173 (2016). PMC4863573

6. Chen, R. & Holmes, E.C. Avian influenza virus exhibits rapid evolutionary dynamics. *Mol Biol Evol* **23**, 2336-2341 (2006).

7. Xu, H., Meng, F., Huang, D., Sheng, X., Wang, Y., Zhang, W., Chang, W., Wang, L. & Qin, Z. Genomic and phylogenetic characterization of novel, recombinant H5N2 avian influenza virus strains isolated from vaccinated chickens with clinical symptoms in China. *Viruses* **7**, 887-898 (2015). PMC4379553

8. Kelly, H., Peck, H.A., Laurie, K.L., Wu, P., Nishiura, H. & Cowling, B.J. The age-specific cumulative incidence of infection with pandemic influenza H1N1 2009 was similar in various countries prior to vaccination. *PLoS One* **6**, e21828 (2011). PMC3151238

9. Palese P, S.M. Fields Virology. In: Knipe DM, H.P., editor. *Orthomyxoviridae: the viruses and their replication*. Philadelphia, PA: Lippicott Williams & Wilkins; 2007. pp. 1647-1689.

10. Tashiro, M., Yamakawa, M., Tobita, K., Klenk, H.D., Rott, R. & Seto, J.T. Organ tropism of Sendai virus in mice: proteolytic activation of the fusion glycoprotein in mouse organs and budding site at the bronchial epithelium. *J Virol* **64**, 3627-3634 (1990). PMC249655

11. Couceiro, J.N., Paulson, J.C. & Baum, L.G. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res* **29**, 155-165 (1993).

12. Byrd-Leotis, L., Cummings, R.D. & Steinhauer, D.A. The Interplay between the Host Receptor and Influenza Virus Hemagglutinin and Neuraminidase. *Int J Mol Sci* **18** (2017). PMC5536029

13. Villenave, R., Touzelet, O., Thavagnanam, S., Sarlang, S., Parker, J., Skibinski, G., Heaney, L.G., McKaigue, J.P., Coyle, P.V., Shields, M.D. & Power, U.F. Cytopathogenesis of Sendai virus in well-differentiated primary pediatric bronchial epithelial cells. *J Virol* **84**, 11718-11728 (2010). PMC2977906

14. Shi, Y., Wu, Y., Zhang, W., Qi, J. & Gao, G.F. Enabling the 'host jump': structural determinants of receptor-binding specificity in influenza A viruses. *Nat Rev Microbiol* **12**, 822-831 (2014).

15. Bouvier, N.M. & Palese, P. The biology of influenza viruses. *Vaccine* **26 Suppl 4**, D49-53 (2008). PMC3074182

16. Kirkpatrick, E., Qiu, X., Wilson, P.C., Bahl, J. & Krammer, F. The influenza virus hemagglutinin head evolves faster than the stalk domain. *Sci Rep* **8**, 10432 (2018). PMC6041311

17. Shao, W., Li, X., Goraya, M.U., Wang, S. & Chen, J.L. Evolution of Influenza A Virus by Mutation and Re-Assortment. *Int J Mol Sci* **18** (2017). PMC5578040

18. Rambaut, A., Pybus, O.G., Nelson, M.I., Viboud, C., Taubenberger, J.K. & Holmes, E.C. The genomic and epidemiological dynamics of human influenza A virus. *Nature* **453**, 615-619 (2008). PMC2441973

19. Gregory, V., Bennett, M., Orkhan, M.H., Al Hajjar, S., Varsano, N., Mendelson, E., Zambon, M., Ellis, J., Hay, A. & Lin, Y.P. Emergence of influenza A H1N2 reassortant viruses in the human population during 2001. *Virology* **300**, 1-7 (2002).

20. Garten, R.J., Davis, C.T., Russell, C.A., Shu, B., Lindstrom, S., Balish, A., Sessions, W.M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C.B., Emery, S.L., Hillman, M.J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D.F., Fouchier, R.A., Pappas, C., Alpuche-Aranda, C.M., Lopez-Gatell, H., Olivera, H., Lopez, I., Myers, C.A., Faix, D., Blair, P.J., Yu, C., Keene, K.M., Dotson, P.D., Jr., Boxrud, D., Sambol, A.R., Abid, S.H., St George, K., Bannerman, T., Moore, A.L., Stringer, D.J., Blevins, P., Demmler-Harrison, G.J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H.F., Belongia, E.A., Clark, P.A., Beatrice, S.T., Donis, R., Katz, J., Finelli, L., Bridges, C.B., Shaw, M., Jernigan, D.B., Uyeki, T.M., Smith, D.J., Klimov, A.I. & Cox, N.J. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **325**, 197-201 (2009). PMC3250984

21. Pappas, C., Aguilar, P.V., Basler, C.F., Solorzano, A., Zeng, H., Perrone, L.A., Palese, P., Garcia-Sastre, A., Katz, J.M. & Tumpey, T.M. Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. *Proc Natl Acad Sci U S A* **105**, 3064-3069 (2008). PMC2268585

22. Morens, D.M., Taubenberger, J.K., Harvey, H.A. & Memoli, M.J. The 1918 influenza pandemic: lessons for 2009 and the future. *Crit Care Med* **38**, e10-20 (2010). PMC3180813

23. Wu, T., Guan, J., Handel, A., Tscharke, D.C., Sidney, J., Sette, A., Wakim, L.M., Sng, X.Y.X., Thomas, P.G., Croft, N.P., Purcell, A.W. & La Gruta, N.L. Quantification of epitope abundance reveals the effect of direct and cross-presentation on influenza CTL responses. *Nat Commun* **10**, 2846 (2019). PMC6599079

24. Terajima, M., Cruz, J., Leporati, A.M., Orphin, L., Babon, J.A., Co, M.D., Pazoles, P., Jameson, J. & Ennis, F.A. Influenza A virus matrix protein 1-specific human CD8+ T-cell response induced in trivalent inactivated vaccine recipients. *J Virol* **82**, 9283-9287 (2008). PMC2546892

25. Taylor, P.M. & Askonas, B.A. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. *Immunology* **58**, 417-420 (1986). PMC1453480

26. Bouvier, N.M. & Lowen, A.C. Animal Models for Influenza Virus Pathogenesis and Transmission. *Viruses* **2**, 1530-1563 (2010). PMC3063653

27. Sanders, C.J., Doherty, P.C. & Thomas, P.G. Respiratory epithelial cells in innate immunity to influenza virus infection. *Cell Tissue Res* **343**, 13-21 (2011).

28. Holt, P.G., Strickland, D.H., Wikstrom, M.E. & Jahnsen, F.L. Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol* **8**, 142-152 (2008).

29. Hammad, H. & Lambrecht, B.N. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* **8**, 193-204 (2008).

30. McGill, J., Heusel, J.W. & Legge, K.L. Innate immune control and regulation of influenza virus infections. *J Leukoc Biol* **86**, 803-812 (2009). PMC2752015

31. Sertl, K., Takemura, T., Tschachler, E., Ferrans, V.J., Kaliner, M.A. & Shevach, E.M. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J Exp Med* **163**, 436-451 (1986). PMC2188026

32. Kim, T.S. & Braciale, T.J. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8+ T cell responses. *PLoS One* **4**, e4204 (2009). PMC2615220

33. del Rio, M.L., Rodriguez-Barbosa, J.I., Kremmer, E. & Forster, R. CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J Immunol* **178**, 6861-6866 (2007).

34. Plantinga, M., Hammad, H. & Lambrecht, B.N. Origin and functional specializations of DC subsets in the lung. *Eur J Immunol* **40**, 2112-2118 (2010).

35. Strickland, D.H., Upham, J.W. & Holt, P.G. Epithelial-dendritic cell interactions in allergic disorders. *Curr Opin Immunol* **22**, 789-794 (2010).

36. Sung, S.S., Fu, S.M., Rose, C.E., Jr., Gaskin, F., Ju, S.T. & Beaty, S.R. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J Immunol* **176**, 2161-2172 (2006).

37. Zhang, Z. & Wang, F.S. Plasmacytoid dendritic cells act as the most competent cell type in linking antiviral innate and adaptive immune responses. *Cell Mol Immunol* **2**, 411-417 (2005).

38. Thomas, J.M., Pos, Z., Reinboth, J., Wang, R.Y., Wang, E., Frank, G.M., Lusso, P., Trinchieri, G., Alter, H.J., Marincola, F.M. & Thomas, E. Differential responses of plasmacytoid dendritic cells to influenza virus and distinct viral pathogens. *J Virol* **88**, 10758-10766 (2014). PMC4178854

39. GeurtsvanKessel, C.H., Willart, M.A., van Rijt, L.S., Muskens, F., Kool, M., Baas, C., Thielemans, K., Bennett, C., Clausen, B.E., Hoogsteden, H.C., Osterhaus, A.D., Rimmelzwaan, G.F. & Lambrecht, B.N. Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *J Exp Med* **205**, 1621-1634 (2008). PMC2442640

40. Wolf, A.I., Buehler, D., Hensley, S.E., Cavanagh, L.L., Wherry, E.J., Kastner, P., Chan, S. & Weninger, W. Plasmacytoid dendritic cells are dispensable during primary influenza virus infection. *J Immunol* **182**, 871-879 (2009).

41. Lin, K.L., Suzuki, Y., Nakano, H., Ramsburg, E. & Gunn, M.D. CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. *J Immunol* **180**, 2562-2572 (2008).

42. McGill, J., Van Rooijen, N. & Legge, K.L. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. *J Exp Med* **205**, 1635-1646 (2008). PMC2442641

43. Tate, M.D., Brooks, A.G. & Reading, P.C. The role of neutrophils in the upper and lower respiratory tract during influenza virus infection of mice. *Respir Res* **9**, 57 (2008). PMC2526083

44. Tate, M.D., Ioannidis, L.J., Croker, B., Brown, L.E., Brooks, A.G. & Reading, P.C. The role of neutrophils during mild and severe influenza virus infections of mice. *PLoS One* **6**, e17618 (2011). PMC3056712

45. Hufford, M.M., Kim, T.S., Sun, J. & Braciale, T.J. Antiviral CD8+ T cell effector activities in situ are regulated by target cell type. *J Exp Med* **208**, 167-180 (2011). PMC3023137

46. Aldridge, J.R., Jr., Moseley, C.E., Boltz, D.A., Negovetich, N.J., Reynolds, C., Franks, J., Brown, S.A., Doherty, P.C., Webster, R.G. & Thomas, P.G. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proc Natl Acad Sci U S A* **106**, 5306-5311 (2009). PMC2664048

47. Seleme, M.C., Kosmac, K., Jonjic, S. & Britt, W.J. Tumor Necrosis Factor Alpha-Induced Recruitment of Inflammatory Mononuclear Cells Leads to Inflammation and Altered Brain Development in Murine Cytomegalovirus-Infected Newborn Mice. *J Virol* **91** (2017). PMC5375689

48. Topham, D.J., Tripp, R.A. & Doherty, P.C. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *J Immunol* **159**, 5197-5200 (1997).

49. Herold, S., Steinmueller, M., von Wulffen, W., Cakarova, L., Pinto, R., Pleschka, S., Mack, M., Kuziel, W.A., Corazza, N., Brunner, T., Seeger, W. & Lohmeyer, J. Lung epithelial

apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *J Exp Med* **205**, 3065-3077 (2008). PMC2605231

50. Bohmwald, K., Galvez, N.M.S., Canedo-Marroquin, G., Pizarro-Ortega, M.S., Andrade-Parra, C., Gomez-Santander, F. & Kalergis, A.M. Contribution of Cytokines to Tissue Damage During Human Respiratory Syncytial Virus Infection. *Front Immunol* **10**, 452 (2019). PMC6431622

51. Zhou, J., Matsuoka, M., Cantor, H., Homer, R. & Enelow, R.I. Cutting edge: engagement of NKG2A on CD8+ effector T cells limits immunopathology in influenza pneumonia. *J Immunol* **180**, 25-29 (2008).

52. Rouse, B.T. & Sehrawat, S. Immunity and immunopathology to viruses: what decides the outcome? *Nat Rev Immunol* **10**, 514-526 (2010). PMC3899649

53. Sun, J., Madan, R., Karp, C.L. & Braciale, T.J. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat Med* **15**, 277-284 (2009). PMC2693210

54. Sun, K., Torres, L. & Metzger, D.W. A detrimental effect of interleukin-10 on protective pulmonary humoral immunity during primary influenza A virus infection. *J Virol* **84**, 5007-5014 (2010). PMC2863832

55. McKinstry, K.K., Strutt, T.M., Buck, A., Curtis, J.D., Dibble, J.P., Huston, G., Tighe, M., Hamada, H., Sell, S., Dutton, R.W. & Swain, S.L. IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge. *J Immunol* **182**, 7353-7363 (2009). PMC2724021

56. Palmer, E.M., Holbrook, B.C., Arimilli, S., Parks, G.D. & Alexander-Miller, M.A. IFNgamma-producing, virus-specific CD8+ effector cells acquire the ability to produce IL-10 as a result of entry into the infected lung environment. *Virology* **404**, 225-230 (2010). PMC2906694

57. Carlson, C.M., Turpin, E.A., Moser, L.A., O'Brien, K.B., Cline, T.D., Jones, J.C., Tumpey, T.M., Katz, J.M., Kelley, L.A., Gauldie, J. & Schultz-Cherry, S. Transforming growth factor-beta: activation by neuraminidase and role in highly pathogenic H5N1 influenza pathogenesis. *PLoS Pathog* **6**, e1001136 (2010). PMC2951376

58. Denney, L., Branchett, W., Gregory, L.G., Oliver, R.A. & Lloyd, C.M. Epithelial-derived TGF-beta1 acts as a pro-viral factor in the lung during influenza A infection. *Mucosal Immunol* **11**, 523-535 (2018). PMC5797694

59. Zhang, N. & Bevan, M.J. Transforming growth factor-beta signaling controls the formation and maintenance of gut-resident memory T cells by regulating migration and retention. *Immunity* **39**, 687-696 (2013). PMC3805703

60. Denney, L., Byrne, A.J., Shea, T.J., Buckley, J.S., Pease, J.E., Herledan, G.M., Walker, S.A., Gregory, L.G. & Lloyd, C.M. Pulmonary Epithelial Cell-Derived Cytokine TGF-beta1 Is a Critical Cofactor for Enhanced Innate Lymphoid Cell Function. *Immunity* **43**, 945-958 (2015). PMC4658339

61. Humphreys, I.R., Walzl, G., Edwards, L., Rae, A., Hill, S. & Hussell, T. A critical role for OX40 in T cell-mediated immunopathology during lung viral infection. *J Exp Med* **198**, 1237-1242 (2003). PMC2194232

62. Haeryfar, S.M., DiPaolo, R.J., Tscharke, D.C., Bennink, J.R. & Yewdell, J.W. Regulatory T cells suppress CD8+ T cell responses induced by direct priming and cross-priming and moderate immunodominance disparities. *J Immunol* **174**, 3344-3351 (2005).

63. Antunes, I. & Kassiotis, G. Suppression of innate immune pathology by regulatory T cells during Influenza A virus infection of immunodeficient mice. *J Virol* **84**, 12564-12575 (2010). PMC3004299

64. Laskin, D.L. & Pendino, K.J. Macrophages and inflammatory mediators in tissue injury. *Annu Rev Pharmacol Toxicol* **35**, 655-677 (1995).

65. Snelgrove, R.J., Goulding, J., Didierlaurent, A.M., Lyonga, D., Vekaria, S., Edwards, L., Gwyer, E., Sedgwick, J.D., Barclay, A.N. & Hussell, T. A critical function for CD200 in

lung immune homeostasis and the severity of influenza infection. *Nat Immunol* **9**, 1074-1083 (2008).

66. Rygiel, T.P., Rijkers, E.S., de Ruiter, T., Stolte, E.H., van der Valk, M., Rimmelzwaan, G.F., Boon, L., van Loon, A.M., Coenjaerts, F.E., Hoek, R.M., Tesselaar, K. & Meyaard, L. Lack of CD200 enhances pathological T cell responses during influenza infection. *J Immunol* **183**, 1990-1996 (2009).

67. Hussell, T., Snelgrove, R., Humphreys, I.R. & Williams, A.E. Co-stimulation: novel methods for preventing viral-induced lung inflammation. *Trends Mol Med* **10**, 379-386 (2004).

68. Humphreys, I.R., Edwards, L., Snelgrove, R.J., Rae, A.J., Coyle, A.J. & Hussell, T. A critical role for ICOS co-stimulation in immune containment of pulmonary influenza virus infection. *Eur J Immunol* **36**, 2928-2938 (2006).

69. Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J. & Ahmed, R. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* **286**, 1377-1381 (1999).

70. Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J. & Ahmed, R. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**, 177-187 (1998).

71. Badovinac, V.P. & Harty, J.T. CD8(+) T-cell homeostasis after infection: setting the 'curve'. *Microbes Infect* **4**, 441-447 (2002).

72. Kaech, S.M. & Ahmed, R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol* **2**, 415-422 (2001). PMC3760150

73. Grayson, J.M., Murali-Krishna, K., Altman, J.D. & Ahmed, R. Gene expression in antigen-specific CD8+ T cells during viral infection. *J Immunol* **166**, 795-799 (2001).

74. Grayson, J.M., Zajac, A.J., Altman, J.D. & Ahmed, R. Cutting edge: increased expression of Bcl-2 in antigen-specific memory CD8+ T cells. *J Immunol* **164**, 3950-3954 (2000).

75. Kaech, S.M., Hemby, S., Kersh, E. & Ahmed, R. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* **111**, 837-851 (2002).

76. Wherry, E.J., Blattman, J.N., Murali-Krishna, K., van der Most, R. & Ahmed, R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* **77**, 4911-4927 (2003). PMC152117

77. Wherry, E.J., Teichgraber, V., Becker, T.C., Masopust, D., Kaech, S.M., Antia, R., von Andrian, U.H. & Ahmed, R. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* **4**, 225-234 (2003).

78. Guo, H., Santiago, F., Lambert, K., Takimoto, T. & Topham, D.J. T cell-mediated protection against lethal 2009 pandemic H1N1 influenza virus infection in a mouse model. *J Virol* **85**, 448-455 (2011). PMC3014175

79. Liang, S.H., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic Immunity to Influenza Type-a Virus in Mice - Effector Mechanisms and Their Longevity. *Journal of Immunology* **152**, 1653-1661 (1994).

80. Mueller, S.N., Gebhardt, T., Carbone, F.R. & Heath, W.R. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol* **31**, 137-161 (2013).

81. Kohlmeier, J.E., Cookenham, T., Roberts, A.D., Miller, S.C. & Woodland, D.L. Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge. *Immunity* **33**, 96-105 (2010). PMC2908370

82. Mueller, S.N. & Mackay, L.K. Tissue-resident memory T cells: local specialists in immune defence. *Nat Rev Immunol* **16**, 79-89 (2016).

83. Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C. & Kupper, T.S. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* **483**, 227-231 (2012). PMC3437663

84. Kohlmeier, J.E. & Woodland, D.L. Immunity to respiratory viruses. *Annu Rev Immunol* **27**, 61-82 (2009).

85. Mackay, L.K., Braun, A., Macleod, B.L., Collins, N., Tebartz, C., Bedoui, S., Carbone, F.R. & Gebhardt, T. Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. *J Immunol* **194**, 2059-2063 (2015).

86. Skon, C.N., Lee, J.Y., Anderson, K.G., Masopust, D., Hogquist, K.A. & Jameson, S.C. Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nat Immunol* **14**, 1285-1293 (2013). PMC3844557

87. Ma, C. & Zhang, N. Transforming growth factor-beta signaling is constantly shaping memory T-cell population. *Proc Natl Acad Sci U S A* **112**, 11013-11017 (2015). PMC4568277

88. Mackay, L.K., Minnich, M., Kragten, N.A., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F.M., Stark, R., Pellicci, D.G., Godfrey, D.I., Belz, G.T., Pellegrini, M., Gebhardt, T., Busslinger, M., Shi, W., Carbone, F.R., van Lier, R.A., Kallies, A. & van Gisbergen, K.P. Hobit and Blimp1 instruct

a universal transcriptional program of tissue residency in lymphocytes. *Science* **352**, 459-463 (2016).

89. Mackay, L.K., Wynne-Jones, E., Freestone, D., Pellicci, D.G., Mielke, L.A., Newman, D.M., Braun, A., Masson, F., Kallies, A., Belz, G.T. & Carbone, F.R. T-box Transcription Factors Combine with the Cytokines TGF-beta and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* **43**, 1101-1111 (2015).

90. Gebhardt, T., Wakim, L.M., Eidsmo, L., Reading, P.C., Heath, W.R. & Carbone, F.R. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* **10**, 524-530 (2009).

91. Bergsbaken, T. & Bevan, M.J. Proinflammatory microenvironments within the intestine regulate the differentiation of tissue-resident CD8(+) T cells responding to infection. *Nat Immunol* **16**, 406-414 (2015). PMC4368475

92. Masopust, D., Choo, D., Vezys, V., Wherry, E.J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K.A., Barber, D.L., Kawamura, K.S., Fraser, K.A., Webby, R.J., Brinkmann, V., Butcher, E.C., Newell, K.A. & Ahmed, R. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* **207**, 553-564 (2010). PMC2839151

93. Schenkel, J.M., Fraser, K.A., Vezys, V. & Masopust, D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* **14**, 509-513 (2013). PMC3631432

94. Wu, T., Hu, Y., Lee, Y.T., Bouchard, K.R., Benechet, A., Khanna, K. & Cauley, L.S. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol* **95**, 215-224 (2014). PMC3896663

95. Pizzolla, A., Nguyen, T.H., Sant, S., Jaffar, J., Loudovaris, T., Mannering, S.I., Thomas, P.G., Westall, G.P., Kedzierska, K. & Wakim, L.M. Influenza-specific lung-resident memory T cells are proliferative and polyfunctional and maintain diverse TCR profiles. *J Clin Invest* **128**, 721-733 (2018). PMC5785253

96. McMaster, S.R., Wilson, J.J., Wang, H. & Kohlmeier, J.E. Airway-Resident Memory CD8 T Cells Provide Antigen-Specific Protection against Respiratory Virus Challenge through Rapid IFN-gamma Production. *J Immunol* **195**, 203-209 (2015). PMC4475417

97. Wakim, L.M., Gupta, N., Mintern, J.D. & Villadangos, J.A. Enhanced survival of lung tissue-resident memory CD8(+) T cells during infection with influenza virus due to selective expression of IFITM3. *Nat Immunol* **14**, 238-245 (2013).

98. Anderson, K.G., Sung, H., Skon, C.N., Lefrancois, L., Deisinger, A., Vezys, V. & Masopust, D. Cutting edge: intravascular staining redefines lung CD8 T cell responses. *J Immunol* **189**, 2702-2706 (2012). PMC3436991

99. Purwar, R., Campbell, J., Murphy, G., Richards, W.G., Clark, R.A. & Kupper, T.S. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One* **6**, e16245 (2011). PMC3027667

100. Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. & Doherty, P.C. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *J Exp Med* **174**, 875-880 (1991). PMC2118969

101. Hou, S., Doherty, P.C., Zijlstra, M., Jaenisch, R. & Katz, J.M. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *J Immunol* **149**, 1319-1325 (1992).

102. Wilkinson, T.M., Li, C.K., Chui, C.S., Huang, A.K., Perkins, M., Liebner, J.C., Lambkin-Williams, R., Gilbert, A., Oxford, J., Nicholas, B., Staples, K.J., Dong, T., Douek, D.C., McMichael, A.J. & Xu, X.N. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nat Med* **18**, 274-280 (2012).

103. Lee, L.Y., Ha do, L.A., Simmons, C., de Jong, M.D., Chau, N.V., Schumacher, R., Peng, Y.C., McMichael, A.J., Farrar, J.J., Smith, G.L., Townsend, A.R., Askonas, B.A., Rowland-Jones, S. & Dong, T. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J Clin Invest* **118**, 3478-3490 (2008). PMC2542885

104. van de Sandt, C.E., Kreijtz, J.H., de Mutsert, G., Geelhoed-Mieras, M.M., Hillaire, M.L., Vogelzang-van Trierum, S.E., Osterhaus, A.D., Fouchier, R.A. & Rimmelzwaan, G.F. Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus. *J Virol* **88**, 1684-1693 (2014). PMC3911609

105. Hogan, R.J., Usherwood, E.J., Zhong, W., Roberts, A.A., Dutton, R.W., Harmsen, A.G. & Woodland, D.L. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J Immunol* **166**, 1813-1822 (2001).

106. Marshall, D.R., Turner, S.J., Belz, G.T., Wingo, S., Andreansky, S., Sangster, M.Y., Riberdy, J.M., Liu, T., Tan, M. & Doherty, P.C. Measuring the diaspora for virus-specific CD8+ T cells. *Proc Natl Acad Sci U S A* **98**, 6313-6318 (2001). PMC33465

107. Kohlmeier, J.E., Miller, S.C. & Woodland, D.L. Cutting edge: Antigen is not required for the activation and maintenance of virus-specific memory CD8+ T cells in the lung airways. *J Immunol* **178**, 4721-4725 (2007).

108. Woodland, D.L. & Kohlmeier, J.E. Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat Rev Immunol* **9**, 153-161 (2009).

109. Slutter, B., Van Braeckel-Budimir, N., Abboud, G., Varga, S.M., Salek-Ardakani, S. & Harty, J.T. Dynamics of influenza-induced lung-resident memory T cells underlie waning heterosubtypic immunity. *Sci Immunol* **2** (2017). PMC5590757

110. Caminschi, I., Lahoud, M.H., Pizzolla, A. & Wakim, L.M. Zymosan by-passes the requirement for pulmonary antigen encounter in lung tissue-resident memory CD8(+) T cell development. *Mucosal Immunol* **12**, 403-412 (2019).

111. Okada, S.F., Nicholas, R.A., Kreda, S.M., Lazarowski, E.R. & Boucher, R.C. Physiological regulation of ATP release at the apical surface of human airway epithelia. *J Biol Chem* **281**, 22992-23002 (2006). PMC2924190

112. Dosch, M., Gerber, J., Jebbawi, F. & Beldi, G. Mechanisms of ATP Release by Inflammatory Cells. *Int J Mol Sci* **19** (2018). PMC5979498

113. Cauwels, A., Rogge, E., Vandendriessche, B., Shiva, S. & Brouckaert, P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death Dis* **5**, e1102 (2014). PMC3973196

114. Lister, M.F., Sharkey, J., Sawatzky, D.A., Hodgkiss, J.P., Davidson, D.J., Rossi, A.G. & Finlayson, K. The role of the purinergic P2X7 receptor in inflammation. *J Inflamm (Lond)* **4**, 5 (2007). PMC1838907

115. Borges da Silva, H., Beura, L.K., Wang, H., Hanse, E.A., Gore, R., Scott, M.C., Walsh, D.A., Block, K.E., Fonseca, R., Yan, Y., Hippen, K.L., Blazar, B.R., Masopust, D., Kelekar, A., Vulchanova, L., Hogquist, K.A. & Jameson, S.C. The purinergic receptor P2RX7 directs metabolic fitness of long-lived memory CD8(+) T cells. *Nature* **559**, 264-268 (2018). PMC6054485

116. Stark, R., Wesselink, T.H., Behr, F.M., Kragten, N.A.M., Arens, R., Koch-Nolte, F., van Gisbergen, K. & van Lier, R.A.W. T RM maintenance is regulated by tissue damage via P2RX7. *Sci Immunol* **3** (2018).

117. Ray, S.J., Franki, S.N., Pierce, R.H., Dimitrova, S., Koteliansky, V., Sprague, A.G., Doherty, P.C., de Fougerolles, A.R. & Topham, D.J. The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* **20**, 167-179 (2004).

118. Goonetilleke, N.P., McShane, H., Hannan, C.M., Anderson, R.J., Brookes, R.H. & Hill, A.V. Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J Immunol* **171**, 1602-1609 (2003).

119. Schneider, J., Gilbert, S.C., Blanchard, T.J., Hanke, T., Robson, K.J., Hannan, C.M., Becker, M., Sinden, R., Smith, G.L. & Hill, A.V. Enhanced immunogenicity for CD8+ T

cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat Med* **4**, 397-402 (1998).

120. Masopust, D., Ha, S.J., Vezys, V. & Ahmed, R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J Immunol* **177**, 831-839 (2006).

