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Dynamic equilibrium of lung T_{rm} dictates waning immunity after Influenza A infection

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Abstract

Lung resident memory (T_{rm}) CD8 T cells induced by influenza A virus (IAV) are pivotal for providing heterosubtypic immunity, but are not maintained long term, causing gradual loss of protection. This contrasts sharply with long-term maintenance of T_{rm} induced by localized infections of the skin and other tissues. Here we show that the decline in lung T_{rm} is determined by an imbalance between apoptosis and lung recruitment and conversion to T_{rm} of circulating memory cells. At the cellular level, circulating effector memory (T_{em}) rather than central memory (T_{cm}) cells are the precursors for conversion to lung T_{rm} . Time-dependent changes in expression of genes critical for lymphocyte trafficking and T_{rm} differentiation, in concert with enrichment of T_{cm} , diminish the capacity of circulating memory CD8 T cells to form T_{rm} with time, explaining why IAV-induced T_{rm} are not stably maintained. Importantly, systemic booster immunization, through increasing the number of circulating T_{em} cells, increases lung T_{rm} , providing a potential new avenue for future IAV vaccines.

Introduction

Seasonal influenza vaccination can provide effective antibody-mediated protection when the main surface antigen (hemagglutinin, HA) in the vaccine, matches that year's circulating influenza A virus (IAV) strains. However, mutations in the globular head region of HA can reduce the neutralizing capacity of vaccine-induced antibodies(1). In the absence of

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Author contributions:

BS, NVBB, GA, SSA, JTY designed experiments. BS and NVBB performed experiments and data analysis. GA performed IN Vac infection experiments and data analysis. SV provided crucial experimental reagents. BS, NVBB, GA, SSA, SMV and JTH contributed to writing and editing of the manuscript.

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neutralizing antibodies, IAV-specific memory CD8 T cells, which are maintained systemically as well as in the lung(2) correlate with some degree of control of viral titers and reduction of disease symptoms in humans(3, 4). Mouse models suggest it is the lung resident memory (T_{rm}) CD8 T cells that enable swift and robust protection against IAV infection(5–8). Thus, establishing a robust long-term T_{rm} population should be an important goal for an IAV vaccine.

Nonetheless, major knowledge gaps remain concerning lung T_{rm} generation, maintenance and effector function, especially compared to T_{rm} found in other non-lymphoid tissues such as skin, intestine and female reproductive tract. Mouse studies suggest that lung T_{rm} share common traits with T_{rm} from other tissues, most notably the expression of the transmembrane C-type lectin CD69 and αE -integrin CD103(7, 9). However at the transcriptome level lung T_{rm} cells are distinct from skin T_{rm} or intestinal T_{rm} (10), suggesting differential adaptation to specific microenvironments. One clear difference is that, compared to T_{rm} populations in other tissues(11), lung T_{rm} have a limited longevity (7), which strongly correlates with waning of subtype transcending heterosubtypic immunity to IAV (7). Therefore understanding the maintenance of lung T_{rm} may provide information that can be used to extend the life span of this population, thus prolonging heterosubtypic immunity to IAV.

Results

Limited longevity of IAV-induced lung T_{rm}

Local infections, such as herpes simplex (HSV) and vaccinia virus (Vac), have been used to study the formation and maintenance of T_{rm} in the skin (12). Skin T_{rm} have a distinct phenotype, co-expressing CD69 and CD103, molecules that have a functional role in retention in the tissues (10). Moreover, compared to non- T_{rm} , skin T_{rm} express low levels of a key transcription factor, Eomesodermin (Eomes) (13). Importantly, the population of skin T_{rm} is stably maintained (11) independently from the circulating memory CD8 T cell pool (12). In contrast, IAV-specific T_{rm} cells are lost from the lung with time(14).