121. Christensen, J.P., Doherty, P.C., Branum, K.C. & Riberdy, J.M. Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8(+) T-cell memory. *J Virol* **74**, 11690-11696 (2000). PMC112451

122. Van Braeckel-Budimir, N., Varga, S.M., Badovinac, V.P. & Harty, J.T. Repeated Antigen Exposure Extends the Durability of Influenza-Specific Lung-Resident Memory CD8(+) T Cells and Heterosubtypic Immunity. *Cell Rep* **24**, 3374-3382 e3373 (2018).

123. Nolz, J.C. & Harty, J.T. Protective capacity of memory CD8+ T cells is dictated by antigen exposure history and nature of the infection. *Immunity* **34**, 781-793 (2011). PMC3103642

124. Jabbari, A. & Harty, J.T. Secondary memory CD8+ T cells are more protective but slower to acquire a central-memory phenotype. *J Exp Med* **203**, 919-932 (2006). PMC2118270

125. Wirth, T.C., Xue, H.H., Rai, D., Sabel, J.T., Bair, T., Harty, J.T. & Badovinac, V.P. Repetitive antigen stimulation induces stepwise transcriptome diversification but preserves

a core signature of memory CD8(+) T cell differentiation. *Immunity* **33**, 128-140 (2010). PMC2912220

126. Gao, W., Soloff, A.C., Lu, X., Montecalvo, A., Nguyen, D.C., Matsuoka, Y., Robbins, P.D., Swayne, D.E., Donis, R.O., Katz, J.M., Barratt-Boyes, S.M. & Gambotto, A. Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *J Virol* **80**, 1959-1964 (2006). PMC1367171

127. Flaherty, D.M., Hinde, S.L., Monick, M.M., Powers, L.S., Bradford, M.A., Yarovinsky, T. & Hunninghake, G.W. Adenovirus vectors activate survival pathways in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **287**, L393-401 (2004).

128. Vemula, S.V. & Mittal, S.K. Production of adenovirus vectors and their use as a delivery system for influenza vaccines. *Expert Opin Biol Ther* **10**, 1469-1487 (2010). PMC2951029

129. Vezys, V., Yates, A., Casey, K.A., Lanier, G., Ahmed, R., Antia, R. & Masopust, D. Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* **457**, 196-199 (2009).

130. Welsh, R.M. & Selin, L.K. Attrition of memory CD8 T cells. *Nature* **459**, E3-4; discussion E4 (2009). PMC3226334

131. Tsai, S.Y., Segovia, J.A., Chang, T.H., Morris, I.R., Berton, M.T., Tessier, P.A., Tardif, M.R., Cesaro, A. & Bose, S. DAMP molecule S100A9 acts as a molecular pattern to enhance inflammation during influenza A virus infection: role of DDX21-TRIF-TLR4-MyD88 pathway. *PLoS Pathog* **10**, e1003848 (2014). PMC3879357

132. Imai, Y., Kuba, K., Neely, G.G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., Ermolaeva, M., Veldhuizen, R., Leung, Y.H., Wang, H., Liu, H., Sun, Y., Pasparakis, M., Kopf, M., Mech, C., Bavari, S., Peiris, J.S., Slutsky, A.S., Akira, S., Hultqvist, M., Holmdahl, R., Nicholls, J., Jiang, C., Binder, C.J. & Penninger, J.M. Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell* **133**, 235-249 (2008).

133. Monticelli, L.A., Sonnenberg, G.F., Abt, M.C., Alenghat, T., Ziegler, C.G., Doering, T.A., Angelosanto, J.M., Laidlaw, B.J., Yang, C.Y., Sathaliyawala, T., Kubota, M., Turner, D., Diamond, J.M., Goldrath, A.W., Farber, D.L., Collman, R.G., Wherry, E.J. & Artis, D. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* **12**, 1045-1054 (2011). PMC3320042

134. Enomoto, Y., Orihara, K., Takamasu, T., Matsuda, A., Gon, Y., Saito, H., Ra, C. & Okayama, Y. Tissue remodeling induced by hypersecreted epidermal growth factor and amphiregulin in the airway after an acute asthma attack. *J Allergy Clin Immunol* **124**, 913-920 e911-917 (2009).

135. Takamura, S., Yagi, H., Hakata, Y., Motozono, C., McMaster, S.R., Masumoto, T., Fujisawa, M., Chikaishi, T., Komeda, J., Itoh, J., Umemura, M., Kyusai, A., Tomura, M., Nakayama, T., Woodland, D.L., Kohlmeier, J.E. & Miyazawa, M. Specific niches for lung-resident memory CD8+ T cells at the site of tissue regeneration enable CD69-independent maintenance. *J Exp Med* **213**, 3057-3073 (2016). PMC5154946

136. Turner, D.L., Bickham, K.L., Thome, J.J., Kim, C.Y., D'Ovidio, F., Wherry, E.J. & Farber, D.L. Lung niches for the generation and maintenance of tissue-resident memory T cells. *Mucosal Immunol* **7**, 501-510 (2014). PMC3965651

Chapter II: Environmental cues regulate epigenetic reprogramming of airway-resident memory CD8 T cells

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Abstract

Tissue resident memory (T_{RM}) is critical for cellular immunity against respiratory pathogens. Lung T_{RM} consist of two different populations that reside in airways or interstitium, and how the microenvironment influences T_{RM} biology in distinct tissue compartments within the lung is unknown. Here, we show the airway environment drives dramatic transcriptional and epigenetic changes that regulate cytolytic functions of airway T_{RM} and promote apoptosis due to amino acid starvation and activation of the integrated stress response. Comparison of airway T_{RM} and splenic T_{EM} transferred into the airways showed that core T_{RM} genes were regulated during their initial differentiation but not through exposure to the tissue environment, whereas cell function and fate was regulated by the environment. In addition, activation of the integrated stress response was reversible when airway T_{RM} were placed in a nutrient-rich environment. Overall, our data define the genetic programs of different lung T_{RM} populations and show how local cues alter airway T_{RM} to limit cytolytic function and promote cell death, ultimately leading to the decline of lung T_{RM}.

Introduction

Tissue resident memory cells (T_{RM}) act as critical sentinels of the immune system within peripheral tissues as they are uniquely positioned to rapidly recognize and respond to invading pathogens[1, 2, 3]. T_{RM} share many properties with effector memory T cells (T_{EM}), including genetic architecture poised for cytokine production and robust cytolytic activity[2, 4]. Despite these functional similarities, T_{RM} are clearly a distinct memory T cell subset defined by a core transcriptional signature that supports long-term tissue residence via regulation of cell trafficking molecules and adaptations that enable survival in the tissue microenvironment[5, 6]. These adaptations allow for long-term protection by T_{RM} in sites such as the skin and gut where they can provide nearly sterilizing immunity when present in sufficient numbers[1, 3]. In contrast, the efficacy of cellular immunity against respiratory pathogens gradually wanes, and this decline is associated with the progressive loss of virus-specific T_{RM} in the lung[7, 8]. However, the mechanisms driving the transient nature of lung T_{RM} are not well defined.

Following clearance of a primary influenza infection, virus-specific memory CD8 T cells are localized to secondary lymphoid organs and peripheral tissues, primarily the lung interstitium and lung airways[9,10, 11]. There is abundant evidence from animal models and humans that memory CD8 T cells confer protective immunity to respiratory viruses by significantly decreasing viral loads, limiting immunopathology, and lowering disease burden[7, 8, 12, 13]. Notably, optimal cellular immunity to influenza requires lung T_{RM}, as systemic infections or vaccinations that fail to induce T_{RM} are largely ineffective against heterosubtypic influenza challenge[14]. The lung CD8 T_{RM} pool is comprised of two distinct populations, airway and interstitial T_{RM}, with unique functional properties. Airway T_{RM} are poorly cytolytic compared to interstitial T_{RM} yet are sufficient to protect against influenza challenge through the rapid production of antiviral cytokines[15]. In

addition, the number of airway T_{RM} correlates with the efficacy of cellular immune protection in the lung[8]. These findings have raised questions about the requirements for differentiation and maintenance of these distinct populations of lung T_{RM}, and about how environmental niches shape the function and lifespan of these cells, but to date the molecular underpinnings of differences between airway and interstitial T_{RM} have not been explored.

It was recently shown that lung T_{RM} were rapidly lost under steady-state conditions and the entire population had to be continually replenished by circulating T_{EM} through an antigen-independent process[16]. However, comparisons of T_{RM} in the lung and other barrier tissues show a remarkably consistent transcriptional profile, raising questions as to why the lifespan of T_{RM} would vary between tissues[6, 17, 18]. Furthermore, several recent reports have shown that antigen encounter in the lung tissue is required for T_{RM} differentiation, preventing circulating cells from replenishing lung T_{RM} following antigen clearance[19]. One potential explanation for these conflicting findings is that lung T_{RM} have often been investigated as a single population and not separated into airway and interstitial T_{RM} subsets. Given the known functional differences between these subsets and the distinct environments where they reside, a detailed comparison of airway and interstitial T_{RM} may shed new light on the mechanisms that control their unique biology and regulate lung T_{RM} decline.

In the present study, we examined the decline of cellular immunity to influenza virus over time, with a focus on comparing flu-specific lung T_{RM} in the airways and interstitium. We observed that the number of airway T_{RM} declined at a faster rate than interstitial T_{RM}. Parabiosis experiments showed that established airway and interstitial T_{RM} subsets do not freely exchange with the circulation, but are gradually lost due to apoptosis in the tissue. Transcriptome and chromatin accessibility analysis comparing flu-specific CD8 airway T_{RM}, interstitial T_{RM}, and circulating splenic T_{EM} revealed that the transcriptional and epigenetic programs of interstitial T_{RM} and splenic

T_{EM} were more closely related than airway T_{RM}. Analysis of airway T_{RM} found an enrichment of genes associated with the integrated stress response (ISR), notably the amino acid starvation pathway. We show that CD98, an amino acid transporter, was upregulated following exposure to the airway environment and deletion of *Ddit3*, a primary mediator of amino acid starvation-induced apoptosis, increased the lifespan of memory CD8 T cells transferred into the airways. Intratracheal transfer of T_{EM} into the airways showed that the induction of genetic programs related to amino acid starvation, apoptosis, and reduced cytolytic activity were due to the airway environment. In contrast, core T_{RM} signature genes were not impacted by the local tissue environment, but rather were regulated during the initial differentiation of airway T_{RM} following infection. The induction of genetic programs related to amino acid starvation and cell stress was unique to the quiescent airway environment, as T_{EM} transferred into inflamed airways failed to induce these programs. Finally, the programs of cell stress and apoptosis in airway T_{RM} could be rescued by transfer to a nutrient-rich environment. Overall, these findings provide new insight into the role of environmental cues in controlling the differential functions and lifespan of airway and interstitial T_{RM} and identify pathways that may be manipulated to improve the longevity of cellular immunity against respiratory pathogens.

Materials and Methods

Data Availability:

All sequencing data can be found in the NCBI Gene Expression Omnibus (GEO) under accession GSE118112. All code and data processing scripts are available from https://github.com/cdschar or upon request.

Mice:

C57BL/6J (WT), B6.SJL-Ptprca Pepc^{b}/BoyJ (CD45.1), and B6.129S(Cg)-*Ddit3*$^{tm2.1Dron}$/J (Ddit3$^{-/-}$) mice were purchased from The Jackson Laboratory and colonies were maintained at Emory University in specific pathogen-free conditions. Mice were between 8 and 13 weeks of age at the time of infection and housed under specific ABSL2 conditions following infection. All experiments were completed in accordance with the Institutional Animal Care and Use Committee guidelines of Emory University and Kindai University.

Infections:

Mice were either anesthetized with 300 mg/kg Avertin (2,2,2-Tribromoethanol, Sigma) or isoflurane (Patterson Veterinary) prior to infection. Mice were infected intranasally with a 30,000 50% egg infectious doses (EID_{50}) Influenza A/HKx31 (x31, H3N2) or 3,000 EID_{50} Sendai Virus in a total volume of 30µl. Heterologous challenge of x31-immune mice was performed with a 10 LD_{50} (3000 PFU) dose of Influenza A/PR8 (PR8, H1N1) in 50µl. Challenged mice were monitored daily for weight loss and humanely euthanized if they fell below 75% of original weight in accordance with Emory IACUC guidelines.

Intravenous labeling, Single Cell isolation and Staining:

Mice were intravenously labeled via tail vein injection under heat lamp with either CD3e (1.5µg of fluorophore-conjugated α-CD3ε antibody in 200µl PBS) or CD45.2 (2µg of fluorophore-conjugated α-CD45.2 antibody in 200µl PBS). Five minutes post i.v. injection mice were euthanized with Avertin and exsanguinated prior to harvest of bronchoalveolar lavage (BAL), lung and other tissues. Lung and other tissues were disassociated as previously described[15]. Single cell isolations were Fc-blocked using 2.4G2. Then surface stained with tetramers at room temperature for one hour, followed by surface staining with listed antibodies. Cell viability was determined by either Zombie NIR (Biolegend) or 7AAD. Tetramers were against influenza epitopes $NP_{366\text{-}374}$ D^b and $PA_{224\text{-}233}$ D^b . For *in vitro* cultures, total BAL and CD8 T cell-enriched spleens from influenza-immune mice were plated in round bottom plates in R10 (RPMI, 10% FBS, and 1% PSG) for 48 hours in a 5% CO2 incubator at 37 degrees prior to sorting for RNA isolation and downstream analysis. For RNA seq samples, $CD8^+$ $CD44^{Hi}$, $CD62L^-$ T cells were sorted from the BAL and spleen pre and post *in vitro* culture.

Antibodies and Flow Cytometry:

Marker	Clone	Manufacturer
CD8a	53-6.7	Biolegend
CD4	RM-45	Biolegend
CD69	H1.2F3	Biolegend
CD103	M290	BD
CD103	2E7	Biolegend
CD11a	M17I4	BD

CD11a	M17I4	Invitrogen
CD44	IM7	Ebioscience
CD44	IM7	Biolegend
CD62L	MEL-14	Biolegend
Zombie NIR		Biolegend
7AAD		Biolegend
CD3e	145-2C11	BD
CD45.2	104	Biolegend
CD98	RL388	Biolegend
CXCR3	CXCR3-173	Biolegend
CD45.1	A20	Biolegend
Annexin V		Biolegend
BCL2	3F11	BD

Tetramers were provided by the NIH Tetramer Core Facility at Emory (H-2D(b) Influenza A NP 366-374 (ASNENMETM) and H-2D(b) Influenza A PA 224-233 (SSLENFRAYV)). All samples were run on an LSRII or Fortessa X20 (BD Biosciences), or sorted on a FACSAria II (BD Biosciences). Flow cytometry data were analyzed with FlowJo v10 software.

Parabiotic Surgery:

Parabiotic surgery was performed as described[20] with the following modification. Each parabiont partner was infected with x31 (30,000 EID_{50}) and allowed to mature to a memory time point (28

days), then stitched together and maintained as parabiont pairs for 3 weeks. Equilibration was confirmed in the peripheral blood before separation, intravital labeling, and analysis.

Mixed bone marrow chimeras:

Mixed BM chimeras were generated as previously described[21] and allowed to reconstitute for 8 weeks before infection. CD45.1 and *Ddit3-/-* mice were used as bone marrow donors, and CD45.2/CD45.1 heterozygous mice were used as recipients. Mice were irradiated using RS2000 x-ray irradiator (Rad Source) and received two doses of 4.75 grays, 6 hours apart.

Intratracheal and Intraperitoneal Transfers:

Intratracheal (IT) and intraperitoneal (IP) transfers were performed as described[15,22]. Cells were isolated from the spleens of x31-immune mice (35-100 days post infection) and sorted on the $CD8^+$ $CD44^{Hi}$, $CD62L^-$ population to isolate T_{EM}. Between 5-10×10^4 cells were transferred intratracheally or intraperitoneally into congenic, infection-matched recipient mice and cells were collected by BAL or peritoneal lavage two days later. Cells were sorted on the basis of congenic marker staining to isolate transferred cells for downstream analysis.

Viral Titers:

Lung viral titers were measured following PR8 infection of naïve congenic mice receiving either WT or *Ddit3*$^{-/-}$ sorted airway T_{RM} as previously described. [23]

RNA-seq:

For each population, 1000 cells were sorted into RLT lysis buffer (Qiagen) containing 1% BME and total RNA purified using the Quick-RNA Microprep kit (Zymo Research). All resulting RNA was used as input for cDNA synthesis using the SMART-Seq v4 kit (Takara Bio) and 10 cycles of PCR amplification. Next, 1ng of cDNA was converted to a sequencing library using the NexteraXT DNA Library Prep Kit and NexteraXT indexing primers (Illumina) with 10 additional cycles of PCR. Final libraries were pooled at equimolar ratio and sequenced on a HiSeq2500 using 50bp paired-end or NextSeq500 using 75bp paired-end sequencing. Raw fastq files were mapped to the mm9 build of the mouse genome using Tophat2[24] with the mm9 UCSC KnownGene reference transcriptome[25]. The overlap of reads with exons was computed and summarized using the GenomicRanges[26] package in R/Bioconductor and data normalized to fragments per kilobase per million (FPKM). Genes that were expressed at a minimum of 3 reads per million in all samples for each cell typed were considered expressed. Differentially expressed genes were determined using the glm function in edgeR[27] using the mouse each cell type originated from a covariate. Genes with an FDR < 0.05 and absolute $\log_2$ fold-change (logFC) > 1 were considered significant. For GSEA[28], all detected genes were ranked by multiplying the sign of the fold change by the -$\log_{10}$ of the P-value between two cell types. The resulting list was used in a GSEA Preranked analysis.

ATAC-seq:

For ATAC-seq[29, 30], 2000 cells were isolated by FACS, resuspended in 50 l Nuclei isolation buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl2, 0.1% IGEPAL CA-630, molecular grade H2O, filter sterilized), and centrifuged for 30 min at 500xg at 4°C. Nuclei were then resuspended in 25 l Tagmentation reaction buffer (2X TD buffer, 1 µl Tagmentation enzyme,

molecular grade H2O, (Illumina, Inc.), incubated for 1 hr at 37°C, and DNA isolated by addition of 25 l Lysis Buffer (326 mM NaCl, 109 mM EDTA, 0.63% SDS) with incubation for 30 min at 40°C. Low molecular weight DNA was purified by SPRI-bead size selection and PCR amplified using Nextera indexing primer (Illumina) and 2x HiFi ReadyMix (KAPA Biosystems). Final libraries were purified by a second size-selection and pooled at equimolar ratio for 50 bp paired-end sequencing on a HiSeq2500. Raw fastq reads were mapped to the mm9 build of the mouse genome using Bowtie[31] with the default settings. For the analysis of accessible regions, first accessible peaks were identified for each sample using MACS2[32]. Second, all unique peaks were merged and the read depth annotated for each sample and normalized to reads per peak (rpm) using GenomicRanges[26] and R/Bioconductor. Differentially accessible regions (DAR) were determined using edgeR[27] and those with an FDR < 0.05 and absolute $\log_2$ fold-change (logFC) > 1 were considered significant. DAR were annotated to the nearest gene transcription start site (TSS) using HOMER[33].

Integrative analysis

To integrate the RNA-seq and ATAC-seq data we used a normalized Euclidean distance K-means clustering pipeline that we have previously described[34, 35]. First, DAR were annotated to the DEG using the overlap of Entrez ID resulting in 1,652 DAR mapping to 704 DEG. Second, for each DAR-DEG combination, data were aggregated by cell type, variance normalized, and a pair-wise Euclidean distance matrix calculated. The resulting matrix was K-means clustered using a K of 3. For each cluster, Gene Ontology (GO) analysis was performed on the DEG using DAVID[36] and enriched motifs in the DAR were identified with HOMER[33] using the 'findMotifsGenome.pl' script. All other data display was done in R/Bionconductor.

Statistical analysis:

Statistical analysis was performed with Prism (GraphPad Software). Each figure legend indicates methods of comparison and corrections.

Results

Airway T_{RM} decline more rapidly than interstitial T_{RM} following influenza infection

To investigate the loss of virus-specific airway and interstitial T_{RM} in the lung, we infected C57Bl/6J (WT) mice with influenza A/HKx31 (x31, H3N2) and analyzed the number of flu-specific CD8 T cells over time in the lung airways (BAL), lung interstitium (INT), and spleen, using intravital labeling, which allows for the identification of extravascular T cells in the tissue. To confirm previous findings that cellular immune protection against heterologous influenza challenge is rapidly lost, some mice were challenged with influenza A/PR8 (PR8, H1N1) at 1, 3, or 8 months post-x31infection. In agreement with prior reports[7, 8], as time increased from the initial x31 infection, the mice showed progressively more weight loss (**Fig. 1a**) and decreased survival (**Fig. 1b**) following PR8 challenge. To examine the loss of flu-specific T_{RM} in different compartments of the lung we performed a time course covering the acute phase and including 6 months post-infection (**Fig. 1c, 1d**). We tracked CD8 T cells specific for two dominant epitopes, influenza nucleoprotein (FluNP) and acid polymerase (FluPA) to control for potential epitope-specific effects. Regardless of specificity, the number of both airway and interstitial T_{RM} gradually declined over time, whereas splenic memory CD8 T cell numbers remained mostly unchanged (**Fig. 1e, 1f**). We observed a similar trend following Sendai virus infection, demonstrating that this effect was not specific to influenza virus (**Supplementary Fig. 1)**. Notably, when comparing the rate of loss among these populations, airway T_{RM} were lost much more rapidly than interstitial T_{RM}, declining nearly 100-fold by 6 months post-infection. Together, these data show that while both lung airway and interstitial T_{RM} are lost over time, the decline of T_{RM} in the airways is significantly accelerated.

Airway and interstitial T_{RM} do not recirculate and express pro-apoptotic markers

To investigate potential mechanisms that could be contributing to the gradual decline of both airway and interstitial T_{RM}, we focused on several possibilities related to egress from the tissue or cell death *in situ*. First, we considered that loss of lung T_{RM} could be limited to a subset of cells. To address this possibility, we examined the expression of the residency markers CD69 and CD103 on flu-specific extravascular CD8 T cells in the lung (**Fig. 2a**). Absolute numbers of all subsets of flu-specific airway and interstitial T_{RM} declined regardless of CD69 or CD103 expression (**Fig. 2b**). Additionally, we examined the possibility that the gradual conversion of the circulating flu-specific memory CD8 T cell pool from T_{EM} to T_{CM} was contributing to the loss of lung T_{RM}. Similar to previous studies of respiratory virus infections[37], the conversion to T_{CM} was very gradual, and the number of flu-specific T_{EM} in the spleen modestly decreased by 6 months post-infection (**Supplementary Fig. 2**). Furthermore, we observed that $CD69^{+}$ and $CD69^{+}CD103^{+}$ lung T_{RM} declined significantly faster than spleen T_{EM}. In contrast, extravascular flu-specific cells in the lung that did not express CD69 and CD103, and were likely transiting T_{EM}, declined at the same rate as T_{EM} in the spleen (**Supplementary Fig. 2**).

To address whether the lung T_{RM} subsets were truly resident populations, or whether they were maintained by replenishment from the circulating memory T cell pool, we performed parabiosis experiments using congenic mice with established airway and interstitial T_{RM}. Four weeks post-infection with x31, mice were joined for three weeks prior to separation and intravascular labeling (**Fig. 2c**). Example staining from the lungs of two partner mice gated on flu-specific, extravascular CD8 T cells in the lung showed that over 90% of the cells were host-derived and expressed CD69 and CD103. In contrast, the few partner-derived cells present were $CD69^{-}$ $CD103^{-}$, suggesting they were transiting T_{EM} (**Fig. 2d**). Comparison across tissues showed that

flu-specific cells were in equilibrium in the spleen and in the lung vasculature (LV), but were significantly enriched for host-derived cells in both airway and interstitial T_{RM} (**Fig. 2e**). Furthermore, analyzing extravascular flu-specific INT T_{RM} using CD69 and CD103 expression found that greater than 95% of $CD69^+$ and $CD69^+CD103^+$ cells were host-derived (**Fig. 2f**).

These data indicated that lung T_{RM} were not exiting the tissue or being replenished by circulating T_{EM}. Therefore, we investigated the possibility that lung T_{RM} were being lost over time due to death in the tissue. On day 35 post-infection with x31, we examined expression of the pro-apoptotic marker Annexin V on flu-specific airway T_{RM}, interstitial T_{RM} and splenic T_{EM} (**Fig. 2g**). Extravascular flu-specific cells in both the airways and interstitium showed increased Annexin V staining compared to the spleen, supporting increased apoptosis of these populations (**Fig. 2h**). Furthermore, we observed a significant increase in apoptotic cells among $CD69^+$ airway T_{RM} (**Fig. 2i**). Together, these data show that the gradual loss of lung T_{RM} is due to increased apoptosis within the tissue. Additionally, the higher rate of cell death among airway T_{RM} suggests that unique microenvironments within the lung differ in their capacity to support T_{RM} survival.

Unique transcriptional programs of airway and interstitial T_{RM}

To gain further insights into the unique properties of each T_{RM} subset we used RNA-seq to compare the transcriptional profiles of flu-specific airway T_{RM}, interstitial T_{RM}, and splenic T_{EM} 35 days post-infection (**Fig. 3a**). Despite their shared residence within the lung, airway and interstitial T_{RM} were surprisingly disparate in terms of their transcriptional signatures. Principal component analysis (PCA) of 9,362 detected genes revealed that interstitial T_{RM} more closely aligned with splenic T_{EM} than with airway T_{RM} (**Fig. 3b**). Differentially expressed genes (DEGs) revealed a distinct expression profile for cells of each population and confirmed that airway T_{RM} were the

most transcriptionally distinct (**Fig. 3c**). We examined the correlation between the change in expression for DEG between airway T_{RM} (BAL) versus spleen T_{EM} and BAL versus interstitial T_{RM} (INT). This identified gene expression changes unique to each population, as well as those shared by both airway and interstitial T_{RM} (**Fig. 3d**). A selection of DEGs upregulated in airway T_{RM} (shown in black) includes genes involved in amino acid transport (*Slc1a4* and *Slc7a5*) and amino acid synthesis, whereas DEGs shared by airway and interstitial T_{RM} (shown in red) include genes associated with T_{RM} programming (*Itgae*) (**Fig. 3d**). Gene set enrichment analysis (GSEA)[28] identified a significant positive enrichment in genes associated with the unfolded protein response (UPR) in airway T_{RM} compared to both interstitial T_{RM} and spleen T_{EM}, suggesting stresses unique to the airway and not present in the lung interstitium or spleen (**Fig. 3e**). Airway T_{RM} were also negatively enriched for genes involved in the CTL pathway, which supports previous findings that airway T_{RM} are poorly cytolytic[15]. The large numbers of DEGs between airway and interstitial T_{RM} led us to confirm that both populations shared expression of a core set of known T_{RM} genes, including *Itgae*, *Cdh1*, *Ahr*, *Cxcr6*, *Klf2*, and *S1p1r*[6, 18] (**Fig. 3f**). Additionally, airway T_{RM} showed decreased expression of genes important for cytolytic function including *Prf1*, *Gzma*, *Gzmb*, and *Gzmk*. Finally, given the increased apoptosis of airway T_{RM} and enrichment for genes associated with cell stress, we compared the expression of DEGs associated with cell survival. Airway T_{RM} showed altered expression of DEGs related to intrinsic cell death, maintenance of cell survival under cell stress, and activation of the integrated stress response (ISR), including *Dusp1*, *Bax*, *Bcl2*, *Bbc3*, *Pim2*, and the pro-apoptotic transcription factor *Ddit3* (**Fig. 3f**). To confirm our RNA-seq findings, we performed a second analysis with cells sorted from additional cohorts of mice and included flu-specific T_{EM} from the lung vasculature to ensure processing of the lung tissue did not impact the genetic signature of interstitial T_{RM} (**Supplementary Fig. 3**). These data show a similar

pattern of gene expression to our initial analysis and confirm that influenza-specific interstitial T_{RM} and lung vascular T_{EM} have unique transcriptional signatures. In addition, protein staining of Bcl2 expression confirms that Bcl2 is significantly upregulated in airway T_{RM} (**Supplementary Fig. 4**). Together, these data show vastly different transcriptional profiles between airway and interstitial T_{RM}, supporting a critical role for the local microenvironment in regulating T_{RM} biology. In addition, the gene expression profile of airway T_{RM} suggests a population under cellular stress balancing both pro- and anti-apoptotic signals.

Differential epigenetic programming regulates lung T_{RM} cell survival and homeostasis

The immediate microenvironment can impact the epigenetic programming and function of immune cells[38], including those in the lung[39]. In order to determine the effect of tissue location on the epigenetic programming of airway and interstitial T_{RM}, we assessed chromatin accessibility using the assay for transposase accessible chromatin (ATAC-seq)[29, 30]. PCA of 47,683 accessible loci revealed that airway T_{RM} separated from both interstitial T_{RM} and spleen T_{EM} (**Fig. 4a**). As with the transcriptome data, these differences were not due to tissue processing and were consistent in a second cohort (**Supplementary Fig. 3**). Differentially accessible regions (DARs) were identified (**Fig. 4b**) and integrated with RNA-seq data to determine coordinated changes in the epigenome and transcriptome (**Fig. 4c**)[35]. Using K-means clustering, we observed three distinct patterns in the data (K1-3) that could be mapped to each of the cell types: K1 - spleen T_{EM}, K2 - interstitial T_{RM}, and K3 - airway T_{RM}. As observed with DEGs, airway T_{RM} have a unique chromatin accessibility landscape compared to both interstitial T_{RM} and spleen T_{EM} that results in coordinated changes in gene expression and suggests that the epigenetic architecture of airway T_{RM} may be impacted by their distinct environment.

We examined each pattern for the enrichment of transcription factor DNA-binding motifs within the DARs. All three cell types were enriched for ETS and RUNX motifs, which are common to memory CD8 T cells[34] (**Fig. 4d**). We found that K3, enriched in airway T_{RM}, was enriched for STAT5 and DDIT3 transcription factor-binding motifs. Airway and interstitial T_{RM} shared enrichment for AP-1 motifs while interstitial T_{RM} were uniquely enriched for FOS and CREM motifs. The unique accessibility footprint surrounding CREM, STAT5, and DDIT3 motifs was analyzed. As predicted by the motif enrichment, CREM motifs were highly accessible in interstitial T_{RM} compared to spleen T_{EM} and airway T_{RM} (**Fig. 4e**). Similarly, both STAT5 and DDIT3 were significantly more accessible in airway T_{RM} cells compared to the other cell types.

To complement the motif analysis, we performed a gene ontology analysis to determine a functional enrichment for genes in each pattern. The spleen T_{EM} pattern was enriched for genes associated with T cell differentiation and activation (**Fig. 4f**). Interestingly, the airway T_{RM} pattern was enriched for genes implicated in cellular homeostasis, ER stress, hypoxia, and glucose starvation. In interstitial T_{RM}, the extrinsic apoptosis pathway was uniquely enriched, which may explain their increased apoptosis compared to spleen T_{EM} and supports differential mechanisms for regulating cell death among lung T_{RM} populations. Airway T_{RM} DARs were enriched for DDIT3 motifs, which is a known to be activated by the integrated stress response and promote apoptosis in cases of unresolved cellular stress[40]. For example, *Slc7a5* and *Asns* contained DARs with increased accessibility and were expressed at higher levels in airway T_{RM} (**Fig. 4g**). Together these data show differential epigenetic and transcriptional programming of pathways related to cell stress and apoptosis in airway T_{RM}.

ISR driven by amino acid starvation controls the lifespan of airway T_{RM}

The ISR is activated by several different indicators of cellular distress, including viral infection, ER stress, and amino acid starvation, with the primary goal of inhibiting protein translation and ultimately inducing apoptosis if the distress is not resolved. Although all ISR triggers lead to a similar outcome of inhibiting protein translation, unique pathways are activated in response to these different stressors in order to restore cellular homeostasis[41]. To determine which of these pathways is primarily responsible for the gradual loss of airway T_{RM}, we focused on a set of DEGs known to be involved in responses to cellular stress. The majority of these transcripts that were upregulated in airway T_{RM} were involved in amino acid transport (*Slc25a22*, *Slc1a4*, *Slc7a5*), amino acid synthesis (*Mars*, *Lars*, *Sars*, *Aars*, *Asns*), recognition of uncharged tRNAs (*Eif2ak4*), cell cycle arrest (*Cdkn1a*), and pro-apoptotic transcriptional regulation in response to stress (*Ddit3*) (**Fig. 5a**). These data suggested that amino acid starvation may be the primary trigger for activation of the ISR in airway T_{RM}[41]. GSEA supported this hypothesis of ISR activation and amino acid starvation, as gene sets involved in intrinsic apoptotic signaling in response to ER stress and amino acid transport were highly enriched in airway T_{RM} compared to interstitial T_{RM} and spleen T_{EM} (**Fig. 5b**). To further investigate the impact of the airway environment on development of the amino acid stress response, we investigated the expression of the neutral amino acid transporter CD98, a heterodimer of Slc7a5 and Slc3a2, on airway T_{RM}, interstitial T_{RM}, and spleen T_{EM}[42]. We examined CD98 expression in combination with CD11a, which is highly expressed on memory CD8 T cells but is downregulated within 36 hours after entry into the airways[43]. Airway T_{RM} expressed significantly higher levels of CD98 compared to interstitial T_{RM} and spleen T_{EM} (**Fig. 5c, 5d**). Furthermore, increased CD98 expression in airway T_{RM} was limited to $CD11a^{Lo}$ cells, showing that upregulation of CD98 occurred shortly after entry into the airway environment (**Fig. 5d**). To confirm the airway environment itself was sufficient to drive upregulation of CD98, we

transferred spleen T_{EM} intratracheally (IT) into congenic recipient mice (**Fig. 5e**). Similar to airway T_{RM}, CD98 expression was significantly increased 4 days following IT transfer (**Fig. 5f**). In addition, CD98 upregulation correlated with duration in the airways as expression steadily increased on transferred cells over time (**Fig. 5g**).

As these data supported a role for amino acid starvation in mediating the loss of airway T_{RM}, we next assessed whether deletion of *Ddit3*, a downstream regulator of apoptosis via amino acid starvation that was specifically induced in airway T_{RM}, could increase the survival of memory CD8 T cells in the airway (**Fig. 5h**).[44, 45] Congenic WT and *Ddit3*$^{-/-}$ mixed bone marrow chimeras were infected with influenza x31 and the number of flu-specific CD8 T cells were assessed in the BAL, lung, and spleen during the peak of acute infection (day 10) and at memory (day 60) (**Fig. 5i**). After normalizing the ratio of WT to *Ddit3*$^{-/-}$ FluNP-specific CD8 T cells in the spleen, we observed no difference in the ratio of WT to *Ddit3*$^{-/-}$ FluNP-specific CD8 T cells between the airway and INT T_{RM} populations (**Fig. 5j**). Although these data show that *Ddit3*-deficiency was not sufficient to allow for the accumulation of flu-specific airway T_{RM} over time, the dynamic balance between cell death and cell recruitment that regulates the number of airway T_{RM} made it difficult to accurately define whether *Ddit3* was impacting the survival of T cells in the airway. Thus, we used a more controlled approach where we could assess cell survival in the absence of new cells being recruited into the airways. T_{EM} from the spleens of congenic WT and *Ddit3*$^{-/-}$ mice previously infected with influenza x31 were sorted and transferred 1:1 into the airways of infection-matched recipients (**Fig. 5k**). Within three days post-transfer, the ratio of transferred cells was significantly skewed towards *Ddit3*$^{-/-}$ cells, indicating increased survival of this population in the airways (**Fig. 5l**). To address whether deletion of *Ddit3* impacted cellular immunity, airway T_{RM} from influenza-immune WT and *Ddit3*$^{-/-}$ mice were transferred

intratracheally into the airways of naïve hosts prior to PR8 influenza challenge. Viral titers were similar between mice that received WT or *Ddit3*$^{-/-}$ airway T_{RM}, showing that *Ddit3* expression did not alter the protective functions of airway T_{RM} (**Supplemental Fig. 5**). Taken together, these data show the profound impact of the airway environment on T_{RM} biology and demonstrate that amino acid starvation contributes to the gradual loss of airway T_{RM}.

Activation of the ISR is driven by the airway environment under homeostatic conditions

Airway and interstitial T_{RM} reside in vastly different environments. The lung airways are nutrient poor, with high oxygen tension and surfactant content that exert stresses on normal cellular homeostasis. Although the epigenetic and transcriptional profiles of airway T_{RM} are consistent with cells exposed to such harsh conditions, we sought to provide a causative link between the microenvironment and the airway T_{RM} transcriptional signature. Therefore, we sorted flu-specific CD8 T_{EM} from the spleen 35 days post-infection and transferred them intratracheally (IT) or intraperitoneally (IP) into infection-matched congenic recipients (**Fig 6a**). Two days later, transferred cells were recovered from the airways or peritoneum, sort purified based on congenic marker expression, and RNA-seq was performed comparing pre- and post-transfer cells. Analysis of DEGs found 375 transcripts altered following airway exposure, whereas only 54 transcripts showed differential expression following transfer into the peritoneum (**Fig. 6b**). Importantly, genes for amino acid transporters, tRNA synthases, mediators of intrinsic cell death, and cell survival were among the upregulated DEGs following airway exposure, but not peritoneal exposure. GSEA comparing pre- and post-transfer samples revealed a significant enrichment for pathways mediating the amino acid starvation response following exposure to the airways, but not the peritoneum (**Fig. 6c)**[40]. Notably, these pathways are enriched specifically in T cells within the

airway environment, as published datasets from alveolar and lung interstitial macrophages showed no enrichment of genes involved in amino acid starvation or ER stress (**Supplementary Fig. 6**).

Cellular stress due to the nutrient-poor conditions of the airway environment during homeostasis may serve to limit the lifespan and effector functions of airway T_{RM} in order to prevent unnecessary immunopathology, but whether similar programming occurs upon entry into inflamed airways during an infection was unclear. In this scenario, death of infected cells and fluid leakage due to tissue damage may supply airway T cells with sufficient local nutrients to limit cellular stress in order to promote optimal effector functions. To test this, influenza-specific T_{EM} were transferred IT into the airways of mice on day 8 of a Sendai virus infection (**Fig. 6d**). As influenza and Sendai viruses are antigenically distinct and share no cross-reactive T cell epitopes, this approach allowed us to directly assess the impact of the inflamed airway environment on the programming of T_{EM}. Comparing T_{EM} transferred into quiescent (Post-IT Mem) versus inflamed airways (Post-IT Acute) found over 400 DEGs, and genes related to amino acid starvation, *Sars* and *Slc7a5*, and genes related to cell survival, *Myc* and *Bbc3*, were significantly increased following transfer into quiescent, but not acutely infected, airways (**Fig 6e**). Combining gene expression from both IT transfer conditions with pre-transfer and post-IP transfer samples shows no change in expression of *Sars* and *Slc7a5* between the pre-transfer, post-IP transfer, and post-IT acute samples, supporting that there are sufficient nutrients in acutely infected airways to prevent induction of the amino acid starvation response (**Fig. 6f**). These differences in gene expression were confirmed by surface expression of the amino acid transporter CD98 following transfer into these different environments (**Fig. 6g**). Together these data show the quiescent airway microenvironment can drive activation of amino acid stress response pathway in memory CD8 T

cells, and that local infection can alter this environment to provide sufficient nutrients which limit cellular stress in airway T cells.

Impact of environment and nutrient availability versus T_{RM} differentiation on the genetic program of airway T_{RM}

To determine the gene signatures that were due solely to the airway environment versus programmed during the initial differentiation of airway T_{RM} following viral clearance we compared all DEGs between airway T_{RM} and spleen T_{EM} (from **Fig. 3**) with DEGs pre- and post-transfer into the airways. We hypothesized that this would separate potential effects of the airway environment from the core transcriptional T_{RM} program induced during differentiation. In agreement with GSEA, a number of genes associated with amino acid starvation, such as *Slc7a5*, *Asns*, and *Myc*, were regulated solely by the airway environment, whereas known T_{RM} genes such as *Itgae* were regulated only during differentiation and independent of the tissue environment (**Fig. 7a**). Expanded comparisons of select DEGs across all cell populations (airway T_{RM}, interstitial T_{RM}, spleen T_{EM}, and pre-and post-transfer) showed that while DEGs associated with cell stress or lack of CTL activity are only observed in cells exposed to the airway environment, DEGs that comprise known T_{RM} genes are similarly expressed only in airway and interstitial T_{RM} (**Fig. 7b**). These data suggest that the initial differentiation program of lung T_{RM} is shared between cells in the airways and interstitium, with subsequent differences being driven by adaptations to the local environment.

With activation of the ISR being a key adaptation of T_{RM} to the quiescent airway environment, it raised the question of whether the cellular stress and pro-apoptotic program of airway T_{RM} could be rescued if nutrients were no longer limiting. To examine the impact of nutrient

restoration, we took airway T_{RM} and splenic T_{EM} from influenza-immune mice and cultured the cells for two days without stimulation *in vitro* (**Fig. 7c**). As shown in the heatmap of DEGs, airway T_{RM} cultured *in vitro* show substantial changes in gene expression, whereas splenic T_{EM} show only minor variations (**Fig. 7d**). Changes in airway T_{RM} gene expression divided into three clusters of DEGs, and gene ontology analyses showed processes involved in apoptosis, tRNA charging, and the response to ER stress being significantly altered in airway T_{RM} following *in vitro* culture (**Fig. 7e**). Notably, a number of genes involved in DNA damage repair are induced in airway T_{RM} following *in vitro* culture, consistent with DNA damage known to occur following serum starvation.[46]. Specific genes related to the ISR, including *Slc7a5*, *Slc3a2*, *Ddit3*, *Sars*, *Aars*, and *Bbc3*, are down-regulated following culture of airway T_{RM} in a nutrient-rich environment, demonstrating this pathway is reversible and driven by the local microenvironment (**Fig. 7f, 7g**). Together these data define the key transcriptional programs induced by T_{RM} differentiation versus the airway environment, and show the ISR driven by amino acid starvation can be rescued by the presence of sufficient nutrients in the local microenvironment.

Discussion

Despite the importance of lung airway and interstitial T_{RM} for effective cellular immunity against respiratory pathogens, the impact of the local environments on their maintenance and effector functions has been largely unexplored. Here we demonstrated that airway and interstitial T_{RM} populations have profoundly different transcriptional and epigenetic profiles due to their unique localization within the tissue. Although both airway and interstitial T_{RM} gradually decline over time, airway T_{RM} show increased apoptosis, decreased expression of genes associated with cytolytic function, and a transcriptional signature indicative of increased cellular stress due to amino acid starvation. The transcriptional and epigenetic profile unique to airway T_{RM} was not programmed during initial T_{RM} differentiation, but by exposure to the airway environment. Conversely, comparisons of naturally generated airway T_{RM} and T_{EM} transferred into the airways showed that expression of known core T_{RM} genes were largely unaffected by the environment, and were instead regulated during initial T_{RM} differentiation. Thus, the cells that comprise the overall lung-resident T cell pool are comprised of functionally and genetically distinct airway T_{RM} and interstitial T_{RM} populations that share a common T_{RM} differentiation program but are further shaped by their respective microenvironments.

The molecular programming of immune responses is controlled by distinct mechanisms that include lineage specification and the local tissue microenvironment[38, 39]. CD8 T_{RM} share a core lineage programming that is driven by interactions between Blimp1 and Hobit transcription factors[6]. Using chromatin accessibility as a readout for epigenetic states, we found that the accessible chromatin of all three subsets of memory CD8 T cells was enriched for ETS and RUNX transcription factor motifs, indicating that these factors contribute to the specification of the CD8 T cell memory lineage. The accessible chromatin of both lung T_{RM} populations was enriched for

AP-1 transcription factor motifs that signal from cytokine receptors, implicating a role for the environment in shaping the epigenome of these populations. However, airway T_{RM} were specifically enriched for *Ddit3* motifs and coordinately upregulated genes in the amino acid starvation response, with distinct accessible chromatin peaks at stress response genes such as *Slc7a5* and *Asns*. Consistent with the concept that the T_{RM} molecular programming is both lineage and environmentally determined, we observed only a partial adoption of the airway T_{RM} program following intratracheal transfer of splenic T_{EM} into the airways. The amino acid stress response, but not core T_{RM} genes, was induced following intratracheal transfer. These data show that core programming of airway T_{RM} is not induced by exposure to the local microenvironment. Rather, regulation of the T_{RM} gene signature in airway T_{RM} is induced during their initial differentiation following pathogen clearance. Thus, these data identify a common lung T_{RM} signature and separate this from the impact of distinct microenvironments within the lung.

Airway T_{RM} were previously shown to have a limited lifespan, and *in situ* labeling experiments have estimated memory CD8 T cells can survive in the airway environment for several weeks[43]. Although airway T_{RM} decline more rapidly than interstitial T_{RM}, airway T_{RM} are nevertheless present for at least 3 months post-infection. This discrepancy in timelines, with the half-life of an individual cell being two weeks whereas the overall population is still present at three months, supports prior studies that show the airway T_{RM} pool is maintained by the continual recruitment of new cells into the airways[22]. The waning of the airway T_{RM} pool over time suggests that the source of these newly recruited cells must also be similarly transient, but whether these cells come predominantly from the circulation or from within the tissue was less clear. However, the parabiosis of influenza-immune mice demonstrated that both airway and interstitial T_{RM} are not maintained by recruitment of circulating cells into the lung T_{RM} pool, suggesting the newly

recruited airway T_{RM} are mostly derived from the interstitial T_{RM} pool. In addition, the small number of flu-specific, extravascular cells derived from the parabiont partner in the lung airways and interstitium did not express CD69 or CD103 and thus are likely T_{EM} migrating through the tissue. The inability of circulating T_{EM} to convert to $CD69^{+}CD103^{+}$ T_{RM} in the lung agrees with several recent studies showing that antigen encounter in the pulmonary environment, and not simply entry to the lung tissue itself, is required for T_{RM} differentiation[14, 20, 47]. These data are in contrast to a recent study showing that circulating T_{EM} were able to re-seed the lung T_{RM} pool.[16] In support of this, it was shown that lung T_{RM} could develop even in the absence of pulmonary antigen encounter under specific inflammatory conditions.[48] This may explain why boosting of the circulating T_{EM} pool was able to enhance lung T_{RM} numbers, while in our study we observed no contribution of the T_{EM} pool under non-inflammatory, homeostatic conditions.

The poor longevity of T_{RM} in the airways and interstitium observed in animal models after respiratory viral infections would be an impediment to the development of cell-mediated vaccines against respiratory pathogens, but several studies have reported approaches that maintained lung T_{RM} and protective cellular immunity for at least one year. Comparison of primary and quaternary CD8 T cell memory showed that lung T_{RM} from repeated influenza infections were resistant to apoptosis in the lung tissue and persisted at much larger numbers[49], suggesting prime-boost strategies may program cells for improved survival in the tissue. Similarly, lung T_{RM} were sustained following vaccination with replication-defective adenoviruses expressing 4-1BBL[50], and it was evident that activation signals in the lung environment were necessary for sustaining lung T_{RM}. Additional research into the mechanisms behind the longevity of lung T_{RM} observed in these studies is needed to better inform the rational design of cell-mediated vaccines.

These findings raise another important question regarding the utility of having a relatively short-lived airway T_{RM} population. Severe influenza infections are accompanied by extensive tissue damage that leads to fluid leakage in the airspaces. Under these conditions, virus-specific CD8 T cells in the airways would be in an environment rich in nutrients and amino acids, and thus able to maintain their cytolytic function to lyse infected cells and promote viral clearance[51]. Indeed, we show that transfer of T_{EM} into acutely infected airways prevents the induction of the ISR and pro-apoptotic pathways, even in the absence of cognate antigen. In contrast, the nutrient poor conditions during homeostasis may serve as a brake on CTL activity by altering the genetic program to avoid unnecessary damage to the epithelial lining of the airways. Notably, this nutrient poor environment does not influence the secretion of antiviral cytokines such as IFN-γ, which is critical for protection mediated by airway CD8 T_{RM}[15]. Therefore, the difference in nutrient availability among distinct microenvironments of the lung may create a division of labor among the lung T_{RM} pool, with T_{RM} in quiescent airways serving primarily a "sensing and alarm" function through the rapid production of cytokines and chemokines that could recruit cytolytic interstitial T_{RM} and other immune cells to the site of infection[2].

In summary, we show that the overall lung T_{RM} pool is comprised of two distinct subsets, airway T_{RM} and interstitial T_{RM}, with different cell functions and cell fates that are shaped by epigenetic reprogramming in response to environmental cues. Our findings show the amino acid starvation response in airway T_{RM} shuts down the cytolytic program and promotes intrinsic apoptosis leading to the gradual homeostatic decline of lung T_{RM}. This may be of particular importance to abnormal settings such as the tumor microenvironment, where alterations in nutrient density could influence the effector functions and longevity of responding T cells. One limitation of our study is that we do not a specific nutrient-molecular axis that primarily drives the re-

programming of T_{RM} in the airways, only that restoration of a complete nutrient-rich milieu is sufficient to restore homeostasis in airway T_{RM}. CD98, a heterodimer composed of Slc3a2 and Slc7a5, preferentially transports large neutral amino acids, and it will be of interest to determine whether deficits in specific amino acids within the airway environment are responsible for inducing cellular stress in airway T_{RM}. Developing a more thorough understanding of how tissue microenvironments in the lung influence the genetic program of resident T cells and defining mechanisms by which cells adapt to and possibly overcome these challenging environments will assist in the rational design of cell-mediated vaccines against respiratory pathogens.

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Author Contributions:

S.L.H., C.D.S., J.M.B., and J.E.K. designed the study. S.L.H., C.D.S., E.K.C., Z-R.T.L. and S.T. performed experiments. S.L.H., C.D.S., E.K.C., Z-R.T.L., S.T., and J.E.K. analyzed data. S.L.H., C.D.S., J.M.B., and J.E.K. wrote the manuscript.

Figures

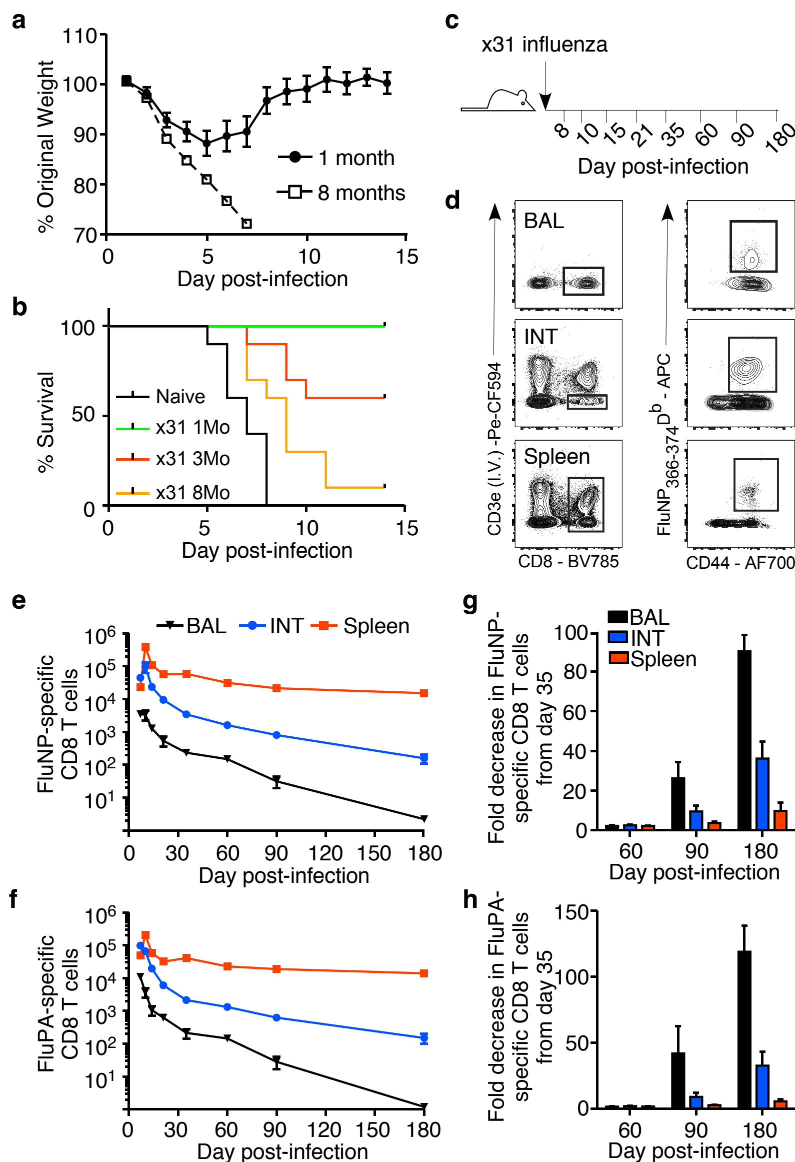


Figure 1. **Decline of airway and interstitial T_{RM} over time. (a)** Morbidity curve examining decline of protection from heterologous challenge. **(b)** Mortality curve examining decline of protection from heterologous challenge over time following a 10x LD_{50} PR8 challenge. **(c)** Experimental design. **(d)** Example intravital staining, showing FluNP-specific T cells derived from resident population. **(e, f)** The number of FluNP-specific **(e)** and FluPA-specific **(f)** CD8 T cells

from the spleen (red line), airway resident (BAL - black line) or lung interstitium resident compartments (INT - blue line) was determined at various times after influenza infection. Data represented as mean ± SEM. **(g, h)** Fold decrease in FluNP-specific CD8 T cells compared to d35 **(h)** and fold decrease in FluPA-specific CD8 T cells to d35. Data represented as mean ± SEM. Derived from data in **(e, f)**. n-10 mice for 1 mo, n=19 mice for 8 mon (a,b). n=9-10 for all time points (except d10 for 5) (e,f) Data further derived from mice used in Fig 1 e,f (d,g,h).