To address this discrepancy under conditions of a fixed TCR response, a small number of naive Thy1.1 P14 transgenic CD8 T cells (specific for the GP33 epitope of LCMV) were transferred into naive Thy1.2 C57Bl/6 recipients followed by epicutaneous (EC) infection of the ear with Vac expressing the LCMV GP33 epitope (Vac-GP33) or intranasal (IN) infection with IAV (PR8-GP33). An intravascular (IV) stain with anti-CD45 antibody was applied prior to euthanasia, to distinguish P14 cells in the tissue (IV–) from those remaining in small capillary beds (IV+)(15, 16). In line with previous work (11), after an initial decline, the number of Vac-GP33-induced skin T_{rm} (defined as CD69+CD103+ P14 cells) and the total number of IV– P14 stabilized out to 200 days post infection (Fig. 1a, b). We also confirmed that Vac-GP33-induced skin T_{rm} display stable and low expression of Eomes (Fig. S1a, b).

Lung T_{rm} however, exhibited a different pattern. After an initial drop, there was an additional ~10-fold reduction in numbers of IV– P14 cells from 50 to 200 days post infection (Fig. 1c, d). In contrast to the skin, the number of P14 T_{rm} in the lung parenchyma

declined ~500-fold over the same time period (Fig. 1c, d). Importantly, IAV-induced lung T_{rm} exhibited the same stable and low Eomes expression as Vac-induced skin T_{rm} (Fig. S1c, d). Thus, despite their transience, IAV-generated lung IV– CD69+CD103+ CD8 T cells represented *bona fide* T_{rm} .

Waning of lung T_{rm} was not limited to P14 TCR-tg cells or recombinant IAV since endogenous T_{rm} specific for an influenza nucleoprotein-derived epitope (NP366) also underwent ~500-fold decrease over the course of 200 days post IN PR8 infection (Fig. 1e, f). Importantly, the dramatic decline of IAV-induced lung T_{rm} was not virus strain dependent as NP366-specific lung T_{rm} declined in a similar manner by day 125 after IN infection with X31 IAV (Fig. 1g). Moreover, the observation of limited lung T_{rm} longevity extended to respiratory infections other than IAV. IN infection of P14 recipients with Vac-GP33 induced GP33- and endogenous Vac B8R-specific lung T_{rm} responses, which declined in a similar fashion as IAV-induced lung T_{rm} (Fig. S2a–d). Thus, the differences between lung and skin T_{rm} are not explained by the use of different viruses for each organ.

Finally, we confirmed that waning of IAV-induced lung T_{rm} correlated with loss of CD8 T cell-mediated heterosubtypic protection. Primary exposure to H3N2 X31 IAV induced protection against challenge with heterosubtypic H1N1 PR8 IAV performed 30 days post X31 infection, measured by reduction of lung PR8 virus titers (Fig. 1h). Depletion of CD8 T cells rendered X31 immunized mice incapable of controlling PR8 virus titers. Heterosubtypic protection was completely lost at 125 days post initial X31 infection, which correlated with substantial decline in lung T_{rm} (Fig. 1g, h).

In summary, these data showed, in models of fixed TCR-specificity with P14 TCR-transgenic T cells, that skin and lung T_{rm} populations exhibit differences in durability and confirmed the observation that IAV-specific T_{rm} populations were not stable in the lung (14). In turn, the loss of T_{rm} correlated with reduced CD8 T cell dependent control of heterosubtypic IAV challenge(14), emphasizing the importance of understanding why the numbers of lung T_{rm} decreased over time.