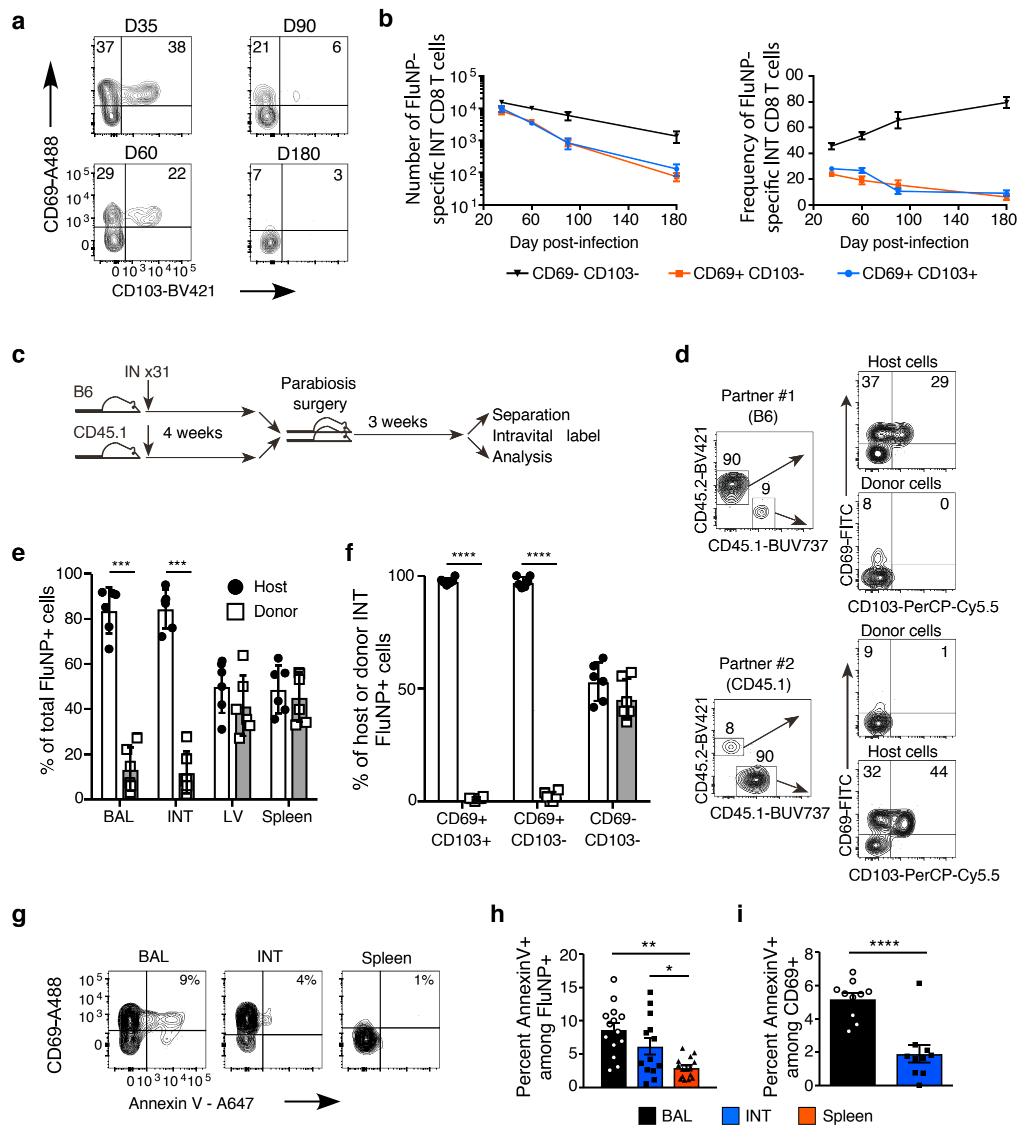


Figure 2: **In situ apoptosis drives lung T$_{RM}$ decline. (a)** Example staining reflecting CD69 and CD103 expression on FluNP-specific INT **(b)** Cell count of CD103/CD69 subsets in resident compartment and frequency of FluNP-specific lung T$_{RM}$ with the indicated phenotype. Data represented as mean ± SEM. **(c)** Experimental design of parabiosis experiment. **(d)** Example staining of host and donor flu-specific lung T$_{RM}$ from one parabiont pair. **(e)** Frequency of donor and host flu-specific CD8 T cells in various tissues of parabiosis partners. Data represented as

mean ± SEM. Multiple t tests were performed. 6 parabiont pairs were sacrificed with an n=12. **(f)** Frequency of host or donor flu-specific INT CD8 T cells from parabiosis partners based on CD69 and CD103 expression. **(g)** Example staining of AnnexinV on $CD69^+$ FluNP-specific CD8 T cells. Mice were infected with x31 (H3N2) and airways (BAL), lung (INT) and spleen were harvested at day 35 PI. **(h)** Frequency of Annexin V on $FluNP^+$ CD8 T cells. Statistical significance determined via paired Student's t test. **(i)** Frequency of Annexin V on $CD69^+$ CD8 T cells. Statistical significance determined via unpaired Student's t test. n=9-10 for all time points (a,b) with data further derived from mice used in Fig 1 e,f . n=12 . n=14 for (g,h,i). P values are as follows: * = $p<0.05$, ** = $p<0.01$, *** =$p<0.001$, **** = $p<0.0001$

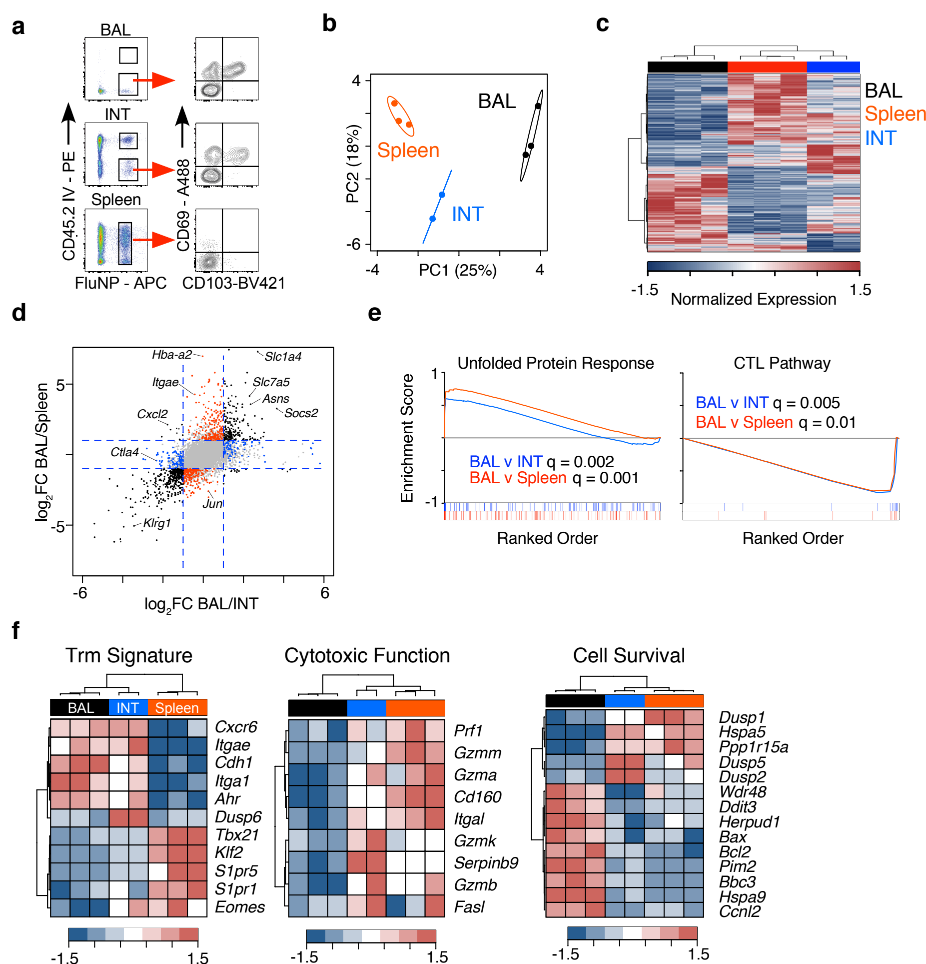


Figure 3: **Influenza-specific lung airway and interstitial T_{RM} have distinct transcriptional profiles. (a)** Example intravital staining and sorting gates for RNA-seq designated with red arrow. **(b)** PCA of 9,362 detected genes. Circles represent 99% confidence intervals. BAL (airway T_{RM}, n = 3), spleen (effector memory T_{EM}, n = 3), and INT (lung interstitium T_{RM}, n = 2). **(c)** Heatmap of 1,622 genes differentially expressed genes (DEG) between any two cell types. **(d)** Scatterplot examining correlation of DEG between BAL versus Spleen and BAL versus INT. Genes in black are DEG in BAL compared to both spleen and INT, red are DEG between only BAL and spleen, and blue are DEG between INT and spleen. **(e)** Gene set enrichment analysis (GSEA) of the unfolded protein response and CTL pathway gene sets comparing BAL to INT and spleen. **(f)**

Heatmap of select genes derived from **(c)** related to a core T_{RM} signature, cytotoxic function, and cell survival.

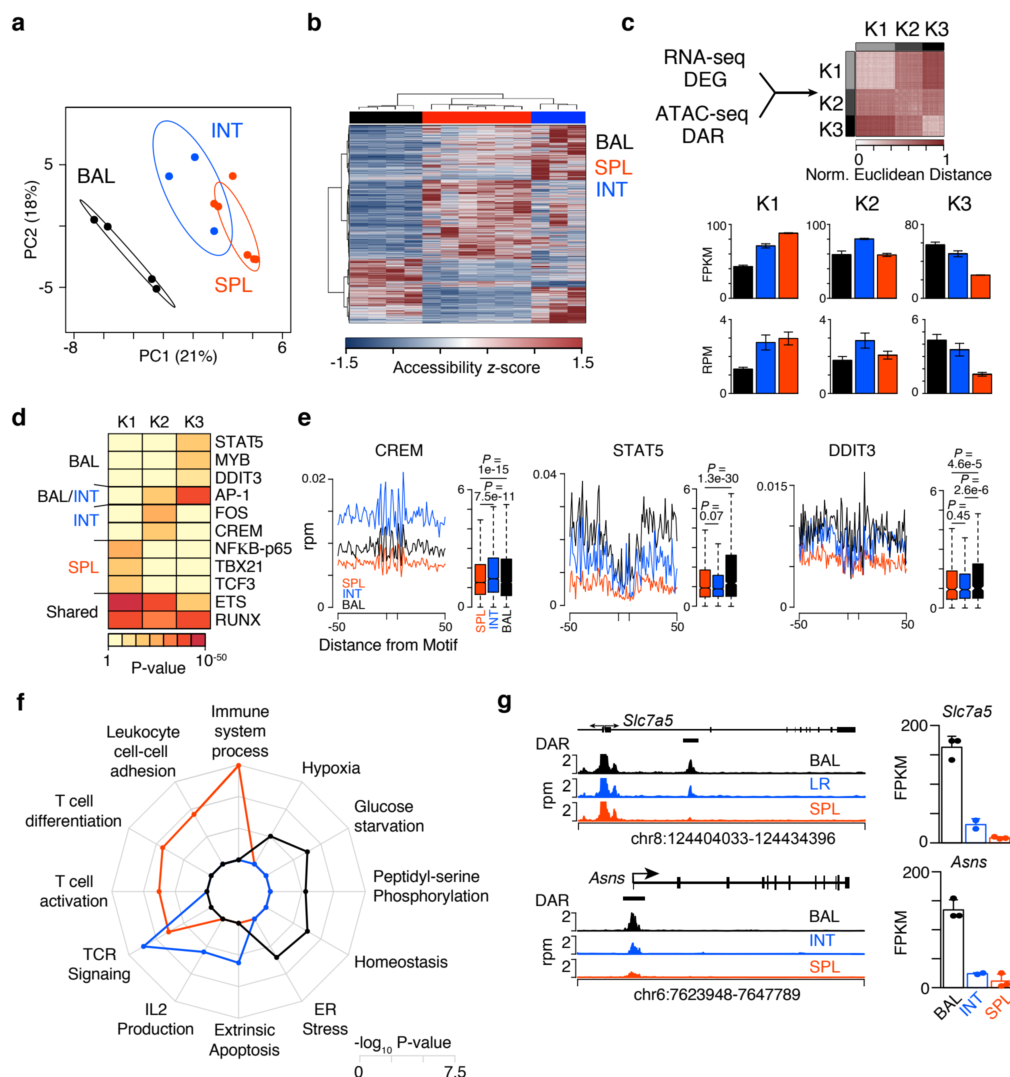


Figure 4: **Chromatin accessibility reveals a distinct epigenetic programming of airway and lung interstitial T_{RM} populations. (a)** PCA of 47,683 accessible loci in each cell type. Circles represent 99% confidence intervals. BAL (airway T_{RM}, n = 4), spleen (effector memory T_{EM}, n = 6), and INT (lung interstitium T_{RM}, n = 3). **(b)** Heatmap of 8,772 differentially accessible regions (DAR) between any two cell types. **(c)** Integrative analysis of DEGs and DARs using k-means clustering. Barplots showing the expression (top) and accessibility (bottom) for each cell type in each of the K-patterns. FPKM, fragments per kilobase per million; RPM, reads per million. Data

represent mean ± SD. **(d)** Heatmap displaying the enrichment of transcription factor motifs for DAR within each of the K-patterns from **(c)**. P-value determined by HOMER using a binomial distribution. **(e)** Histogram (left) and boxplot (right) of accessibility surrounding the indicated motif for each of the cell types. Boxplot center line indicates data median, lower and upper bounds of boxes the 1st and 3rd quartile ranges, and whiskers the upper and lower ranges of the data. Data represents the mean accessibility for each cell type. Significance determined by two-tailed Student's *t*-test. **(f)** Radar plot of GO pathway enrichment for each of the K-patterns from **(c)**. Significance determined by Fisher's exact test. **(g)** Genome plot (left) showing the accessibility pattern at the indicated loci for each cell type. For each gene, the direction of transcription and location of DAR are annotated. A barplot showing the expression of each gene is also depicted (right). Data represent mean ± SD.

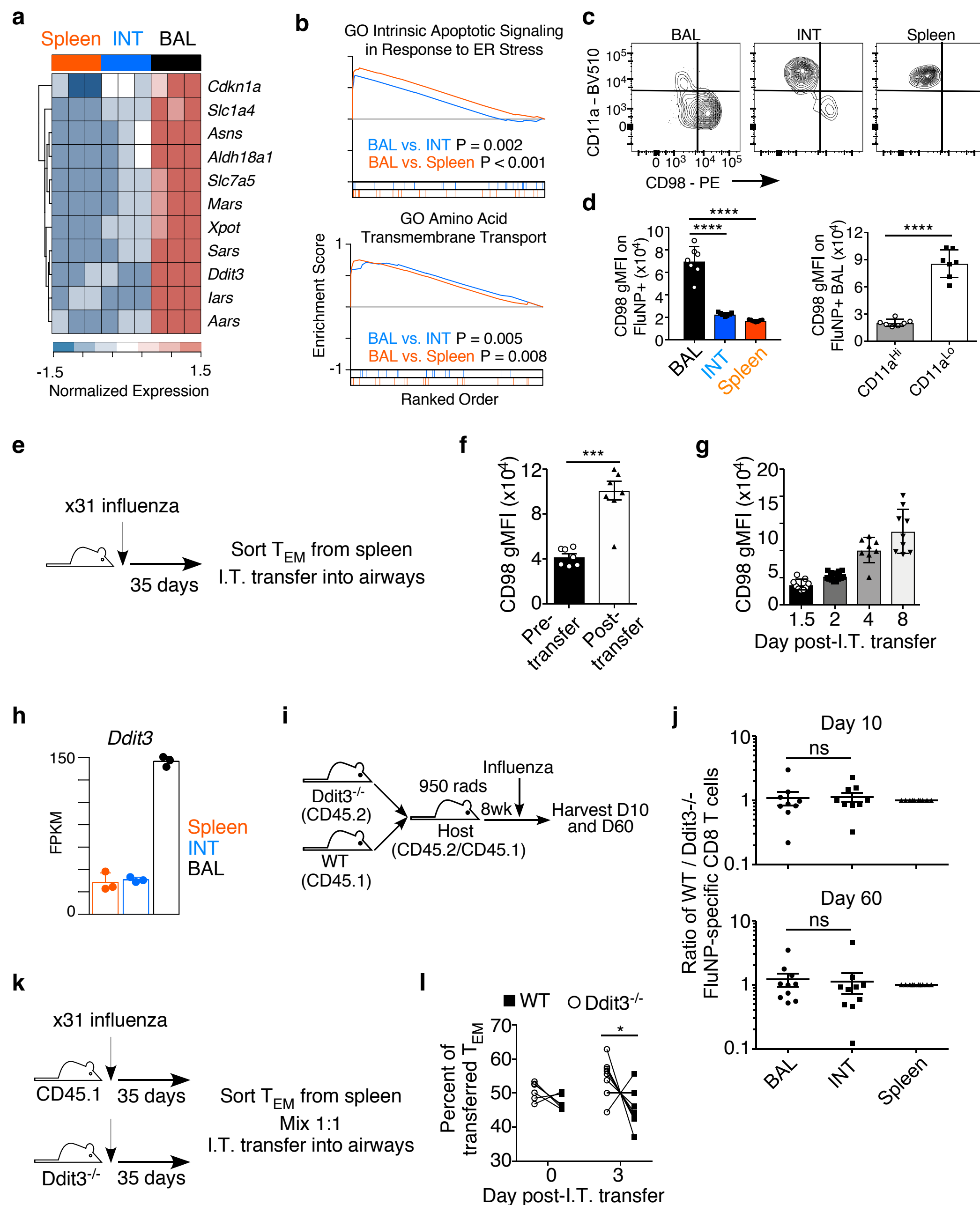


Figure 5: **Exposure to the airway environment drives activation of the integrated stress response.** (**a**) Heatmap for selected ISR genes associated with the amino acid starvation response. (**b**) GSEA of intrinsic apoptotic signaling in response to ER stress and amino acid trans-membrane transport gene sets comparing of BAL to INT and BAL to spleen. (**c**) Example CD11a by CD98 staining from mice infected with x31 (H3N2) 35 days prior. Samples are gated on flu-specific T_{RM}

(BAL and INT) or flu-specific T_{EM} (spleen) (**d**) gMFI of CD98 measured on FluNP-specific $CD69^+$ cells from BAL (airway T_{RM}) compared to spleen T_{EM} and INT (lung interstitial T_{RM}), and on $CD11a^{Hi}$ or $CD11a^{Lo}$ cells in the BAL. n=7 (c,d). A Kruskal Wallis Test on gMFI between BAL, INT, and spleen determined statistical significance and Mann Whitney U test was performed to compare $CD11a^{Hi}$ or $CD11a^{Lo}$ cells in the BAL. (**e**) Experimental design of intratracheal transfer experiment. (**f**) $CD44^{Hi}$ CD8 T_{EM} from spleen were isolated from influenza infected memory mice, intratracheally transferred into congenic recipients, and isolated by airway lavage (BAL) four days later. gMFI of CD98 was determined on pre-sort and transferred cells n=8. Statistical significance was determined by Mann Whitney U test. (**g**) gMFI of CD98 over time following transfer into the airways n=8-15 per day. (**h**) Expression of *Ddit3* from RNA-seq in BAL, spleen, and INT, shown as FPKM (Fragments Per Kilobase Per Million reads). Data represent mean ±SD. (i) Experimental design of 50:50 (Ddit3-/- : WT) mixed bone marrow chimeras. (j) Ratio of WT to Ddit3-/- FluNP specific CD8 T cells in BAL and INT normalized to the ratio in spleen (n=9-10 mice per timepoint). (**k**) Experimental design of 1:1 intratracheal transfer of $Ddit3^{-/-}$ and congenic WT cells. (**l**) Frequency of $Ddit3^{-/-}$ and WT cells prior to (day 0) or 3 days following intratracheal transfer. Data represented as mean ± SD. Statistical significance was determined via multiple (**d, f, j**) or paired (**i**) t-tests. P values are as follows: * = $p<0.05$, ** = $p<0.01$, *** =$p<0.001$, **** = $p<0.0001$.

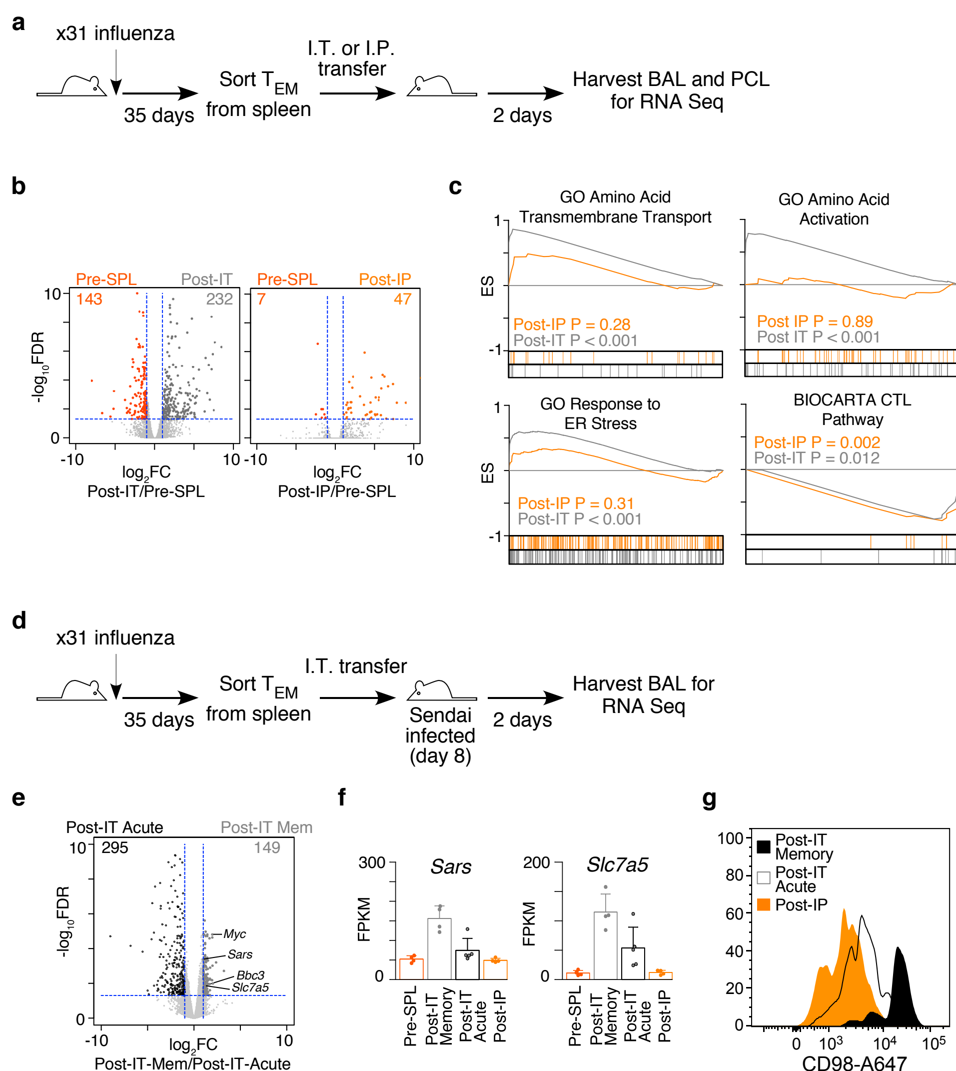


Figure 6: **The airway environment is sufficient to alter the transcriptional program of memory CD8 T cells but does not induce core T$_{RM}$ programming.** (**a**). Experimental design of intratracheal (IT) and intraperitoneal (IP) transfer experiment. (**b**) Volcano plot of DEG between pre-transfer spleen TEM (pre-SPL) (n=4) and post IT (n=4) and IP (n=4) transferred T$_{EM}$ into the airway. Example genes are highlighted and the number of DEG are indicated. **(c)** GSEA on pre- and post-transfer using the indicated gene sets. (**d**) Experimental design of intratracheal acute experiment (acute). (**e**) Volcano plot of DEG comparing between post intratracheal transfer into

acute Sendai virus infection airway and post intratracheal transfer into memory airway. Example genes are highlighted and the number of DEG are indicated. (n=5 for acute transfer samples) (**f**) Barplot showing the expression of cell stress genes (*Sars*, *Slc7a5*, *Ddit3)*. Data represent mean ±SD. *(***g**) Example histogram of CD98 staining on CD44 high CD8 T cells on all post transfer samples (memory, acute, and IP).

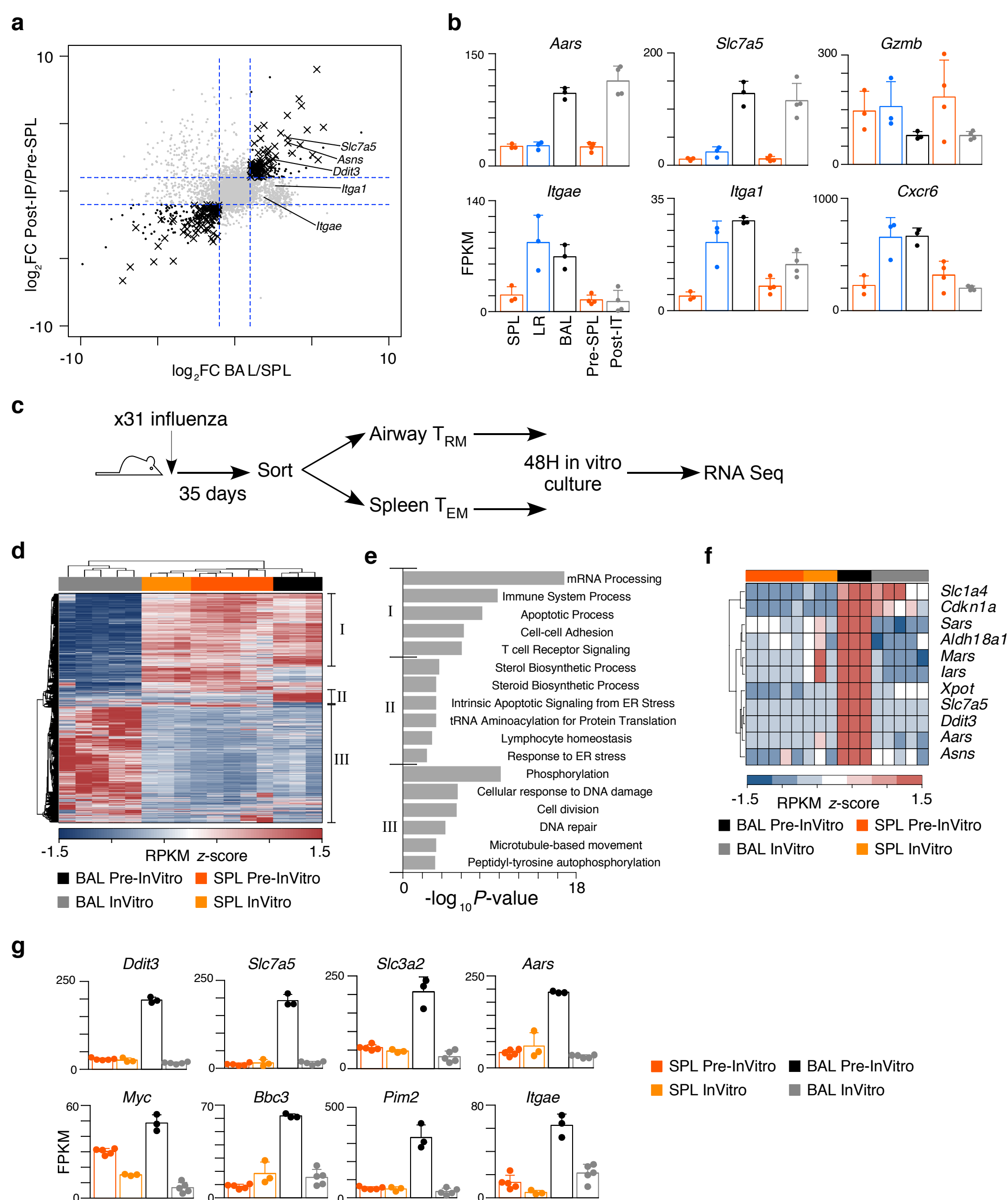
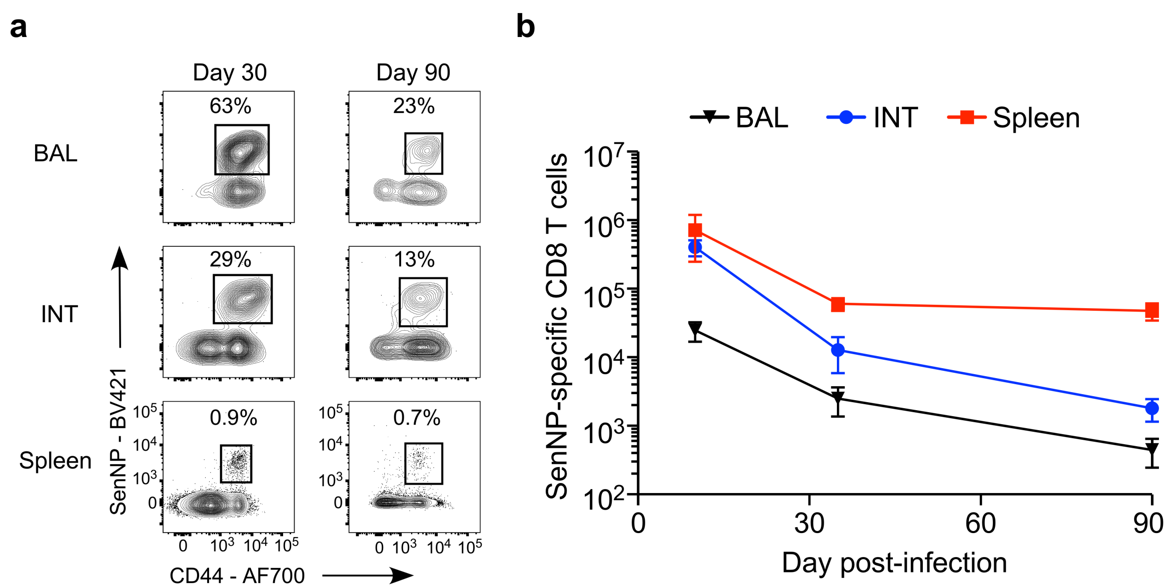


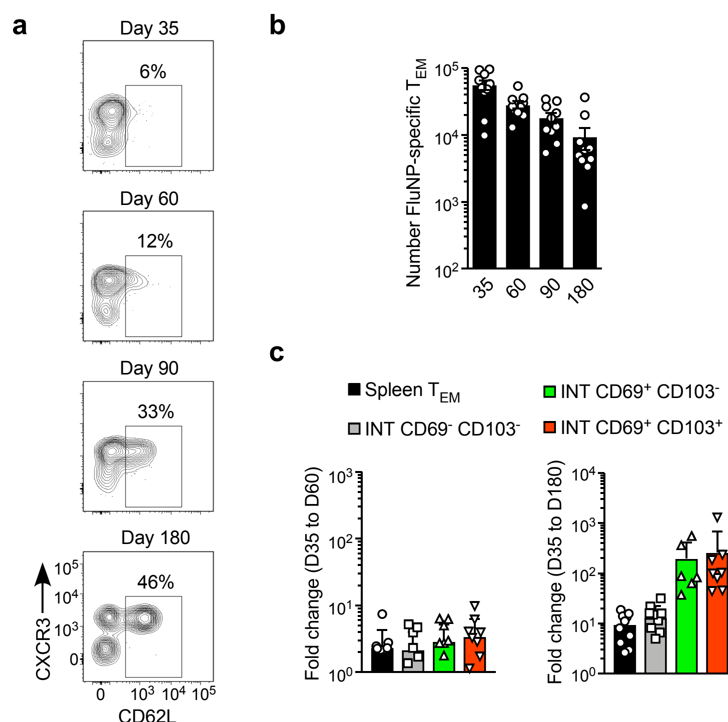
Figure 7: **Restoration of a nutrient-rich environment resolves environmentally driven cellular stress in airway T_{RM}. (a)** Scatterplot correlating the DEG between pre- and post-transferred T_{EM} and BAL T_{RM} versus spleen T_{EM} (SPL). The location of select genes are indicated. X indicates DEG in both comparisons. **(b)** Barplot showing the expression of cell stress (*Aars*, *Slc7a5*, *Ddit3*) and T_{RM} (*Itgae*, *Itga1*, *Gzmb*) signature genes. FPKM (Fragments Per Kilobase Per Million reads).

Data represent mean ± SD. (**c**) Experimental design. (**d**) Heatmap of 5,715 genes differentially expressed genes (DEG) between any two cell types comparing spleen and BAL pre and post in vitro (n=5 for BAL, n=3 for SPL). (**e**) GO pathway analysis for three distinct regions of gene expression. Significance determined by Fisher's exact test. (**f**) Heatmap for selected ISR genes associated with the amino acid starvation response. (**g**) Barplot showing the expression of amino acid stress *(Slc7a5, Slc3a2, Aars*,), pro-apoptotic (*Ddit3, Bbc3),* cell stress *(Myc, Pim2)* and cell adhesion (*Itgae*) genes. FPKM (Fragments Per Kilobase Per Million reads). Data represent mean ± SD.

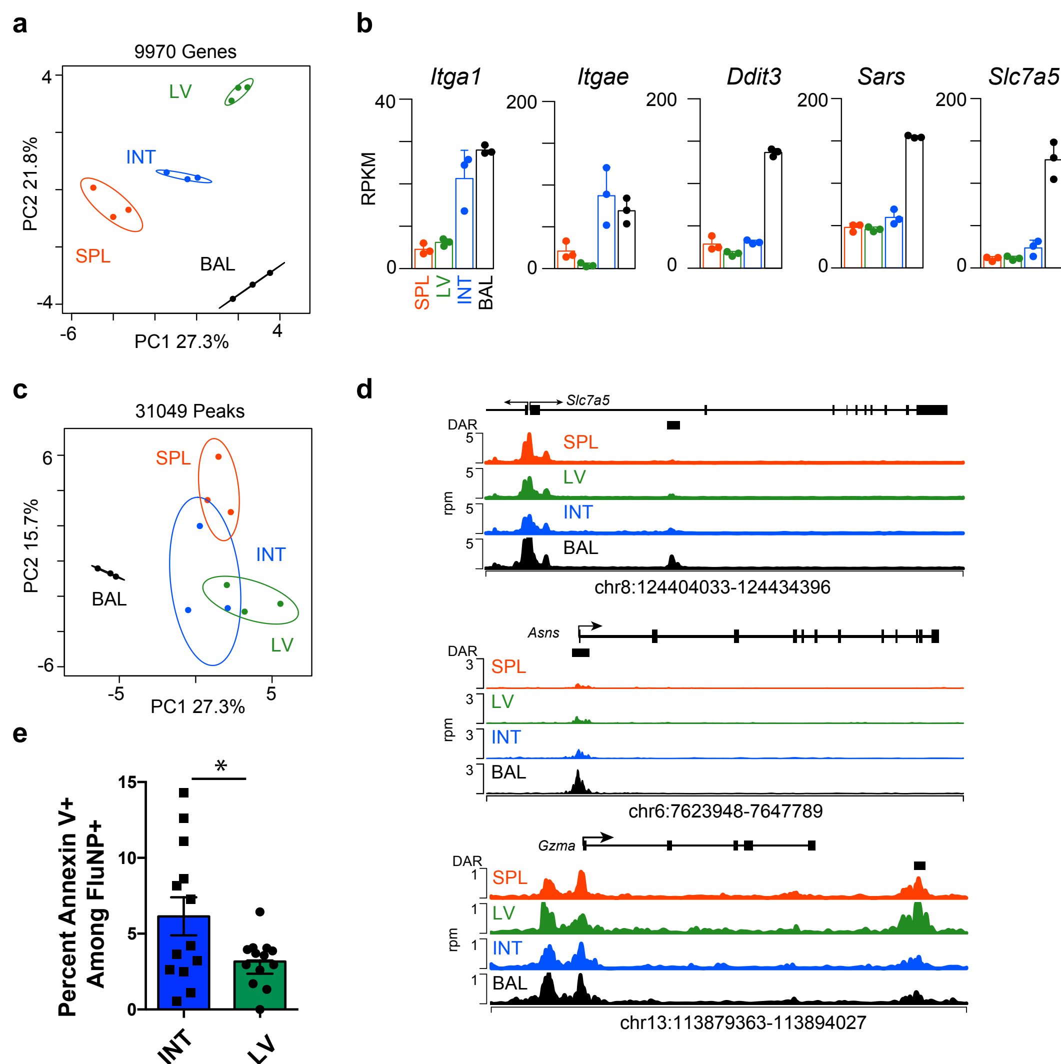
Supplementary Information



Supplementary Fig 1: Sendai-specific CD8 TRM in the airways and interstitium gradually decline over time. (a) Example staining reflecting Sendai NP tetramer staining of BAL, INT, and Spleen and CD44. **(b)** Number of Sendai NP-specific CD8 T cells in the airway (BAL), lung interstitium (INT), and spleen following infection with Sendai virus. n=10 for all timepoints. Data represented as mean ± SEM.

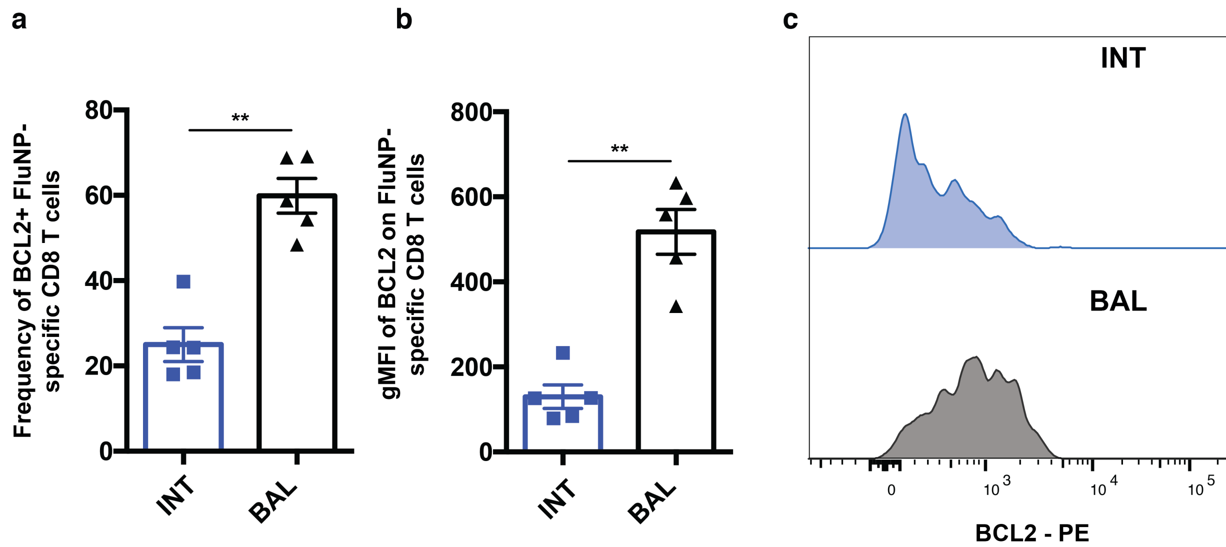


Supplementary Fig 2: Lung T_{RM} decline more rapidly than circulating T_{EM}. (a) Example staining of CXCR3 and CD62L on flu-specific splenic T_{EM} over time. **(b)** Number of flu-specific splenic T_{EM}. **(c)** Fold change in spleen T_{EM} and subsets of lung interstitium (INT) T cells defined by CD69 and CD103 expression between day 35 and day 60 (left graph) and between day 35 and day 180 (right graph). n=10 for all time points and data are derived from mice in Fig. 1. Data represented as mean ± SEM.

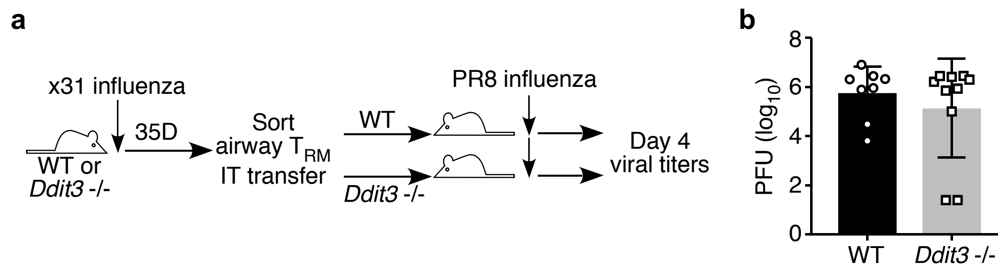


Supplementary Fig 3. Validation of BAL-specific epigenomic and transcriptome profiles. (a) Principal component analysis of 9,970 detected genes in SPL (n=3), LV (N=3), INT (N=3) and BAL (n=3). Points denote samples and circles show 99% confidence intervals for each cell type. **(b)** Bar plots of FPKM normalized gene expression for the indicated genes. Data represent mean ± SD. **(c)** Principal component analysis of 31,049 accessible peaks in SPL (n=3), LV (N=3), INT (N=3) and BAL (n=3). Points denote samples and circles show 99% confidence intervals for each

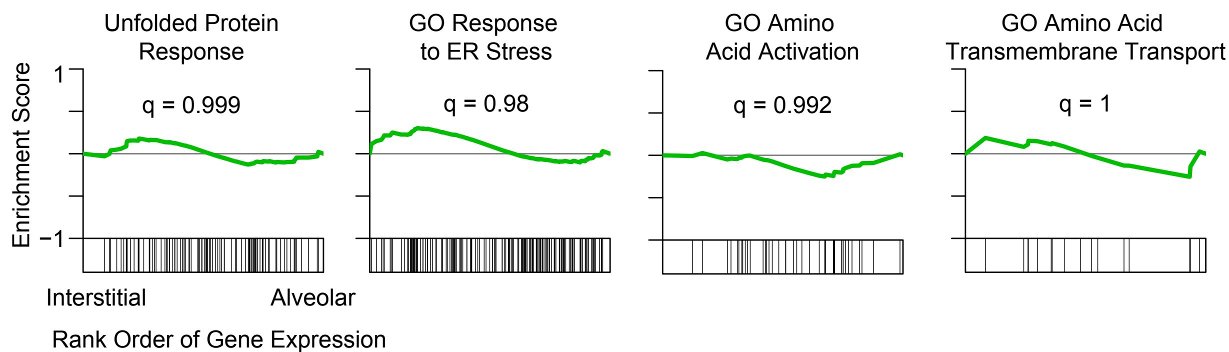
cell type. **(d)** Genome plot showing the *Slc7a5*, *Asns*, and *Gzma* loci. Accessibility for the indicated sample is shown along with gene structure and transcription direction. Locations of DAR are boxed. Data represent the mean of three replicates for each group. **(e)** Percent Annexin V+ among FluNP+ CD44+ CD8 T cells in INT and LV in the lung. INT data taken from Fig 2h. $p<0.05$. Data represented as mean ± SEM.



Supplementary Fig 4: BCL2 is up regulated in airway T$_{RM}$. (a) Frequency of BCL2 on FluNP-specific CD8 T cells in Spleen, INT and BAL (n=5). **(b)** gMFI of BCL2 on FluNP-specific CD8 T cells in Spleen, INT and BAL (n=5). Data represented as mean ± SEM. Significance determined by Mann-whitney U test. **(c)** Example histogram of BCL2 on FluNP-specific CD8 T cells in Spleen, INT and BAL.



Supplementary Fig 5: Airway T_{RM} from WT and *Ddit3-/-* mice provide similar protection following influenza challenge. (a) Experimental design for intratracheal transfer (IT) of WT or *Ddit3-/-* airway T_{RM} into naïve recipient mice. **(b)** Viral titers measured on day 4 post-challenge in mice receiving WT (black bar, open circles) or *Ddit3-/-* (grey bar, open squares) airway T_{RM}. Data represented as mean ± SD.



Supplementary Fig 6. Alveolar macrophages do not up regulate stress response pathways. Gene Set Enrichment Analysis comparing transcriptome profiles of Alveolar versus Interstitial macrophages[52] for the indicated gene sets. The FDR q-value for each comparison is indicated.

References

1. Gebhardt, T., Wakim, L.M., Eidsmo, L., Reading, P.C., Heath, W.R. & Carbone, F.R. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* **10**, 524-530 (2009).

2. Schenkel, J.M., Fraser, K.A., Vezys, V. & Masopust, D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* **14**, 509-513 (2013). PMC3631432

3. Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C. & Kupper, T.S. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* **483**, 227-231 (2012). PMC3437663

4. Kohlmeier, J.E., Cookenham, T., Roberts, A.D., Miller, S.C. & Woodland, D.L. Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge. *Immunity* **33**, 96-105 (2010). PMC2908370

5. Mackay, L.K., Wynne-Jones, E., Freestone, D., Pellicci, D.G., Mielke, L.A., Newman, D.M., Braun, A., Masson, F., Kallies, A., Belz, G.T. & Carbone, F.R. T-box Transcription Factors Combine with the Cytokines TGF-beta and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* **43**, 1101-1111 (2015).

6. Mackay, L.K., Minnich, M., Kragten, N.A., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F.M., Stark, R., Pellicci,

D.G., Godfrey, D.I., Belz, G.T., Pellegrini, M., Gebhardt, T., Busslinger, M., Shi, W., Carbone, F.R., van Lier, R.A., Kallies, A. & van Gisbergen, K.P. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science* **352**, 459-463 (2016).

7. Liang, S.H., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic Immunity to Influenza Type-a Virus in Mice - Effector Mechanisms and Their Longevity. *Journal of Immunology* **152**, 1653-1661 (1994).

8. Wu, T., Hu, Y., Lee, Y.T., Bouchard, K.R., Benechet, A., Khanna, K. & Cauley, L.S. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol* **95**, 215-224 (2014). PMC3896663

9. Purwar, R., Campbell, J., Murphy, G., Richards, W.G., Clark, R.A. & Kupper, T.S. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One* **6**, e16245 (2011). PMC3027667

10. Lee, L.Y., Ha do, L.A., Simmons, C., de Jong, M.D., Chau, N.V., Schumacher, R., Peng, Y.C., McMichael, A.J., Farrar, J.J., Smith, G.L., Townsend, A.R., Askonas, B.A., Rowland-Jones, S. & Dong, T. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J Clin Invest* **118**, 3478-3490 (2008). PMC2542885

11. Hogan, R.J., Usherwood, E.J., Zhong, W., Roberts, A.A., Dutton, R.W., Harmsen, A.G. & Woodland, D.L. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J Immunol* **166**, 1813-1822 (2001).

12. Hou, S., Doherty, P.C., Zijlstra, M., Jaenisch, R. & Katz, J.M. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *J Immunol* **149**, 1319-1325 (1992).

13. Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. & Doherty, P.C. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *J Exp Med* **174**, 875-880 (1991). PMC2118969

14. McMaster, S.R., Wein, A.N., Dunbar, P.R., Hayward, S.L., Cartwright, E.K., Denning, T.L. & Kohlmeier, J.E. Pulmonary antigen encounter regulates the establishment of tissue-resident CD8 memory T cells in the lung airways and parenchyma. *Mucosal Immunol* **11**, 1071-1078 (2018). PMC6030505

15. McMaster, S.R., Wilson, J.J., Wang, H. & Kohlmeier, J.E. Airway-Resident Memory CD8 T Cells Provide Antigen-Specific Protection against Respiratory Virus Challenge through Rapid IFN-gamma Production. *J Immunol* **195**, 203-209 (2015). PMC4475417

16. Slutter, B., Van Braeckel-Budimir, N., Abboud, G., Varga, S.M., Salek-Ardakani, S. & Harty, J.T. Dynamics of influenza-induced lung-resident memory T cells underlie waning heterosubtypic immunity. *Sci Immunol* **2** (2017). PMC5590757

17. Farber, D.L., Yudanin, N.A. & Restifo, N.P. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* **14**, 24-35 (2014). PMC4032067

18. Kumar, B.V., Ma, W., Miron, M., Granot, T., Guyer, R.S., Carpenter, D.J., Senda, T., Sun, X., Ho, S.H., Lerner, H., Friedman, A.L., Shen, Y. & Farber, D.L. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Rep* **20**, 2921-2934 (2017). PMC5646692

19. Hombrink, P., Helbig, C., Backer, R.A., Piet, B., Oja, A.E., Stark, R., Brasser, G., Jongejan, A., Jonkers, R.E., Nota, B., Basak, O., Clevers, H.C., Moerland, P.D., Amsen, D. & van Lier, R.A. Programs for the persistence, vigilance and control of human CD8(+) lung-resident memory T cells. *Nat Immunol* **17**, 1467-1478 (2016).

20. Takamura, S., Yagi, H., Hakata, Y., Motozono, C., McMaster, S.R., Masumoto, T., Fujisawa, M., Chikaishi, T., Komeda, J., Itoh, J., Umemura, M., Kyusai, A., Tomura, M., Nakayama, T., Woodland, D.L., Kohlmeier, J.E. & Miyazawa, M. Specific niches for lung-resident memory CD8+ T cells at the site of tissue regeneration enable CD69-independent maintenance. *J Exp Med* **213**, 3057-3073 (2016). PMC5154946

21. Kohlmeier, J.E., Reiley, W.W., Perona-Wright, G., Freeman, M.L., Yager, E.J., Connor, L.M., Brincks, E.L., Cookenham, T., Roberts, A.D., Burkum, C.E., Sell, S., Winslow, G.M., Blackman, M.A., Mohrs, M. & Woodland, D.L. Inflammatory chemokine receptors regulate CD8(+) T cell contraction and memory generation following infection. *J Exp Med* **208**, 1621-1634 (2011). PMC3149221

22. Kohlmeier, J.E., Miller, S.C. & Woodland, D.L. Cutting edge: Antigen is not required for the activation and maintenance of virus-specific memory CD8+ T cells in the lung airways. *J Immunol* **178**, 4721-4725 (2007).

23. Kohlmeier, J.E., Miller, S.C., Smith, J., Lu, B., Gerard, C., Cookenham, T., Roberts, A.D. & Woodland, D.L. The chemokine receptor CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections. *Immunity* **29**, 101-113 (2008). PMC2519120

24. Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R. & Salzberg, S.L. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* **14**, R36 (2013). 4053844

25. Hsu, F., Kent, W.J., Clawson, H., Kuhn, R.M., Diekhans, M. & Haussler, D. The UCSC Known Genes. *Bioinformatics (Oxford, England)* **22**, 1036-1046 (2006).

26. Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., Morgan, M.T. & Carey, V.J. Software for Computing and Annotating Genomic Ranges. *PLoS Comput Biol* **9** (2013).

27. Robinson, M.D., McCarthy, D.J. & Smyth, G.K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26**, 139-140 (2010). 2796818

28. Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S. & Mesirov, J.P. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550 (2005).

29. Scharer, C.D., Blalock, E.L., Barwick, B.G., Haines, R.R., Wei, C., Sanz, I. & Boss, J.M. ATAC-seq on biobanked specimens defines a unique chromatin accessibility structure in naive SLE B cells. *Sci Rep* **6**, 27030 (2016). PMC4888756

30. Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y. & Greenleaf, W.J. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* **10**, 1213-1218 (2013). 3959825

31. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S.L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10**, R25 (2009). PMC2690996

32. Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nussbaum, C., Myers, R.M., Brown, M., Li, W. & Liu, X.S. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**, R137 (2008). 2592715

33. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H. & Glass, C.K. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. *Mol Cell* **38**, 576-589 (2010).

34. Scharer, C.D., Bally, A.P., Gandham, B. & Boss, J.M. Cutting Edge: Chromatin Accessibility Programs CD8 T Cell Memory. *J Immunol* (2017).

35. Barwick, B.G., Scharer, C.D., Bally, A.P. & Boss, J.M. Plasma cell differentiation is coupled to division-dependent DNA hypomethylation and gene regulation. *Nat Immunol* **17**, 1216-1225 (2016). 5157049

36. Huang da, W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44-57 (2009).

37. Hikono, H., Kohlmeier, J.E., Takamura, S., Wittmer, S.T., Roberts, A.D. & Woodland, D.L. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J Exp Med* **204**, 1625-1636 (2007). PMC2118640

38. Gosselin, D., Link, V.M., Romanoski, C.E., Fonseca, G.J., Eichenfield, D.Z., Spann, N.J., Stender, J.D., Chun, H.B., Garner, H., Geissmann, F. & Glass, C.K. Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. *Cell* **159**, 1327-1340 (2014). PMC4364385

39. Lavin, Y., Winter, D., Blecher-Gonen, R., David, E., Keren-Shaul, H., Merad, M., Jung, S. & Amit, I. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. *Cell* **159**, 1312-1326 (2014). PMC4437213

40. Teske, B.F., Fusakio, M.E., Zhou, D., Shan, J., McClintick, J.N., Kilberg, M.S. & Wek, R.C. CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Mol Biol Cell* **24**, 2477-2490 (2013). PMC3727939

41. Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A. & Gorman, A.M. The integrated stress response. *EMBO Rep* **17**, 1374-1395 (2016). PMC5048378

42. de la Ballina, L.R., Cano-Crespo, S., Gonzalez-Munoz, E., Bial, S., Estrach, S., Cailleteau, L., Tissot, F., Daniel, H., Zorzano, A., Ginsberg, M.H., Palacin, M. & Feral, C.C. Amino

Acid Transport Associated to Cluster of Differentiation 98 Heavy Chain (CD98hc) Is at the Cross-road of Oxidative Stress and Amino Acid Availability. *J Biol Chem* **291**, 9700-9711 (2016). PMC4850307

43. Ely, K.H., Cookenham, T., Roberts, A.D. & Woodland, D.L. Memory T cell populations in the lung airways are maintained by continual recruitment. *J Immunol* **176**, 537-543 (2006).

44. Li, T., Su, L., Lei, Y., Liu, X., Zhang, Y. & Liu, X. DDIT3 and KAT2A Proteins Regulate TNFRSF10A and TNFRSF10B Expression in Endoplasmic Reticulum Stress-mediated Apoptosis in Human Lung Cancer Cells. *J Biol Chem* **290**, 11108-11118 (2015). PMC4409269

45. Pino, S.C., O'Sullivan-Murphy, B., Lidstone, E.A., Yang, C., Lipson, K.L., Jurczyk, A., diIorio, P., Brehm, M.A., Mordes, J.P., Greiner, D.L., Rossini, A.A. & Bortell, R. CHOP mediates endoplasmic reticulum stress-induced apoptosis in Gimap5-deficient T cells. *PLoS One* **4**, e5468 (2009). PMC2674944

46. Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C., Trent, J.M., Staudt, L.M., Hudson, J., Jr., Boguski, M.S., Lashkari, D., Shalon, D., Botstein, D. & Brown, P.O. The transcriptional program in the response of human fibroblasts to serum. *Science* **283**, 83-87 (1999).

47. Pizzolla, A., Nguyen, T.H.O., Smith, J.M., Brooks, A.G., Kedzieska, K., Heath, W.R., Reading, P.C. & Wakim, L.M. Resident memory CD8(+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection. *Sci Immunol* **2** (2017).

48. Caminschi, I., Lahoud, M.H., Pizzolla, A. & Wakim, L.M. Zymosan by-passes the requirement for pulmonary antigen encounter in lung tissue-resident memory CD8(+) T cell development. *Mucosal Immunol* **12**, 403-412 (2019).

49. Van Braeckel-Budimir, N., Varga, S.M., Badovinac, V.P. & Harty, J.T. Repeated Antigen Exposure Extends the Durability of Influenza-Specific Lung-Resident Memory CD8(+) T Cells and Heterosubtypic Immunity. *Cell Rep* **24**, 3374-3382 e3373 (2018).

50. Zhou, A.C., Wagar, L.E., Wortzman, M.E. & Watts, T.H. Intrinsic 4-1BB signals are indispensable for the establishment of an influenza-specific tissue-resident memory CD8 T-cell population in the lung. *Mucosal Immunol* **10**, 1294-1309 (2017).

51. Mintern, J.D., Guillonneau, C., Carbone, F.R., Doherty, P.C. & Turner, S.J. Cutting edge: Tissue-resident memory CTL down-regulate cytolytic molecule expression following virus clearance. *J Immunol* **179**, 7220-7224 (2007).

52. Yu, X., Buttgereit, A., Lelios, I., Utz, S.G., Cansever, D., Becher, B. & Greter, M. The Cytokine TGF-beta Promotes the Development and Homeostasis of Alveolar Macrophages. *Immunity* **47**, 903-912 e904 (2017).

Chapter III: Unrelated respiratory infections compromise established cellular immunity by promoting loss of pre-existing lung-resident memory CD8 T cells

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Abstract

Lung-resident memory T cells (lung T_{RM}) are critical for heterosubtypic protection against influenza viruses, but the efficacy of this immunity wanes over time due to the gradual loss of flu-specific lung T_{RM}. One possible mechanism for this decline is frequent exposure to environmental and biological insults that promote the death of established lung T_{RM}. We investigated whether unrelated infections could exacerbate the loss of pre-existing, flu-specific lung T_{RM} in mice and reduce the efficacy of cellular immunity to subsequent influenza challenge. Infection of influenza-immune mice with Sendai virus, an unrelated murine parainfluenza virus, resulted in significantly higher viral titers and greater morbidity following subsequent influenza challenge, and this loss of protective cellular immunity corresponded to a significant decrease of the number of pre-existing flu-specific lung CD8 T_{RM}. Notably, Sendai infection had no impact on the number of systemic flu-specific memory CD8 T cells in the spleen. The loss of lung T_{RM} required a local infection, as intranasal delivery of TLR agonists did not significantly reduce the number of pre-existing flu-specific lung CD8 T_{RM}. In addition, the loss of pre-existing lung T_{RM} following Sendai infection was not due to competition for limited resources or tissue niches between flu- and Sendai-specific lung T_{RM}. However, blocking extracellular ATP during Sendai infection prevented the accelerated loss of pre-existing flu-specific lung T_{RM}. Together, these data suggest that tissue damage induced by unrelated respiratory infections can promote the loss of pre-existing lung T_{RM} and compromise cellular immunity against respiratory pathogens.

Introduction

Virus-specific memory CD8 T cells are formed and localized to peripheral tissues and secondary lymphoid organs following pathogen clearance. In the case of influenza and parainfluenza virus infections, these peripheral tissues include the lung interstitium and the lung airways [1, 2, 3, 4]. Memory CD8 T cells confer protective immunity against respiratory viruses through the elimination of infected cells, thereby decreasing viral loads and limiting immunopathology which together lower disease burden [5, 6, 7, 8, 9, 10]. A subset of these memory CD8 T cells, known as tissue-resident memory T cells (T_{RM}), remain permanently lodged in the lung due to a unique transcriptional program that inhibits cell egress and promotes tissue retention [11, 12, 13, 14, 15, 16]. Here, they act as sentinels of the adaptive immune system within peripheral tissues due to their location at the site of pathogen entry, and their ability to rapidly recognize and counteract invading pathogens is essential for optimal cellular immunity [17, 18, 19, 20, 21].

Cellular immunity mediated by memory CD8 T cells is known to be protective against several respiratory pathogens, such as influenza, RSV, and coronaviruses [6, 22, 23, 24]. We and others have shown that optimal protection is provided by T_{RM} in the airways and interstitium of the lung, but the efficacy of protection slowly wanes over time [23, 25]. Notably, waning cellular immunity is accompanied by the gradual loss of virus-specific lung T_{RM} over time [6, 26]. This is in contrast to other mucosal tissues, where T_{RM} are long-lived and can maintain cellular immunity for many months [27, 28, 29]. The mechanisms driving this gradual loss of lung T_{RM} are not well understood, but may include local environmental conditions that induce cellular stress, limited access to nutrients and/or survival signals, loss of tissue niches capable of supporting T_{RM}, or inflammation and/or tissue damage resulting from environmental or infectious exposures.

Controlled animal experiments investigating the longevity of T cell memory subsets and protective cellular immunity following respiratory viral infection do not reflect the complex exposure history that occurs naturally, where individuals can be infected with multiple respiratory pathogens over a single year. The effect of repeated, unrelated infections on pre-existing cellular immunity has not been well studied or characterized. T_{RM} are exposed at the front lines of barrier tissues where multiple infections can occur, and are thus repeatedly exposed to pathogen- and damage/danger-associated molecular patterns (PAMPs and DAMPs), inflammatory cytokines, and tissue damage and remodeling. It was recently shown that the purinergic receptor P2RX7, which recognizes the DAMP extracellular ATP (eATP), is highly expressed on T_{RM}, and in models of sterile tissue damage resulted in the selective cell death of T_{RM} [30]. However, the impact of these multiple signals on pre-existing lung T_{RM} maintenance following unrelated respiratory infections is still largely unexplored.

In this paper we illustrate that infection of influenza-immune mice with an unrelated paramyxovirus, Sendai virus, compromises a protective cellular memory response to heterologous influenza challenge, and this corresponds with a loss of influenza-specific CD8 lung T_{RM}. Inducing inflammation in the lung by TLR agonist administration is insufficient to induce loss of pre-existing T_{RM}, and the decline in pre-existing lung T_{RM} following Sendai infection is not due to competition for limited tissue niches for T_{RM} maintenance. However, an acute systemic viral infection also resulted in a loss of pre-existing lung T_{RM}, suggesting a requirement for immune-mediated tissue damage in this process. In support of this, Sendai virus infection leads to increased extracellular ATP (eATP) in the lung, a danger signal and known agonist of purinergic receptors linked to cell death [31]. Finally, hydrolysis of eATP by apyrase treatment during Sendai infection is sufficient to prevent loss of pre-existing $FluNP^+$ lung T_{RM}. Overall this study shows that tissue

damage by unrelated respiratory infections drives the loss of pre-existing lung T_{RM}, and amelioration of these danger signals may provide a mechanism to extend the longevity of protective cellular immunity in the lung.

Materials and Methods

Mice: C57BL/6J (WT) mice were purchased from The Jackson Laboratory and colonies were maintained at Emory University in specific pathogen-free conditions. Mice were between 8 and 13 weeks of age at the time of infection and housed under specific ABSL2 conditions following infection. All experiments were completed in accordance with the Institutional Animal Care and Use Committee guidelines of Emory University.

Infections: Mice were either anesthetized with isoflurane (Patterson Veterinary) or with 300mg/kg (2,2,2-Tribromoethanol, Sigma) prior to infection. Mice were infected intranasally with a 30,000 50% egg infectious doses (EID_{50}) Influenza A/HKx31 (x31, H3N2) or 3,000 EID50 Sendai Virus in a total volume of 30µl. Heterologous challenge of x31-immune mice was performed with a 2.5LD_{50} (750 PFU) or a 5LD_{50} (1500 PFU) dose of Influenza A/PR8 (PR8, H1N1) in 50µl. Challenged mice were monitored daily for weight loss and humanely euthanized if they fell below 75% of original weight in accordance with Emory IACUC guidelines. LCMV Armstrong was kindly provided by Dr. Rafi Ahmed, and mice were infected intraperitoneally with 2×10^5 PFU.

Intravital labeling, cell isolation and flow cytometry: Mice were intravenously labeled via tail vein injection under heat lamp with either CD3e (clone 145-2C11, 1.5µg of fluorophore-conjugated α-CD3ε antibody in 200µl PBS). Five minutes post-intravital labeling, mice were euthanized with an overdose of avertin and exsanguinated prior to harvest of bronchoalveolar lavage (BAL), lung and spleen. Lung and spleen were disassociated as previously described [25]. For flow cytometry, single cell suspensions were treated with Fc-block (2.4G2 antibody), then stained with tetramers at room temperature for one hour, followed by antibody staining. Tetramers (H-

2D(b) Influenza A NP 366-374 (ASNENMETM) and H-2K(b) Sendai NP 324-332 (FAPGNYPAL)) were provided by the NIH Tetramer Core Facility. Antibodies used for flow cytometry were anti-CD8α (clone 53-6.7, BioLegend), anti-CD4 (clone RM-45, BioLegend, anti-CD69 (H1.2F3, BioLegend), anti-CD103 (clone M290, BD), anti-CD103 (clone 2E7, BioLegend), anti-CD11a (clone M17I4, BD), anti-CD44 (clone IM7, Ebioscience), anti-CD62L (clone MEL-14, BioLegend), and anti-CXCR3 (clone CXCR3-173, BioLegend). Cell viability was determined by Zombie NIR (Biolegend). All samples were run on an LSRII or Fortessa X20 (BD Biosciences). Flow cytometry data were analyzed with FlowJo v10 software.

Gating strategy: Influenza- or Sendai-specific CD8 T cells were gated as follows: Singlets, lymphocytes, live cells, CD4- CD8α+, then lung resident cells were gated on CD3e IV- CD8+ (for spleen total CD8+ was taken), followed by gating on tetramer+ $CD44^{hi}$ cells to identify antigen-specific cells. Additional markers for gating include CD69 and CD103.

Extracellular ATP assay: Mice were sacrificed at the indicated time points and BAL was collected in 1ml PBS. Lungs were placed in 2ml of PBS and minced to allow PBS to permeate the tissue, and rested on ice for 1 hour. Following resting, BAL and lungs were spun to clear the supernatant of cells. The supernatant was collected and aliquots stored at -80C. ATP levels were measured using a bioluminescent ATP assay detection kit (Abcam).

Apyrase dosing: Mice received 15 units of Apyrase [32] from potatoes (Sigma) in 200ul of PBS via daily intraperitoneal injections from d-1 to d10 of Sendai infection.

CpG and SenNP peptide dosing: Mice were dosed intranasally with 5µg CpG (ODN 1826 InvivoGen) every other day for 6 days, for a total of 3 treatments. Some mice received 5µg of Sendai $NP_{324-332}$ peptide in combination with CpG using the same dosing schedule.

Statistical analysis: All figure legends indicate methods of comparison and corrections. Statistical analysis was performed with Prism v6.

Results

Parainfluenza virus infection reduces the efficacy of pre-existing cellular immunity to influenza

To investigate how pre-existing immunity is impacted by a secondary, unrelated respiratory infection, we employed a model of Sendai virus infection. Sendai virus is a parainfluenza which shares no cross-reactive T or B cell epitopes with influenza virus. We infected C57Bl/6J (WT) mice with influenza A/HKx31 (x31, H3N2) and at day 35 post-infection (p.i.) mice were challenged with either Sendai virus or PBS intranasally (i.n.). Thirty five days p.i. with Sendai virus or PBS, mice were challenged with heterologous influenza A/PR8 (PR8, H1N1), and weight loss, mortality and viral titers were assessed (**Fig. 1A**). Following 2.5LD_{50} PR8 challenge, x31-immune mice that received a Sendai virus showed significantly greater weight loss than PBS controls on days 6 and 7 following challenge (**Fig. 1B**). Additionally these mice had slightly increased mortality, although this difference was not significant (**Fig. 1C**). However, increasing the PR8 challenge dose to 5LD_{50} resulted in earlier and increased weight loss in x31-immune mice that received Sendai virus, and also a significant increase in mortality in mice that received Sendai virus as opposed to PBS controls (**Supplemental Fig. 1**). Furthermore, if the viral titers are examined following 2.5LD50 PR8 challenge we find that at both day 4 and day 6 post challenge x31-immune mice that received Sendai virus had significantly increased PR8 viral titers on days 4 and 6 p.i. compared to PBS controls, demonstrating pre-existing cellular immunity to influenza virus was comprised (**Fig. 1D**).

Unrelated respiratory infections induce loss of pre-existing lung T_{RM}

Following the loss of protection due to an unrelated respiratory infection, we sought to understand the effect this infection had on the memory T cell populations mediating heterosubtypic protection to influenza. Our lab and many others have shown tissue resident memory (T_{RM}) CD8 T cells in the lung are critical to mediating cellular immunity to respiratory pathogens [24, 25], therefore we sought to examine the effect of Sendai infection on pre-existing flu-specific lung T_{RM} (**Fig. 2A**). Example flow cytometry staining shows that x31-immune mice that received Sendai virus had reduced frequencies of $FluNP^+$ $CD44^{hi}$ cells in the lung resident compartment as defined by intravital labeling. In contrast, the splenic $FluNP^+$ effector memory frequencies remained stable (**Fig 2B**). Further examination showed there is a significant decrease in the number of FluNP-specific lung T_{RM} in mice that had received Sendai virus versus those that received PBS (**Fig 2C**). Comparison of $FluNP^+$ lung T_{RM} numbers from day 35 to day 90 in Sendai infected or PBS control mice found that mice receiving Sendai infection had a 55-fold loss of pre-existing $FluNP^+$ lung T_{RM} compared to a 15-fold loss in mice that received PBS (**Fig 2D**). To determine if this phenomenon was unique to a Sendai virus infection, we reversed the system and primed mice with Sendai virus followed by an x31 influenza infection or PBS at day 35 post-Sendai, and then examined Sendai-specific lung T_{RM} (**Fig 2E**). Similar to our previous results, infection of Sendai-immune mice with x31 influenza significantly reduced the number of SenNP+ $CD44^{hi}$ lung T_{RM} compared to PBS, suggesting the loss of pre-existing lung T_{RM} was a common feature following infection with an unrelated respiratory virus (**Fig 2F**).

Inflammation and limited availability of tissue niches do not contribute to loss of pre-existing lung T_{RM}

Several factors, including inflammation and availability of tissue niches that support T_{RM} maintenance, could be contributing to the loss of a pre-existing lung T_{RM} population. In order to examine whether sterile inflammation was sufficient to induce the loss of pre-existing T_{RM}, x31-immune mice were dosed three times i.n. with the TLR agonist CpG, or CpG plus SenNP peptide in order to induce a secondary SenNP^{+} lung T_{RM} population as previously described (**Fig. 3A**)[33]. As shown in **Fig. 3B**, the frequency of FluNP-specific lung T_{RM} is unaffected by i.n. administration of CpG with or without the addition of Sendai peptide. Examination of the number of FluNP^{+} lung T_{RM} confirms that there is no significant loss of pre-existing T_{RM} following CpG administration (**Fig. 3C**). Additionally, we have confirmed that the dose of CpG used induces infiltration of neutrophils into the airways, and therefore is sufficient to induce immunologically relevant inflammation (**Supplemental Fig 2**). We confirmed that CpG + SenNP peptide induced formation of a de novo Sen NP^{+} lung T_{RM} population (**Fig. 3D and 3E**). As expected, mice that received CpG+SenNP peptide and formed SenNP^{+} lung T_{RM} also had a significant increase in the number of total memory CD8 CD44^{hi} T cells in the lung compared to those receiving PBS or CpG alone (**Fig. 3F**). These data suggested the loss of pre-existing lung T_{RM} observed following a Sendai infection was not due to a competition for limited tissue niches capable of supporting lung T_{RM} maintenance. To further investigate whether "space" limitations impacted development of lung T_{RM}, mice were infected with x31 influenza, or mock infected with PBS, to assess whether increased numbers of pre-existing lung T_{RM} lead to reduced numbers of de novo lung T_{RM} following an unrelated respiratory infection (**Fig. 3G**). At day 35, prior to Sendai virus infection, as expected the x31-immune mice had significantly increased memory CD8 T cells in the lung compared to PBS controls (**Fig 3H**). Following Sendai virus infection of both x31-immune and PBS control mice, there was no difference in the number of SenNP^{+} lung T_{RM} (**Fig. 3I**), suggesting

pre-existing lung T_{RM} did not preclude the formation of a *de novo* lung T_{RM} population via competition for limited tissue niches.

Local infection alone induces loss of pre-existing T cell memory populations

Given that neither inflammation alone nor limited availability of tissue niches was sufficient to drive the loss of pre-existing influenza-specific lung T_{RM}, we reasoned that a viral infection within the tissue may be required. LCMV results in a systemic infection that generates robust inflammation and immune damage in many tissues, and we used this model to test the ability of an acute systemic viral infection to drive lung T_{RM} loss. X31-immune mice were infected with LCMV intraperitoneally or given PBS as a control, and we assessed the effect of LCMV infection on pre-existing flu-specific lung T_{RM} (**Fig. 4A**). Example flow cytometry staining shows, that following LCMV infection, the frequency of pre-existing $FluNP^+$ CD8 T cells in both spleen and lung resident compartments were reduced compared to PBS controls (**Fig. 4B**). This reduction was confirmed by significant decrease in the numbers of both $FluNP^+$ T_{EM} from the spleen and $FluNP^+$ lung T_{RM} (**Fig. 4C**), suggesting that acute infection within a tissue results in changes to the local microenvironment that are necessary to induce the loss of pre-existing T_{RM}.

Limiting danger signals during an unrelated respiratory infection increases the survival of pre-existing lung T_{RM}

It has been shown that danger signals resulting from immune-mediated damage, such as extracellular ATP (eATP), can induce the attrition of tissue resident memory CD8 T cells in the liver through activation of receptors such as P2RX7 [30]. Additionally, it was demonstrated that eATP contributed to pulmonary inflammation and lung fibrosis, and that loss of P2RX7 reduces

inflammation within the lung during injury [34]. To examine whether danger signals may be playing a role in the loss of pre-existing T_{RM} in the context of an unrelated respiratory infection, we first investigated the release of extracellular ATP during an acute Sendai virus infection in x31-immune mice using a luminescent ATP assay (**Fig 5A**). On day 7, there was a significant increase of eATP in the lungs of mice that received Sendai virus compared to PBS. In the airway lavage (BAL), there was also increased eATP at day 7, although this difference was not significant (**Fig. 5B**). Together these data show that the danger signal eATP is released during Sendai virus infection. To determine if eATP was playing a role in the loss of pre-existing influenza-specific lung T_{RM}, x31-immune mice were treated with apyrase or PBS during Sendai infection (**Fig. 5C**). Apyrase hydrolyzes ATP to ADP and AMP [35], rendering it unable to signal through the purinergic receptors linked to memory CD8 T cell attrition. As shown by example flow cytometry staining, administration of apyrase through the acute stages of Sendai virus infection resulted in an increased frequency of $FluNP^+$ lung T_{RM} compared to PBS-treated controls (**Fig. 5D**). Additionally, mice that received apyrase had significantly larger numbers of $FluNP^+$ lung T_{RM} compared to PBS-treated controls (**Fig 5E**). However, the number of $FluNP^+$ T_{EM} in the spleen were unaffected by apyrase dosing during Sendai infection, as expected given that an infection limited to the respiratory tract is unlikely to generate eATP in the spleen. These data show that the ability of an unrelated respiratory infection to drive the loss of pre-existing lung T_{RM} is reduced when eATP is neutralized, and that extracellular danger signals play a critical role in the maintenance of lung T_{RM}.

Discussion

During the course of an influenza season, individuals can be exposed to multiple respiratory infections from different pathogens that drive local inflammation, immune system activation, and tissue damage and remodeling. However, the effect of these repeated, unrelated infections on pre-existing memory CD8 T cell subsets had been studied very little. In this paper, we show that unrelated, acute viral infections in the lung deplete the pre-existing influenza-specific CD8 T_{RM} and compromise the effectiveness of cellular immunity to heterologous influenza challenge. This effect was not due to sterile inflammation alone, nor to competition for limited tissue niches necessary to support T_{RM} maintenance. Rather, tissue damage from immune-mediated pathology or lytic viral infection resulted in the local production of DAMPs, such as eATP, that was necessary for the loss of pre-existing lung T_{RM}. These findings are supported by the observations that an unrelated respiratory infection did not impact the number of pre-existing, circulating influenza-specific T_{EM}, and that hydrolysis of eATP protected pre-existing lung T_{RM} from loss during an unrelated respiratory infection.

These findings parallel a recent study from van Lier and colleagues where signaling through the purinergic receptor P2RX7 resulted in the rapid death of T_{RM} in a model of sterile tissue damage [30]. Additionally, it has been shown that P2RX7 promotes the development of memory CD8 T cells by regulating mitochondrial homeostasis [36]. Interestingly, TCR stimulation protected T_{RM} from P2RX7-mediated cell death through downregulation of the receptor, providing a means to protect only those CD8 T_{RM} that can recognize the invading pathogen. These findings, and the data presented here, raise interesting questions regarding the potential benefit of deleting pre-existing lung T_{RM} in an antigen-independent manner. It was recently shown that lung T_{RM}, while critical for protection against respiratory virus challenges, must temper their effector

functions in order to limit immune-mediated damage and fibrosis [37]. Thus, perhaps purinergic receptor signaling in lung T_{RM} acts to prevent the antigen-independent release of pro-inflammatory mediators in order to maintain the fine balance between immune protection and pathology within the infected tissue.

The delicate balance of ATP regulation and release has been well documented within the lung environment under homeostatic conditions. ATP regulates epithelial mucociliary clearance activities within the airways and ATP release with the airways regulates cell volume critical to lung functionality [38, 39]. In the context of an infection or inflammation this balance can be dysregulated resulting in the increased release of eATP, which can amplify the inflammatory program induced by pattern recognition receptors and drive additional pro-inflammatory cytokine production from antigen presenting cells and neutrophils [40, 41, 42] This release of eATP is capable of driving systemic inflammation through the release pro-inflammatory cytokines such as TNF and Il-1β, and the removal of eATP via the use of apyrase has been shown to reduce these signals and ameliorate damage [32, 43]. In our system, the TLR agonist CpG was insufficient to drive loss of pre-existing T_{RM}, this may in part be due to the tightly controlled regulation of inflammatory cytokine genes following TLR stimulation in the absence of additional danger signals [44]. Together these studies, combined with our data, suggest that differential amounts of eATP may be released in the lung by sterile inflammation versus an active infection, and only during an infection is eATP present in sufficient concentrations to drive the P2RX7-mediated attrition of pre-existing T_{RM}.

Another possibility is that this system provides a mechanism to ensure that T_{RM} being seeded in the tissue immediately following clearance of an acute respiratory infection have access to sufficient nutrients and specific niches required for their development. For example, areas of tissue damage and repair in the lung serve as niches for developing lung T_{RM} [45, 46], and high

concentrations of extracellular nucleotides at these sites during the infection may serve to deplete non-specific, established T_{RM} to eliminate competition for this niche. Although we did not observe a role for limited tissue niches in our study using two respiratory virus infections, it is important to note that this model fails to recapitulate the many unrelated respiratory infections that an individual is exposed to over a lifetime. Indeed, examination of T cells mucosal tissues in adults showed that T_{RM} are the dominant memory CD8 T cell subset in the lung [47], raising the possibility that competition for tissue niches could become a limiting factor for the development of de novo T_{RM} over time. Additional studies are necessary to determine the importance of P2RX7-mediated T_{RM} deletion for the generation of niches capable for supporting de novo T_{RM} development.

In summary, we have shown that unrelated respiratory infections can compromise pre-existing cellular immunity in the lung through the depletion of pre-existing lung T_{RM}. This required local tissue damage and the production of DAMPs such as eATP, as sterile inflammation alone was insufficient to drive T_{RM} loss, and hydrolysis of eATP during an unrelated infection protected pre-existing lung T_{RM}. These findings have important implications for the design of cell-mediated vaccines against respiratory pathogens.

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Author contributions:

S.L.H. and J.E.K. designed the study. S.L.H., Z-R.T.L., J.L.L., and J.O.P.E performed experiments. S.L.H., Z-R.T.L., J.L.L., J.O.P.E., and J.E.K. analyzed data. S.L.H. and J.E.K. wrote the manuscript.

Figures

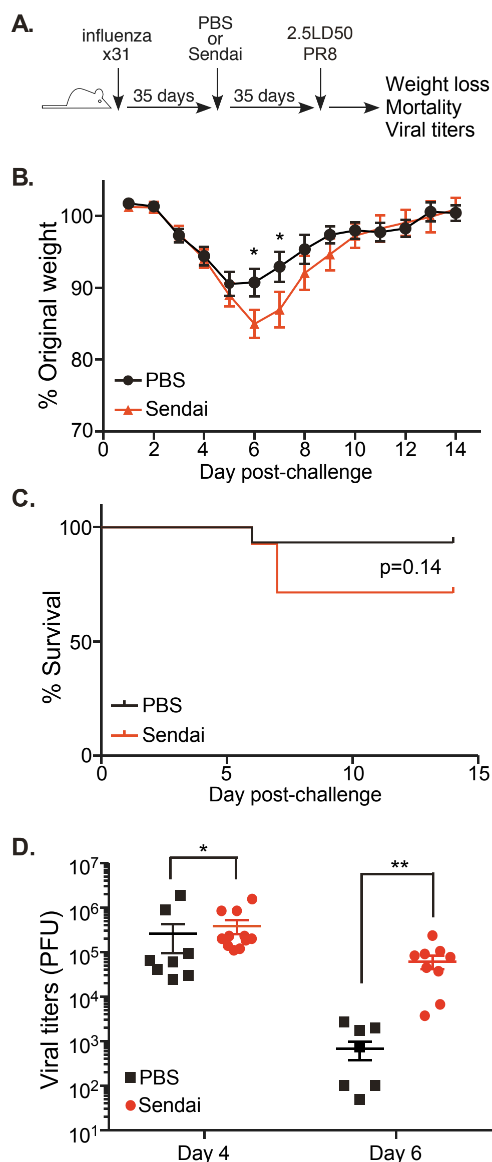


Figure 1. Infection with Sendai virus reduces protection against heterologous influenza challenge in influenza immune mice (A) Experimental design. (**B**) Percent of original weight following 2.5LD50 PR8 challenge in x31-immune mice infected with Sendai virus or given PBS. (**C**) Percent survival following 2.5LD50 PR8 challenge in x31-immune mice infected with Sendai virus or given PBS. For PBS n=15, for Sendai n=14. (**D**) Viral titers at day 4 and day 6 following

heterologous challenge with 2.5LD50 PR8 in x31-immune mice infected with Sendai virus or given PBS. For PBS n=12 per time point, for Sendai n=11 per time point. P values are as follows: * = $p<0.05$, ** = $p<0.01$, *** =$p<0.001$, **** = $p<0.0001$. Data represent mean ± SEM

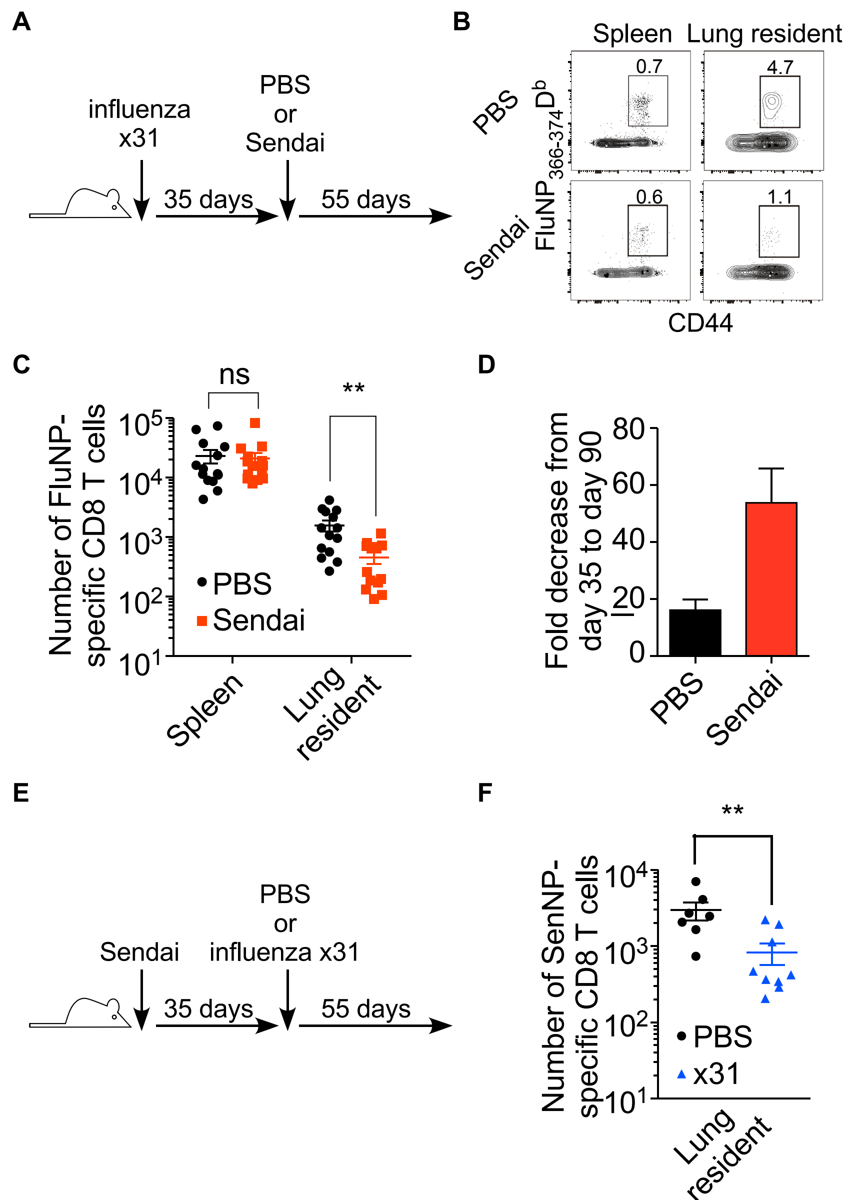


Figure 2: Infection with an unrelated respiratory virus accelerates the decline of pre-existing flu-specific lung T_{RM}. (**A**) Experimental design. (**B**) Examples of FluNP tetramer frequencies in the lung (IV-) and spleen from Sendai and PBS treatment groups. (**C**) Number of FluNP-specific splenic T_{EM} and lung T_{RM} in x31-immune mice 55 days after infection with Sendai virus or PBS treatment. Significance was determined using a Mann-whitney U test. For PBS n= 10, for Sendai

n=10. (**D**) Fold decrease in FluNP-specific from day 35 to day 90 following Sendai infection or PBS treatment (**E**) Experimental design. (**F**) Number of SenNP-specific lung T_{RM} in Sendai-immune mice 55 days after infection with x31 influenza or PBS treatment. Significance was determined using a Mann-whitney U test. For PBS n=8, for x31 n=9. Data represent mean ± SEM .

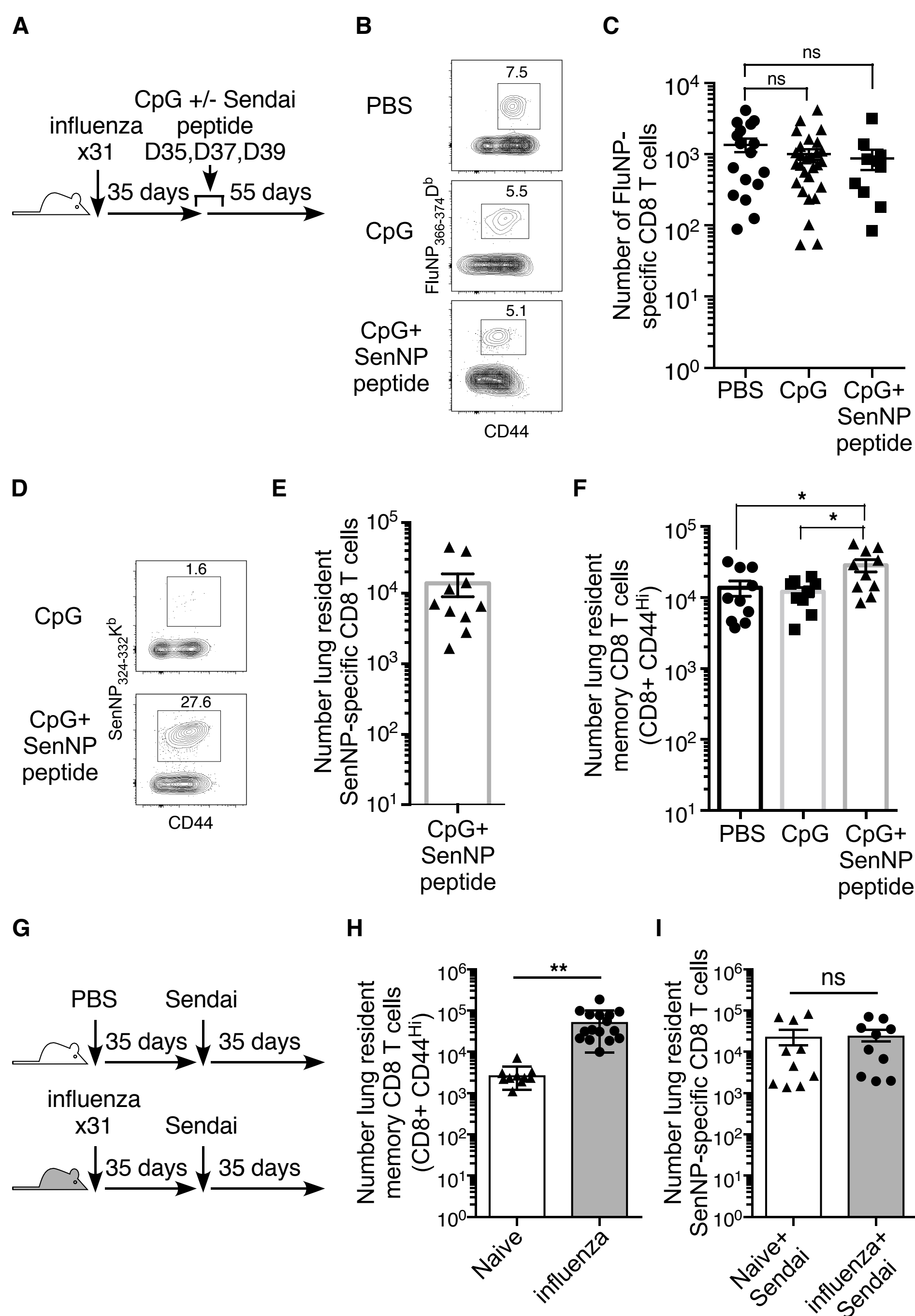


Figure 3: Inflammation and limited availability of tissue niches do not contribute to loss of pre-existing lung T_{RM}. (**A**) Experimental design. (**B**) Example staining of FluNP-specific lung

T_{RM} in x31-immune mice on day 55 post-treatment. (**C**) Number of pre-existing FluNP-specific lung T_{RM} following PBS, CpG, or CpG+SenNP peptide treatment. (**D**) Example staining of Sendai-virus specific lung TRM in mice given CpG alone or CpG+SenNP peptide. (**E**) Number of SenNP-specific lung TRM in x31-immune mice 55 days after CpG+SenNP peptide treatment. (**F**) Number of total memory CD8 $CD44^{hi}$ T cells in the lung (IV-) in x31-immune mice 55 days following PBS (n=11), CpG (n=9) or CpG+SenNP peptide (n=10). Significance was determined using a Mann-whitney U test, $p=0.029$ (CpG+SenNP vs PBS) and $p=0.044$ (CpG+SenNP vs CpG). (**G**) Experimental design. (**H)** Number of total memory CD8 $CD44^{hi}$ T cells in the lung (IV-) in naive SPF mice (n=9) and x31-immune mice 35 days post-infection (n=15). Significance was determined using a Mann-whitey U test. (**I**) Number of SenNP-specific lung T_{RM} in previously naïve or x31-immune mice 55 day post-Sendai infection. Data represent mean ± SEM

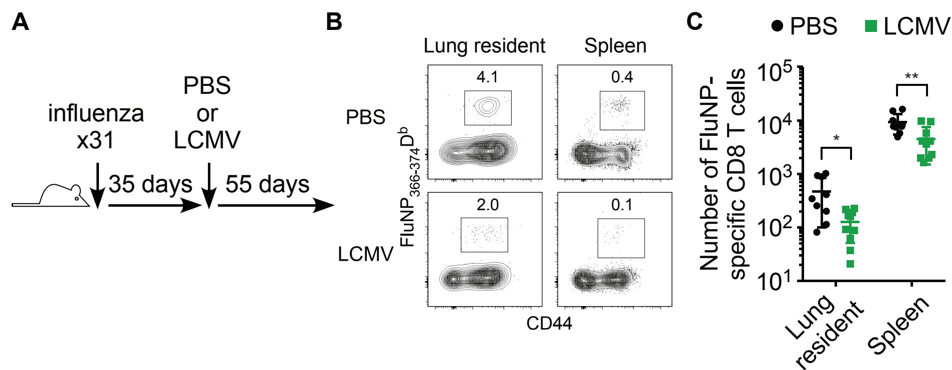


Figure 4: Local infection alone induces loss of pre-existing T cell memory populations. (**A**) Experimental design. (**B**) Example staining of FluNP-specific lung T_{RM} and spleen T_{EM} 55 days after infection with LCMV-Armstrong or PBS treatment. (**C**) Number of FluNP-specific lung T_{RM} (p=0.019) and splenic T_{EM} (p=0.01) in x31-immune mice 55 days following LCMV-Armstrong infection (n=10) or PBS treatment (n=9). Significance was determined using a Mann-whitey U test. Data represent mean ± SEM

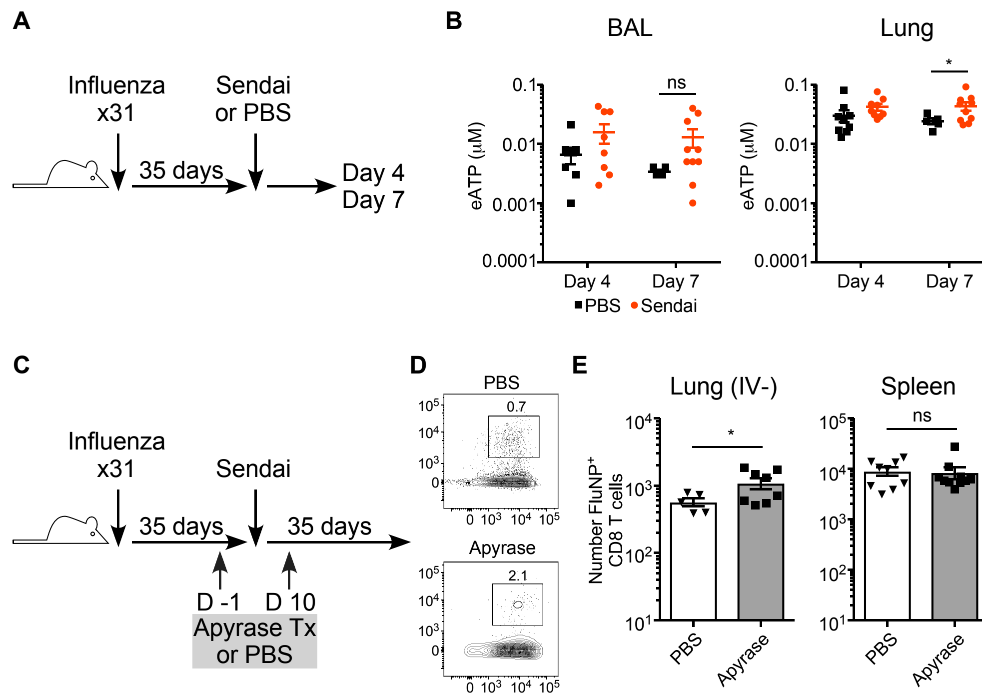
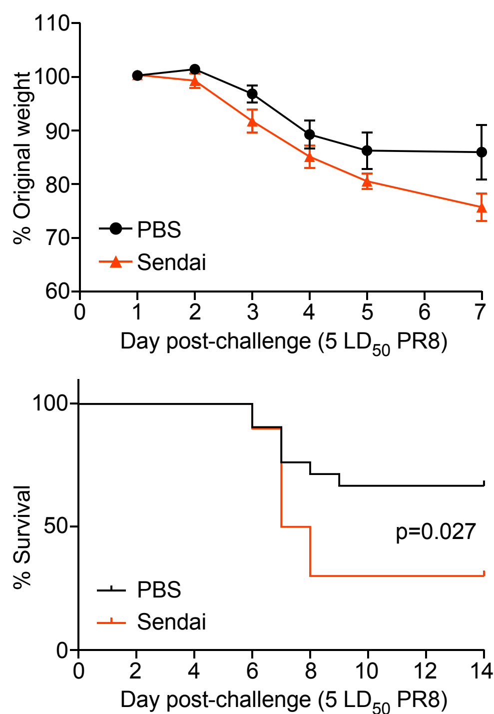
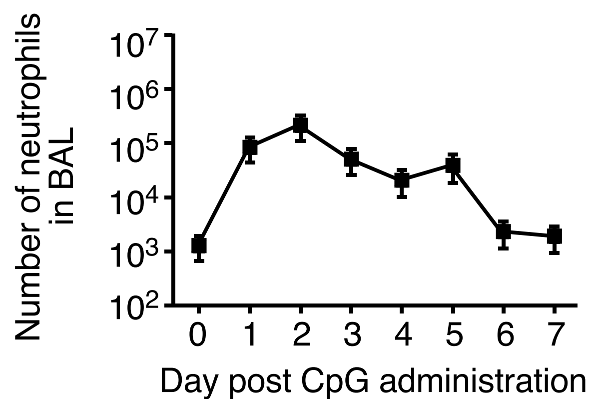


Figure 5: Limiting danger signals during an unrelated respiratory infection increases the survival of pre-existing lung T_{RM}. (**A**) Experimental design. (**B**) Extracellular ATP in broncho-alveolar lavage (BAL) of x31-immune mice on day 4 and day 7 following infection with Sendai virus or PBS treatment. PBS d4 n=8, d7 n=5. Sendai d4 n=8, d7 n=10 (**C**) Experimental design. (**D**) Example staining of FluNP-specific lung T_{RM} in x31-immune mice that received either PBS or apyrase treatment during Sendai infection. (**E**) Number of FluNP-specific lung T_{RM} and splenic T_{EM} in x31-immune mice that received either PBS (n=5) or apyrase (n=8) treatment during Sendai infection. Data represent mean ± SEM

Supplementary information:



Supplemental Figure 1: **5LD50 PR8 challenge induces significant mortality in Sendai exposed mice.** x31-immune mice infected with Sendai virus (n=21) show increased weight loss and significant decrease in survival compared to PBS controls (n=20) following 5LD50 PR8 challenge. Significance determined by log-rank (Mantel-cox) test.



Supplemental Figure 2: **CpG administration induces migration of neutrophils into the airways of mice**. Mice received 5ug of CpG (n=5, representative of 3 experiments) and the number of neutrophils in the BAL was measured by flow cytometry.

References

1. Purwar, R., Campbell, J., Murphy, G., Richards, W.G., Clark, R.A. & Kupper, T.S. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One* **6**, e16245 (2011). PMC3027667

2. Lee, L.Y., Ha do, L.A., Simmons, C., de Jong, M.D., Chau, N.V., Schumacher, R., Peng, Y.C., McMichael, A.J., Farrar, J.J., Smith, G.L., Townsend, A.R., Askonas, B.A., Rowland-Jones, S. & Dong, T. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. *J Clin Invest* **118**, 3478-3490 (2008). PMC2542885

3. Hogan, R.J., Usherwood, E.J., Zhong, W., Roberts, A.A., Dutton, R.W., Harmsen, A.G. & Woodland, D.L. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J Immunol* **166**, 1813-1822 (2001).

4. Hombrink, P., Helbig, C., Backer, R.A., Piet, B., Oja, A.E., Stark, R., Brasser, G., Jongejan, A., Jonkers, R.E., Nota, B., Basak, O., Clevers, H.C., Moerland, P.D., Amsen, D. & van Lier, R.A. Programs for the persistence, vigilance and control of human CD8(+) lung-resident memory T cells. *Nat Immunol* **17**, 1467-1478 (2016).

5. Liang, S., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *J Immunol* **152**, 1653-1661 (1994).

6. Wu, T., Hu, Y., Lee, Y.T., Bouchard, K.R., Benechet, A., Khanna, K. & Cauley, L.S. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol* **95**, 215-224 (2014). PMC3896663

7. Hou, S., Doherty, P.C., Zijlstra, M., Jaenisch, R. & Katz, J.M. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. *J Immunol* **149**, 1319-1325 (1992).

8. Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. & Doherty, P.C. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *J Exp Med* **174**, 875-880 (1991). PMC2118969

9. Doherty, P.C., Topham, D.J., Tripp, R.A., Cardin, R.D., Brooks, J.W. & Stevenson, P.G. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. *Immunol Rev* **159**, 105-117 (1997).

10. Olson, J.A., McDonald-Hyman, C., Jameson, S.C. & Hamilton, S.E. Effector-like CD8(+) T cells in the memory population mediate potent protective immunity. *Immunity* **38**, 1250-1260 (2013). PMC3703254

11. Mackay, L.K., Minnich, M., Kragten, N.A., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F.M., Stark, R., Pellicci,

D.G., Godfrey, D.I., Belz, G.T., Pellegrini, M., Gebhardt, T., Busslinger, M., Shi, W., Carbone, F.R., van Lier, R.A., Kallies, A. & van Gisbergen, K.P. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science* **352**, 459-463 (2016).

12. Kumar, B.V., Ma, W., Miron, M., Granot, T., Guyer, R.S., Carpenter, D.J., Senda, T., Sun, X., Ho, S.H., Lerner, H., Friedman, A.L., Shen, Y. & Farber, D.L. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Rep* **20**, 2921-2934 (2017). PMC5646692

13. Mackay, L.K., Wynne-Jones, E., Freestone, D., Pellicci, D.G., Mielke, L.A., Newman, D.M., Braun, A., Masson, F., Kallies, A., Belz, G.T. & Carbone, F.R. T-box Transcription Factors Combine with the Cytokines TGF-beta and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* **43**, 1101-1111 (2015).

14. Zhou, A.C., Wagar, L.E., Wortzman, M.E. & Watts, T.H. Intrinsic 4-1BB signals are indispensable for the establishment of an influenza-specific tissue-resident memory CD8 T-cell population in the lung. *Mucosal Immunol* **10**, 1294-1309 (2017).

15. Mackay, L.K., Braun, A., Macleod, B.L., Collins, N., Tebartz, C., Bedoui, S., Carbone, F.R. & Gebhardt, T. Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. *J Immunol* **194**, 2059-2063 (2015).

16. Skon, C.N., Lee, J.Y., Anderson, K.G., Masopust, D., Hogquist, K.A. & Jameson, S.C. Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nat Immunol* **14**, 1285-1293 (2013). PMC3844557

17. Gebhardt, T., Wakim, L.M., Eidsmo, L., Reading, P.C., Heath, W.R. & Carbone, F.R. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* **10**, 524-530 (2009).

18. Schenkel, J.M., Fraser, K.A., Vezys, V. & Masopust, D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* **14**, 509-513 (2013). PMC3631432

19. Wakim, L.M., Woodward-Davis, A. & Bevan, M.J. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc Natl Acad Sci U S A* **107**, 17872-17879 (2010). PMC2964240

20. Davies, B., Prier, J.E., Jones, C.M., Gebhardt, T., Carbone, F.R. & Mackay, L.K. Cutting Edge: Tissue-Resident Memory T Cells Generated by Multiple Immunizations or Localized Deposition Provide Enhanced Immunity. *J Immunol* **198**, 2233-2237 (2017).

21. Fernandez-Ruiz, D., Ng, W.Y., Holz, L.E., Ma, J.Z., Zaid, A., Wong, Y.C., Lau, L.S., Mollard, V., Cozijnsen, A., Collins, N., Li, J., Davey, G.M., Kato, Y., Devi, S., Skandari, R., Pauley, M., Manton, J.H., Godfrey, D.I., Braun, A., Tay, S.S., Tan, P.S., Bowen, D.G., Koch-Nolte, F., Rissiek, B., Carbone, F.R., Crabb, B.S., Lahoud, M., Cockburn, I.A.,

Mueller, S.N., Bertolino, P., McFadden, G.I., Caminschi, I. & Heath, W.R. Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. *Immunity* **45**, 889-902 (2016).

22. Kohlmeier, J.E., Cookenham, T., Roberts, A.D., Miller, S.C. & Woodland, D.L. Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge. *Immunity* **33**, 96-105 (2010). PMC2908370

23. Liang, S.H., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic Immunity to Influenza Type-a Virus in Mice - Effector Mechanisms and Their Longevity. *Journal of Immunology* **152**, 1653-1661 (1994).

24. Pizzolla, A., Nguyen, T.H.O., Smith, J.M., Brooks, A.G., Kedzieska, K., Heath, W.R., Reading, P.C. & Wakim, L.M. Resident memory CD8(+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection. *Sci Immunol* **2** (2017).

25. McMaster, S.R., Wilson, J.J., Wang, H. & Kohlmeier, J.E. Airway-Resident Memory CD8 T Cells Provide Antigen-Specific Protection against Respiratory Virus Challenge through Rapid IFN-gamma Production. *J Immunol* **195**, 203-209 (2015). PMC4475417

26. Slutter, B., Van Braeckel-Budimir, N., Abboud, G., Varga, S.M., Salek-Ardakani, S. & Harty, J.T. Dynamics of influenza-induced lung-resident memory T cells underlie waning heterosubtypic immunity. *Sci Immunol* **2** (2017). PMC5590757

27. Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C. & Kupper, T.S. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* **483**, 227-231 (2012). PMC3437663

28. Zaid, A., Mackay, L.K., Rahimpour, A., Braun, A., Veldhoen, M., Carbone, F.R., Manton, J.H., Heath, W.R. & Mueller, S.N. Persistence of skin-resident memory T cells within an epidermal niche. *Proc Natl Acad Sci U S A* **111**, 5307-5312 (2014). PMC3986170

29. Park, S.L., Zaid, A., Hor, J.L., Christo, S.N., Prier, J.E., Davies, B., Alexandre, Y.O., Gregory, J.L., Russell, T.A., Gebhardt, T., Carbone, F.R., Tscharke, D.C., Heath, W.R., Mueller, S.N. & Mackay, L.K. Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses. *Nat Immunol* **19**, 183-191 (2018).

30. Stark, R., Wesselink, T.H., Behr, F.M., Kragten, N.A.M., Arens, R., Koch-Nolte, F., van Gisbergen, K. & van Lier, R.A.W. T RM maintenance is regulated by tissue damage via P2RX7. *Sci Immunol* **3** (2018).

31. Surprenant, A., Rassendren, F., Kawashima, E., North, R.A. & Buell, G. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* **272**, 735-738 (1996).

32. Cauwels, A., Rogge, E., Vandendriessche, B., Shiva, S. & Brouckaert, P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death Dis* **5**, e1102 (2014). PMC3973196

33. McMaster, S.R., Wein, A.N., Dunbar, P.R., Hayward, S.L., Cartwright, E.K., Denning, T.L. & Kohlmeier, J.E. Pulmonary antigen encounter regulates the establishment of tissue-resident CD8 memory T cells in the lung airways and parenchyma. *Mucosal Immunol* **11**, 1071-1078 (2018). PMC6030505

34. Riteau, N., Gasse, P., Fauconnier, L., Gombault, A., Couegnat, M., Fick, L., Kanellopoulos, J., Quesniaux, V.F., Marchand-Adam, S., Crestani, B., Ryffel, B. & Couillin, I. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *Am J Respir Crit Care Med* **182**, 774-783 (2010).

35. Cohn, M. & Meek, G.A. The mechanism of hydrolysis of adenosine di- and tri-phosphate catalysed by potato apyrase. *Biochem J* **66**, 128-130 (1957). PMC1199975

36. Borges da Silva, H., Beura, L.K., Wang, H., Hanse, E.A., Gore, R., Scott, M.C., Walsh, D.A., Block, K.E., Fonseca, R., Yan, Y., Hippen, K.L., Blazar, B.R., Masopust, D., Kelekar, A., Vulchanova, L., Hogquist, K.A. & Jameson, S.C. The purinergic receptor P2RX7 directs metabolic fitness of long-lived memory CD8(+) T cells. *Nature* **559**, 264-268 (2018). PMC6054485

37. Wang, Z., Wang, S., Goplen, N.P., Li, C., Cheon, I.S., Dai, Q., Huang, S., Shan, J., Ma, C., Ye, Z., Xiang, M., Limper, A.H., Porquera, E.C., Kohlmeier, J.E., Kaplan, M.H., Zhang, N., Johnson, A.J., Vassallo, R. & Sun, J. PD-1(hi) CD8(+) resident memory T cells balance immunity and fibrotic sequelae. *Sci Immunol* **4** (2019).

38. Okada, S.F., Nicholas, R.A., Kreda, S.M., Lazarowski, E.R. & Boucher, R.C. Physiological regulation of ATP release at the apical surface of human airway epithelia. *J Biol Chem* **281**, 22992-23002 (2006). PMC2924190

39. Clarke, L.L. & Boucher, R.C. Chloride secretory response to extracellular ATP in human normal and cystic fibrosis nasal epithelia. *Am J Physiol* **263**, C348-356 (1992).

40. Dosch, M., Gerber, J., Jebbawi, F. & Beldi, G. Mechanisms of ATP Release by Inflammatory Cells. *Int J Mol Sci* **19** (2018). PMC5979498

41. Zhang, C., He, H., Wang, L., Zhang, N., Huang, H., Xiong, Q., Yan, Y., Wu, N., Ren, H., Han, H., Liu, M., Qian, M. & Du, B. Virus-Triggered ATP Release Limits Viral Replication through Facilitating IFN-beta Production in a P2X7-Dependent Manner. *J Immunol* **199**, 1372-1381 (2017).

42. Shieh, C.H., Heinrich, A., Serchov, T., van Calker, D. & Biber, K. P2X7-dependent, but differentially regulated release of IL-6, CCL2, and TNF-alpha in cultured mouse microglia. *Glia* **62**, 592-607 (2014).

43. Lister, M.F., Sharkey, J., Sawatzky, D.A., Hodgkiss, J.P., Davidson, D.J., Rossi, A.G. & Finlayson, K. The role of the purinergic P2X7 receptor in inflammation. *J Inflamm (Lond)* **4**, 5 (2007). PMC1838907

44. Foster, S.L., Hargreaves, D.C. & Medzhitov, R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447**, 972-978 (2007).

45. Takamura, S., Yagi, H., Hakata, Y., Motozono, C., McMaster, S.R., Masumoto, T., Fujisawa, M., Chikaishi, T., Komeda, J., Itoh, J., Umemura, M., Kyusai, A., Tomura, M., Nakayama, T., Woodland, D.L., Kohlmeier, J.E. & Miyazawa, M. Specific niches for lung-resident memory CD8+ T cells at the site of tissue regeneration enable CD69-independent maintenance. *J Exp Med* **213**, 3057-3073 (2016). PMC5154946

46. Turner, D.L., Bickham, K.L., Thome, J.J., Kim, C.Y., D'Ovidio, F., Wherry, E.J. & Farber, D.L. Lung niches for the generation and maintenance of tissue-resident memory T cells. *Mucosal Immunol* **7**, 501-510 (2014). PMC3965651

47. Thome, J.J., Yudanin, N., Ohmura, Y., Kubota, M., Grinshpun, B., Sathaliyawala, T., Kato, T., Lerner, H., Shen, Y. & Farber, D.L. Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell* **159**, 814-828 (2014). PMC4243051

Chapter IV: Discussion

Tissue resident memory (T_{RM}) CD8 T cells are an important subset of memory CD8 T cells. They provide protection against previously experienced antigen and considered to be the sentinels of the adaptive immune system. They are isolated from the circulation due to their location within the peripheral tissues away from the vasculature. They conically express CD103 and CD69 which are markers that aid in their retention in the tissue[1, 2]. T_{RM} share expression of a set of core genes and transcription factors regardless of the peripheral tissue in which they reside[3]. We show these core genes are conserved within both the airway and interstitium T_{RM} populations of the lung in chapter II of this dissertation. T_{RM} provide protection at common sites of infection through their recognition of cognate antigens. They provide both cytokine-mediated protection (activating the immune system through alarmins and the recruitment of additional immune cells to the infected site) and cytotoxic protection through killing of infected cells [4, 5]. T_{RM} have been shown to be critically important to the response and protection from respiratory viruses including influenza, respiratory syncytial virus, and tuberculosis[6, 7, 8]. Additionally, examining the loss of immunity to influenza, Walter Gerhard and others have previously shown that protection from heterologous influenza infection wanes over time, resulting in increased morbidity and mortality as time from initial priming increases[9, 10, 11]. Our laboratory and many others have long suspected this loss of immunity was due to a loss of influenza-specific tissue resident memory from the lung environment.

In chapter II, we examine mechanisms driving the loss of influenza-specific tissue resident memory T cell populations in the lung under homeostatic conditions. This chapter focuses on two distinct populations of tissue resident memory within the lung; airway resident CD8 T cells (BAL) found to be loosely associated with the epithelium of airways in the lung and interstitium resident

CD8 T cells (INT) found within the interstitial tissue between the epithelium and endothelium of the lung tissue[7, 11, 12]. There locational differences can be further seen in Figure 1. Tissue resident memory CD8 T cells in the lung can be split between these two populations via experimental technique that isolate these populations separately, and through their phenotypic characteristics. In order to isolate airway resident cells, bronco-alveolar lavage is used to pull airway resident T cells out of the airway before the lung tissue is mechanically and enzymatically digested[7]. Phenotypically, airway resident cells downregulate CD11a, which is one subunit of the integrin αL, within 48 hours of entering the airway environment[13] . Here, we show that it is very important to separate and study BAL and INT populations individually, as unique lung influenza-specific populations serving differential and complementary purposes.

Repeating past studies, we find that protection from heterologous PR8 influenza infection wanes as time from initial x31 influenza infection passes. By 3-months post-infection, mortality increases to almost double that of 1-month post-infection, and by 8-months post-infection protection is completely lost, with all mice succumbing to a $10LD_{50}$ infectious challenge. Further investigating this loss of T cell mediated heterologous immunity, we found there was a 40-fold decrease in interstitium resident CD8 T cells and an over 100-fold loss in airway resident CD8 T cells between 1 and 8 months following initial infection. This loss is not a result of a loss of specific subsets as defined by CD69 and CD103. Additionally, through a parabiosis model we found that this loss is not due to T_{RM} exiting the tissue and re-entering the circulation, which would have suggested they were not truly resident. Additionally, we found BAL and INT to be differential in their expression the pro-apoptotic marker annexin V, with BAL cells showing increased apoptosis compared to INT cells. This differential apoptosis and the unique environments in which these cells reside lead us to examine these two populations separately using global approaches, namely

RNA and ATAC-seq. We found that BAL and INT have vastly different transcriptional and epigenetic profiles. Both populations share expression of core genes previously defined as hallmarks of tissue resident CD8 T cells. However, they were unique in their expression of genes related to cell stress and cell survival. In BAL cells, these genes were found to be associated with the integrated stress response, specifically the amino acid stress response pathway. When the stressors are not resolved the ultimate result is cell death, and this mechanism is a primary mediator driving the loss of tissue resident memory in the lung over time [14, 15].

The amino acid stress response is a pathway activated when cells are placed in an environment lacking the amino acid necessary for cell survival[14]. In order to examine this phenomenon in more detail, TEM were transferred intra-tracheally into the airways of congenic mice to examine the effect of the airway environment alone on the activation of the amino acid stress response pathway. Using a combination of RNA-seq and flow cytometry, we were able to determine this pathway was activated as a result of the environment and was not due general properties of the development of T_{RM} in the airways. Additionally, we were able to show that the nutrients present in the airways during an active infection are sufficient to prevent activation of genes related to the amino acid stress response pathway, enabling BAL T_{RM} to persist during an infection so that they can mediate their antiviral functions. Given the loss of BAL over time, and the implication of the amino acid stress response pathway in driving this cell death, there may be therapeutic options for increasing the lifespan of BAL T_{RM}. One possible option is the restoration of nutrients to the BAL cells. Additionally, in this chapter we examined how the BAL cells responded when placed into a nutrient-rich environment in vitro. Using RNA-seq, we found the restoration of nutrients there resulted in a rapid down-regulation of gene expression related to the amino acid stress response pathway. Furthermore, there were a large number of genes activated

which are involved in DNA repair and other repair functions, further suggestion that the airway environment is extremely harsh and showing that several mechanisms may be at play in the decline of BAL. However, nutrient deprivation is an important factor that ties these cell death mechanisms together. Overall, the data in this chapter suggests a unique, environmentally-driven loss of T_{RM} from the airways contributes to the homeostatic loss of T_{RM} from the lung.

In chapter III, we further explore another scenario of loss of tissue resident T cell memory in the lung by examining the loss of pre-existing influenza-specific lung resident T_{RM} following secondary parainfluenza infection. Typically, T_{RM} in the lungs and airways are examined in the context of single infection or inflammatory stimuli. This does not reflect the reality in which multiple respiratory infections are experienced yearly. Recent data suggests that inflammatory stimuli are capable of deleting pre-existing immune cells through the activation of P2RX7[16] . These purinergic receptors are activated via ATP released extracellularly in the context of an inflammatory assault [17, 18] . While the activation of such receptors are shown to regulate metabolism, it was also shown that tissue resident memory was cleared from the liver via a P2RX7-dependent mechanism [16, 19]. Given the effect of non-specific inflammation on pre-existing T cells, we chose to examine how an unrelated secondary infection affected pre-existing T_{RM} within the tissue of the lung. First of all, we examined how parainfluenza infection effected protection provided by pre-existing influenza-specific T_{RM}. An unrelated Sendai infection resulted in an increase in morbidity and mortality in the face of a heterologous influenza challenge. Additionally, there was a significant increase in PR8 viral titers in the lungs of mice who had previously seen parainfluenza virus compared to PBS control. This suggested that parainfluenza infection had resulted in a decline in influenza-specific immunity. We found that parainfluenza infection resulted in a significant decrease in influenza-specific T_{RM} in the lung, but not in other tissues such as the

spleen. Additionally, if the system was reversed, in which parainfluenza-specific memory was allowed to form, followed by an unrelated influenza infection, there was a significant loss in the parainfluenza-specific memory from the lung. Together these data suggested that an unrelated respiratory infection leads to a decline in pre-existing immunity through the deletion of established lung T_{RM}.

The loss of pre-existing immunity due to an unrelated respiratory infection suggested several possible mechanisms, including inflammation, space limitations, and damage from infection may be playing a role. We found that sterile inflammation alone was insufficient to drive loss of pre-existing T_{RM}. We then found that the presence of pre-existing T_{RM} in the lung, compared to a naïve mouse, had no effect on the expansion of *de novo* parainfluenza-specific T_{RM}. This suggested that there was not competition for niche space among T_{RM} within the lung. Since inflammation and availability of niches were not implicated in the loss of pre-existing T_{RM}, an examination of the effect of local infection was necessary. Using the systemic acute viral infection LCMV, it was revealed that local infection was sufficient to induce loss of pre-existing CD8 T cells not only from the lung resident compartment, but also from the splenic effector memory compartment. This suggested that local infection was capable of clearing pre-existing immunity from multiple compartments, but only when there was an active infection in that tissue. Given recent studies implicating purinergic receptors in the clearance of T_{RM}, we found that parainfluenza virus infection resulted in significant release of eATP within the lung. Using apyrase, eATP was quenched during an unrelated infection, resulting in the maintenance of pre-existing lung T_{RM} following parainfluenza infection. Together, these data suggested that local infection drives eATP release, which activated P2RX7 signaling on lung T_{RM}, leading to cell death and the loss of pre-existing lung T_{RM}. Together, the data in this chapter present an examination of how unrelated

infections can drive the loss of pre-existing T_{RM}, suggesting dynamic changes in the local environment can modulate the lifespan of lung T_{RM}.

Implications of environmental stressors on tissue resident memory:

The data within this dissertation bring together a picture of complicated environmental stressors which affect the survival of tissue resident memory CD8 T cells in the lung. Both the environment at homeostasis and the environment during an infection can contribute to the loss of lung T_{RM}, which together suggest there are multiple scenarios that can have downstream effects on the lifespan of CD8 tissue resident memory. Notably, the loss of T_{RM} over time seems to be specific to the lung, as T_{RM} in many other peripheral tissues are shown to be stable over time [20, 21, 22].

This lack of stability may in part be due to the structure and function of the lung. The lung is highly susceptible to damage due to uncontrolled pro-inflammatory cytokine release and tissue destruction from uncontrolled immune responses. In severe influenza infection, damage results in secondary bacterial pneumonia and other conditions as the lung attempts to heal and remodel following infection[23, 24, 25]. Following asthma, severe damage due to eATP can lead to fibrosis and other pathologic conditions which further impair the abilities of the lung tissue [17, 18]. Due to the susceptibility of the lung to damage following inflammatory insult, it is critical that mechanisms exist to modulate and prevent the damage.

It has previously been shown that following metapneumovirus infection, CD8 T cells in the lung upregulate PD-1, Lag-3 and 2B4 in order to mitigate the potential damage by effector memory T cells in the lung[26]. It was also shown that inhibitory ligands were expressed on APCs and airway epithelial cells to provide further control of effector mechanisms. Recently, it was

shown that PD-1 acts as a break on tissue resident memory cells following influenza infection to prevent pulmonary fibrosis[27]. Together, these examples show an effort by multiple mechanisms in the lung to limit the pathological effects of CD8 T cells. Through the data presented in this dissertation, we present two further mechanisms of preventing deleterious immunopathology via the culling of T_{RM} from the airways and intersititium of the lung.

Designing vaccines in face of constitutive loss of protection:

In the face of continual loss of tissue resident memory CD8 T cells, vaccine design must change to either account for, or to ameliorate, this loss. T cell-directed vaccines are critical for heterologous protection against multiple seasonal strains[28, 29]. T cell protection does not prevent infection but is important for limiting viral replication and this preventing immunopathology[30] . If T_{RM} are continually lost as shown by multiple mechanisms in this dissertation, there is an immediate concern that T cell-based vaccines for respiratory infection may have limited effectiveness and may not be able to provide long-term protection over multiple seasons.

The continual loss of T_{RM} suggests a couple of alternatives that can be explored in order to prevent the loss of cell-mediated protection. An initial suggestion is a continual boosting strategy, or an examination of whether secondary or tertiary memory populations, memory T cells which have seen cognate antigen multiple times, show a more stable persistence in the lung, or whether the number of these cells can be boosted to such a degree that gradual loss did not have a significant impact on protection[31]. For example, in a transfer model in which primary and secondary influenza memory cells were transferred into a naïve host and challenged, it was shown that primary memory was better able to migrate to the site of infection to provide protection, however secondary memory induces greater numbers of protective memory CD8 T cells[32]. Together, these findings suggest

that multiple encounters with antigen do increase protection, and prime-boost approaches need to be further explored, and they may have profound effects on the intrinsic properties of memory CD8 T cells that can enhance their survival in the lung.

An alternative to prime-boosting strategies is the use of recombinant adenovirus vectors expressing T cell antigens as vaccine platforms. These vaccine approaches have been shown to provide long-term protective immunity in several settings and have the potential to be extremely useful in infections where protection is mediated by T cells. In HCV, adenoviruses have been shown to produce strong and prolonged T cell responses in human hosts. Additionally, using multiple adenovirus serotypes, the response can be boosted further driving the prolonged T cell response to immunodominant HCV epitopes and preventing infection[33]. Additionally, it was shown that a replication deficient adenovirus expressing influenza nucleoprotein in combination with 4-1BBL is able to induce stable and protective T_{RM} within the lung[34]. These lung T_{RM} formed with this approach are persistent to a much greater degree than lung T_{RM} generated during an influenza infection. The mechanisms of this persistence of T_{RM} in the lung have not yet been defined but have been suggested to be due to persistent antigen that serves to constantly replenish and re-stimulate the T_{RM} pool, furthering their proliferation and maintenance within the lung tissue.

Future studies in tissue resident memory:

Tissue resident memory T cells are becoming increasingly important in many fields of study outside of infectious disease. The presence of tissue resident memory CD8 T cells are implicated in improved suppression of cancer in melanoma, with their immunosurveillance being critical to maintaining the dormancy of epicutaneous cancer cells, and T_{RM} being critical to maintaining durable immunity to melanoma [35, 36]. Additionally, CD103 "T_{RM} like" tumor

infiltrating lymphocytes, have been associated with favorable prognosis in solid tumors, with the critical T_{RM} development cytokine TGF-β being critical to the accumulation of CD103+ T cells in lung tumors[37, 38]. Tissue resident memory stability in the context of cancer has not been thoroughly examined. Depending on the tumor type, tissue resident memory T cells may be starved of nutrients in a similar manner to which airway resident T cells are shown to be starved of amino acids in chapter II. Solid tumors are known to be hypoxic and nutrient starved[39]. In fact, tumor cells are shown to take advantage of the amino acid stress response pathway in order to further their growth through the GCN2 – ATF4 pathway, allowing them to subvert nutrient deprivation[40]. Tissue resident memory CD8 T cells will be exposed to these starvation conditions and mechanisms which lead to their adaption to the environment, or subsequent loss, are of great interest to the tissue resident memory field. It is also of interest whether tissue resident memory T cells specific for cancer neo-antigens are critical in simply preventing the spread of tumors by staying at the more nutrient-dense edges of the solid tumor, or whether they perform a more selfless duty within the tumors aiding in the tumor destruction while also limiting their lifespan due to nutrient deprivation. Further studies examining how T_{RM} interact with different tumor types, and under different nutritional conditions, will be extremely important in determining how T_{RM} adapt to tissue-specific stress pathways.

T_{RM} have also been implicated in the formation and pathogenesis of autoimmune disease[41]. Their role in mediating the damage associated with autoimmune diseases, and mitigating their formation or suppression, is becoming increasingly important as a potential treatment for disease. In multiple sclerosis, T_{RM} have been associated with active brain lesions and implicated in the inflammatory response and reactivation of lesions[42]. In psoriasis, it has been shown that relapses preferentially occurs in areas of skin which has been previously affected and contain T_{RM} cells

which are pathogenically poised to produced IL-17A and IL-22[43]. Additionally, patients can be stratified in their clinical outcomes by the presence of T_{RM} in resolved psoriasis lesions[44]. Further studies examining how and under what certain circumstances T_{RM} can be suppressed therapeutically will be important in controlling pathogenic T_{RM}.

Together these data suggest that the waning of tissue resident memory is a unique phenomenon to the lung, and both homeostatic and pathogenic factors can contribute to this loss. These data develop a mechanism of homeostatic loss in which the airway microenvironment drives the loss of airway resident CD8 T cells via the activation of the amino acid stress response pathway. This loss over time may play a role in mediating and preventing unnecessary and deleterious immune activation due to non-specific stimuli. Ameliorating this loss through therapeutic methods such as amino acid restoration or limiting loss by inducing a larger CD8 T_{RM} response through various vaccine strategies, may provide alternatives to prevent this loss and increase the longevity of protective cellular immunity to respiratory pathogens. These data additionally examine a method of clearing pre-existing T_{RM} in an antigen-independent manner, where an unrelated infection with a parainfluenza virus causes tissue damage, leading to the release of extracellular ATP, resulting in the loss of T_{RM} via local infection. These mechanisms implicate another mechanism of T_{RM} clearance, which may also be critical in preventing the unwanted, non-specific immune activation of T_{RM}, thus preventing immunopathology and maintaining critical gas exchange functions of the lung. Additionally, this may be a method of clearance in order to ensure that T_{RM} are able to be seeded following infection by providing adequate nutrients and specific niches for T_{RM} to form. Overall, this dissertation serves to define mechanisms of lung T_{RM} decay and examines how this deterioration leads to the gradual decline of cellular immunity within the lung.

Figure 1

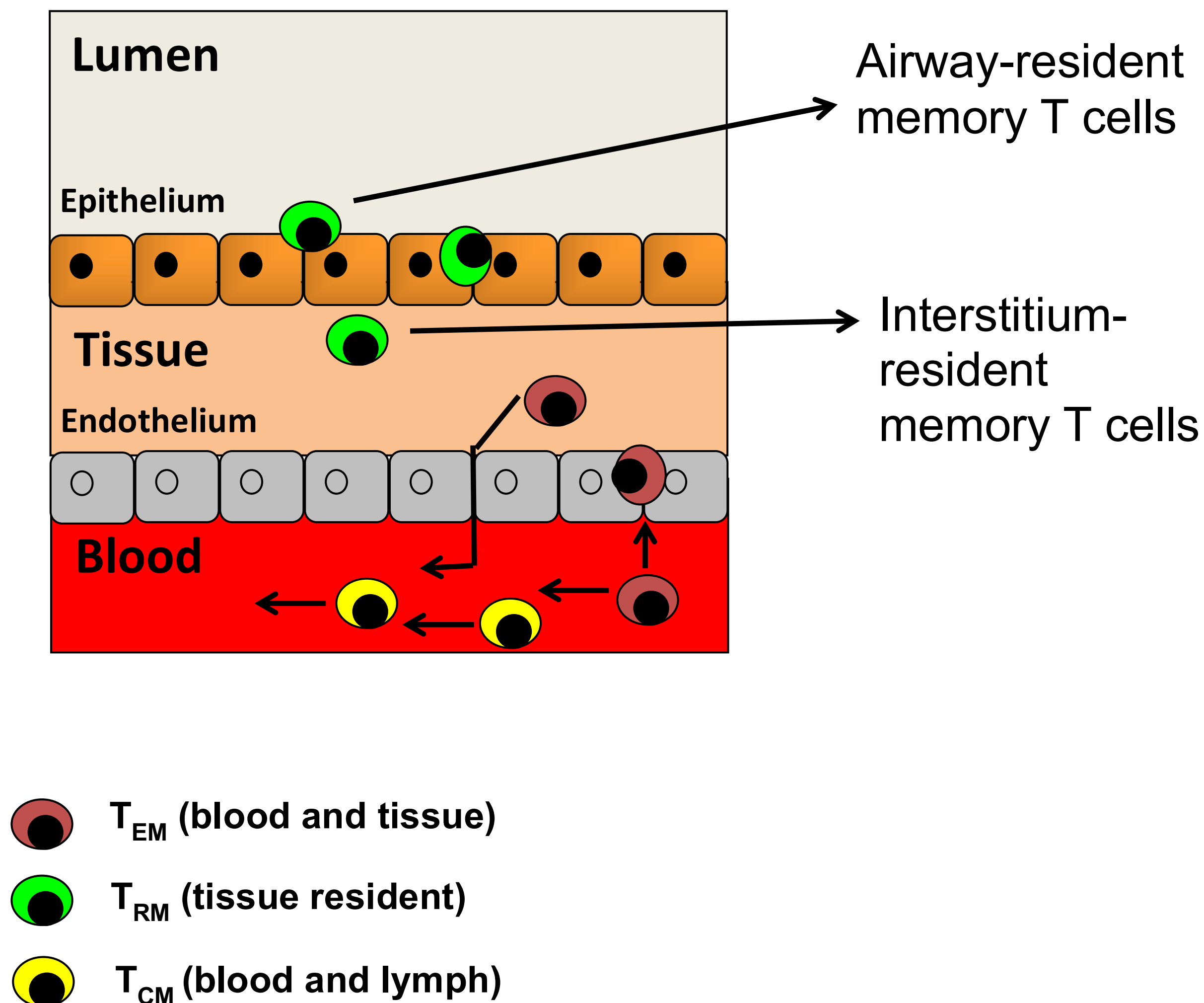


Figure 1. Summary figure distinguishing airway and interstitium tissue resident T cell population via distinct location within lung environment. Airway resident T_{RM} shown to be loosely associated with epithelium. Interstitium resident found between epithelium and endothelium. T_{EM} and T_{CM} found in blood and trafficking through tissue.

References:

1. Kohlmeier, J.E. & Woodland, D.L. Immunity to respiratory viruses. *Annu Rev Immunol* **27**, 61-82 (2009).

2. Mackay, L.K., Braun, A., Macleod, B.L., Collins, N., Tebartz, C., Bedoui, S., Carbone, F.R. & Gebhardt, T. Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. *J Immunol* **194**, 2059-2063 (2015).

3. Mackay, L.K., Minnich, M., Kragten, N.A., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F.M., Stark, R., Pellicci, D.G., Godfrey, D.I., Belz, G.T., Pellegrini, M., Gebhardt, T., Busslinger, M., Shi, W., Carbone, F.R., van Lier, R.A., Kallies, A. & van Gisbergen, K.P. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science* **352**, 459-463 (2016).

4. Gebhardt, T., Wakim, L.M., Eidsmo, L., Reading, P.C., Heath, W.R. & Carbone, F.R. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* **10**, 524-530 (2009).

5. Schenkel, J.M., Fraser, K.A., Vezys, V. & Masopust, D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* **14**, 509-513 (2013). PMC3631432

6. Morabito, K.M., Ruckwardt, T.R., Redwood, A.J., Moin, S.M., Price, D.A. & Graham, B.S. Intranasal administration of RSV antigen-expressing MCMV elicits robust tissue-resident effector and effector memory CD8+ T cells in the lung. *Mucosal Immunol* **10**, 545-554 (2017). PMC5123975

7. McMaster, S.R., Wilson, J.J., Wang, H. & Kohlmeier, J.E. Airway-Resident Memory CD8 T Cells Provide Antigen-Specific Protection against Respiratory Virus Challenge through Rapid IFN-gamma Production. *J Immunol* **195**, 203-209 (2015). PMC4475417

8. Pizzolla, A., Nguyen, T.H.O., Smith, J.M., Brooks, A.G., Kedzieska, K., Heath, W.R., Reading, P.C. & Wakim, L.M. Resident memory CD8(+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection. *Sci Immunol* **2** (2017).

9. Liang, S.H., Mozdzanowska, K., Palladino, G. & Gerhard, W. Heterosubtypic Immunity to Influenza Type-a Virus in Mice - Effector Mechanisms and Their Longevity. *Journal of Immunology* **152**, 1653-1661 (1994).

10. Marshall, D.R., Turner, S.J., Belz, G.T., Wingo, S., Andreansky, S., Sangster, M.Y., Riberdy, J.M., Liu, T., Tan, M. & Doherty, P.C. Measuring the diaspora for virus-specific CD8+ T cells. *Proc Natl Acad Sci U S A* **98**, 6313-6318 (2001). PMC33465

11. Wu, T., Hu, Y., Lee, Y.T., Bouchard, K.R., Benechet, A., Khanna, K. & Cauley, L.S. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol* **95**, 215-224 (2014). PMC3896663

12. Pizzolla, A., Nguyen, T.H., Sant, S., Jaffar, J., Loudovaris, T., Mannering, S.I., Thomas, P.G., Westall, G.P., Kedzierska, K. & Wakim, L.M. Influenza-specific lung-resident memory T cells are proliferative and polyfunctional and maintain diverse TCR profiles. *J Clin Invest* **128**, 721-733 (2018). PMC5785253

13. Ely, K.H., Cookenham, T., Roberts, A.D. & Woodland, D.L. Memory T cell populations in the lung airways are maintained by continual recruitment. *J Immunol* **176**, 537-543 (2006).

14. Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A. & Gorman, A.M. The integrated stress response. *EMBO Rep* **17**, 1374-1395 (2016). PMC5048378

15. Teske, B.F., Fusakio, M.E., Zhou, D., Shan, J., McClintick, J.N., Kilberg, M.S. & Wek, R.C. CHOP induces activating transcription factor 5 (ATF5) to trigger apoptosis in response to perturbations in protein homeostasis. *Mol Biol Cell* **24**, 2477-2490 (2013). PMC3727939

16. Stark, R., Wesselink, T.H., Behr, F.M., Kragten, N.A.M., Arens, R., Koch-Nolte, F., van Gisbergen, K. & van Lier, R.A.W. T RM maintenance is regulated by tissue damage via P2RX7. *Sci Immunol* **3** (2018).

17. Cauwels, A., Rogge, E., Vandendriessche, B., Shiva, S. & Brouckaert, P. Extracellular ATP drives systemic inflammation, tissue damage and mortality. *Cell Death Dis* **5**, e1102 (2014). PMC3973196

18. Riteau, N., Gasse, P., Fauconnier, L., Gombault, A., Couegnat, M., Fick, L., Kanellopoulos, J., Quesniaux, V.F., Marchand-Adam, S., Crestani, B., Ryffel, B. & Couillin, I. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. *Am J Respir Crit Care Med* **182**, 774-783 (2010).

19. Borges da Silva, H., Beura, L.K., Wang, H., Hanse, E.A., Gore, R., Scott, M.C., Walsh, D.A., Block, K.E., Fonseca, R., Yan, Y., Hippen, K.L., Blazar, B.R., Masopust, D., Kelekar, A., Vulchanova, L., Hogquist, K.A. & Jameson, S.C. The purinergic receptor P2RX7 directs metabolic fitness of long-lived memory CD8(+) T cells. *Nature* **559**, 264-268 (2018). PMC6054485

20. Mackay, L.K., Stock, A.T., Ma, J.Z., Jones, C.M., Kent, S.J., Mueller, S.N., Heath, W.R., Carbone, F.R. & Gebhardt, T. Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc Natl Acad Sci U S A* **109**, 7037-7042 (2012). PMC3344960

21. Masopust, D., Choo, D., Vezys, V., Wherry, E.J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K.A., Barber, D.L., Kawamura, K.S., Fraser, K.A., Webby, R.J., Brinkmann, V., Butcher, E.C., Newell, K.A. & Ahmed, R. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* **207**, 553-564 (2010). PMC2839151

22. Woodland, D.L. & Kohlmeier, J.E. Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat Rev Immunol* **9**, 153-161 (2009).

23. Ivanov, S., Renneson, J., Fontaine, J., Barthelemy, A., Paget, C., Fernandez, E.M., Blanc, F., De Trez, C., Van Maele, L., Dumoutier, L., Huerre, M.R., Eberl, G., Si-Tahar, M., Gosset, P., Renauld, J.C., Sirard, J.C., Faveeuw, C. & Trottein, F. Interleukin-22 reduces lung inflammation during influenza A virus infection and protects against secondary bacterial infection. *J Virol* **87**, 6911-6924 (2013). PMC3676141

24. Narasaraju, T., Ng, H.H., Phoon, M.C. & Chow, V.T. MCP-1 antibody treatment enhances damage and impedes repair of the alveolar epithelium in influenza pneumonitis. *Am J Respir Cell Mol Biol* **42**, 732-743 (2010). PMC2891499

25. Herold, S., Becker, C., Ridge, K.M. & Budinger, G.R. Influenza virus-induced lung injury: pathogenesis and implications for treatment. *Eur Respir J* **45**, 1463-1478 (2015).

26. Erickson, J.J., Rogers, M.C., Tollefson, S.J., Boyd, K.L. & Williams, J.V. Multiple Inhibitory Pathways Contribute to Lung CD8+ T Cell Impairment and Protect against Immunopathology during Acute Viral Respiratory Infection. *J Immunol* **197**, 233-243 (2016). PMC4933524

27. Wang, Z., Wang, S., Goplen, N.P., Li, C., Cheon, I.S., Dai, Q., Huang, S., Shan, J., Ma, C., Ye, Z., Xiang, M., Limper, A.H., Porquera, E.C., Kohlmeier, J.E., Kaplan, M.H., Zhang, N., Johnson, A.J., Vassallo, R. & Sun, J. PD-1(hi) CD8(+) resident memory T cells balance immunity and fibrotic sequelae. *Sci Immunol* **4** (2019).

28. Guo, H., Santiago, F., Lambert, K., Takimoto, T. & Topham, D.J. T cell-mediated protection against lethal 2009 pandemic H1N1 influenza virus infection in a mouse model. *J Virol* **85**, 448-455 (2011). PMC3014175

29. Morens, D.M., Taubenberger, J.K., Harvey, H.A. & Memoli, M.J. The 1918 influenza pandemic: lessons for 2009 and the future. *Crit Care Med* **38**, e10-20 (2010). PMC3180813

30. Sun, J., Madan, R., Karp, C.L. & Braciale, T.J. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat Med* **15**, 277-284 (2009). PMC2693210

31. Kardani, K., Bolhassani, A. & Shahbazi, S. Prime-boost vaccine strategy against viral infections: Mechanisms and benefits. *Vaccine* **34**, 413-423 (2016).

32. Van Braeckel-Budimir, N., Martin, M.D., Hartwig, S.M., Legge, K.L., Badovinac, V.P. & Harty, J.T. Antigen Exposure History Defines CD8 T Cell Dynamics and Protection during Localized Pulmonary Infections. *Front Immunol* **8**, 40 (2017). PMC5269565

33. Barnes, E., Folgori, A., Capone, S., Swadling, L., Aston, S., Kurioka, A., Meyer, J., Huddart, R., Smith, K., Townsend, R., Brown, A., Antrobus, R., Ammendola, V., Naddeo, M., O'Hara, G., Willberg, C., Harrison, A., Grazioli, F., Esposito, M.L., Siani, L., Traboni, C., Oo, Y., Adams, D., Hill, A., Colloca, S., Nicosia, A., Cortese, R. & Klenerman, P. Novel adenovirus-based vaccines induce broad and sustained T cell responses to HCV in man. *Sci Transl Med* **4**, 115ra111 (2012). PMC3627207

34. Zhou, A.C., Wagar, L.E., Wortzman, M.E. & Watts, T.H. Intrinsic 4-1BB signals are indispensable for the establishment of an influenza-specific tissue-resident memory CD8 T-cell population in the lung. *Mucosal Immunol* **10**, 1294-1309 (2017).

35. Malik, B.T., Byrne, K.T., Vella, J.L., Zhang, P., Shabaneh, T.B., Steinberg, S.M., Molodtsov, A.K., Bowers, J.S., Angeles, C.V., Paulos, C.M., Huang, Y.H. & Turk, M.J. Resident memory T cells in the skin mediate durable immunity to melanoma. *Sci Immunol* **2** (2017). PMC5525335

36. Park, S.L., Buzzai, A., Rautela, J., Hor, J.L., Hochheiser, K., Effern, M., McBain, N., Wagner, T., Edwards, J., McConville, R., Wilmott, J.S., Scolyer, R.A., Tuting, T.,

Palendira, U., Gyorki, D., Mueller, S.N., Huntington, N.D., Bedoui, S., Holzel, M., Mackay, L.K., Waithman, J. & Gebhardt, T. Tissue-resident memory CD8(+) T cells promote melanoma-immune equilibrium in skin. *Nature* **565**, 366-371 (2019).

37. Boutet, M., Gauthier, L., Leclerc, M., Gros, G., de Montpreville, V., Theret, N., Donnadieu, E. & Mami-Chouaib, F. TGFbeta Signaling Intersects with CD103 Integrin Signaling to Promote T-Lymphocyte Accumulation and Antitumor Activity in the Lung Tumor Microenvironment. *Cancer Res* **76**, 1757-1769 (2016).

38. Komdeur, F.L., Prins, T.M., van de Wall, S., Plat, A., Wisman, G.B.A., Hollema, H., Daemen, T., Church, D.N., de Bruyn, M. & Nijman, H.W. CD103+ tumor-infiltrating lymphocytes are tumor-reactive intraepithelial CD8+ T cells associated with prognostic benefit and therapy response in cervical cancer. *Oncoimmunology* **6**, e1338230 (2017). PMC5599086

39. DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G. & Thompson, C.B. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* **7**, 11-20 (2008).

40. Ye, J., Kumanova, M., Hart, L.S., Sloane, K., Zhang, H., De Panis, D.N., Bobrovnikova-Marjon, E., Diehl, J.A., Ron, D. & Koumenis, C. The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation. *EMBO J* **29**, 2082-2096 (2010). PMC2892366

41. Wu, H., Liao, W., Li, Q., Long, H., Yin, H., Zhao, M., Chan, V., Lau, C.S. & Lu, Q. Pathogenic role of tissue-resident memory T cells in autoimmune diseases. *Autoimmun Rev* **17**, 906-911 (2018).

42. Machado-Santos, J., Saji, E., Troscher, A.R., Paunovic, M., Liblau, R., Gabriely, G., Bien, C.G., Bauer, J. & Lassmann, H. The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain* **141**, 2066-2082 (2018). PMC6022681

43. Cheuk, S., Wiken, M., Blomqvist, L., Nylen, S., Talme, T., Stahle, M. & Eidsmo, L. Epidermal Th22 and Tc17 cells form a localized disease memory in clinically healed psoriasis. *J Immunol* **192**, 3111-3120 (2014). PMC3962894

44. Martini, E., Wiken, M., Cheuk, S., Gallais Serezal, I., Baharom, F., Stahle, M., Smed-Sorensen, A. & Eidsmo, L. Dynamic Changes in Resident and Infiltrating Epidermal Dendritic Cells in Active and Resolved Psoriasis. *J Invest Dermatol* **137**, 865-873 (2017).