Apoptosis rather than migration drives the loss of IAV-generated T_{rm} from the lung

To assess the possibility that some T_{rm} migrate out of the lung and to determine the persistence of lung T_{rm} , we labeled lung-residing cells using an *in vivo* CFSE labeling approach (17, 18). Intranasal inoculation of CFSE into mice previously infected with PR8-GP33 labeled all P14 cells in the lung parenchyma, but led to negligible CFSE staining of P14 cells in the draining lymph nodes and the spleen (Fig. S3), allowing for specific fate tracking of lung T_{rm} . *In vivo* CFSE labeling of lung IV– P14 generated by intraperitoneal (IP) infection with LCMV Armstrong, an approach that does not generate lung T_{rm} (Fig. S4), showed loss of labeled cells within 24 hours (Figure 2a, b). In contrast, IV– P14 cells generated through IN PR8-GP33 infection persisted with a half-life of ~5 days (Figure 2a, b). Loss of CFSE did not result from proliferation, as no discernable BrdU incorporation was detected in the CFSE^{lo} cells during this time period (Fig. S5). To assess whether T_{rm} are lost by migration, entrance of lung T_{rm} to the afferent lymphatics was blocked using the S1PR1 agonist FTY720 (19). FTY720 treatment did not prevent the loss of CFSE+ P14

from the lung parenchyma (Figure 2c), strongly suggesting that migration to the afferent lymph was not the primary mode of loss of IAV-generated T_{rm} .

Alternatively, impaired maintenance of lung T_{rm} could result from an increased apoptosis. In support of this notion we observed a higher percentage of IAV-induced IV– lung memory P14 expressing active caspases 3/7 (Flica stain) relative to IV+ lung memory P14, suggesting elevated pro-apoptotic activity in tissue-embedded cells (Figure 2d). Increased apoptosis is not a general feature of T_{rm} , as we observed no difference in Flica staining between skin IV– memory P14 cells and their IV+ (blood) counterparts (Figure 2d). Furthermore, IV– CD103+ P14 cells displayed higher caspase 3/7 activity than CD103– P14 cells (Fig. 2e). Additionally, protein expression of the anti-apoptotic molecule Bcl-2 was substantially reduced in IV– memory P14 cells relative to the IV+ cells in the lung or in blood (Figure 2f). Importantly, decreased Bcl-2 protein expression in IV– relative to IV+ memory P14 cells was also observed at day 90 post infection, suggesting that compromised survival may be a sustained characteristic of lung T_{rm} (Fig. S6). In contrast, skin IV– and IV+ circulating memory P14 cells exhibited similar level of Bcl-2 expression at day 30 post infection (Figure 2f). Of note, within the IV– P14 population, CD103+ cells had substantially decreased Bcl-2 expression compared to IV+ P14, while CD103– P14 cells had similar Bcl-2 expression to IV+ cells (Fig. 2g). Finally, we assessed the maintenance of mitochondrial membrane potential (Ψ mito), to evaluate the proapoptotic state of memory CD8 T cells in the lung after IAV infection (Fig. 2h, i). In line with increased Flica staining and decreased Bcl-2 expression, a larger fraction of IV– P14 cells exhibited decreased Ψ mito fluorescence compared to IV+ P14 (Fig. 2h). Additionally, IV– CD103+ cells showed lower Ψ mito fluorescence compared to their IV– CD103– counterparts (Fig. 2i). Together, these data showed that lung T_{rm} were prone to apoptosis, suggesting that cell death, rather than migration to draining lymph nodes was the reason for the limited longevity of this CD8 T cell population.

Continuous seeding of lung T_{rm} by precursors in the circulation in antigen-independent fashion

Despite the increased signature of apoptosis in the T_{rm} population (Fig. 2d–i) and a steady decline of CFSE labeled T_{rm} P14 cells in the lung parenchyma (Figure 2b), the total number of T_{rm} cells did not decrease over the six day CFSE-chase interval (Fig. S7). As depicted in Figure 3a, the emergence of CFSE–, CD69+ or CD103+ P14 over time compensated for the loss of CFSE labeled memory P14 from the lung T_{rm} pool. As proliferation was eliminated as a cause of disappearance of CFSE+ memory P14 cells (Fig. S5), these data suggest *de novo* generation of lung T_{rm} from a memory population outside of the lung parenchyma. Given previous reports showing continuous recruitment of circulating memory CD8 T cells into the lung even weeks after the clearance of the infection(20, 21), we hypothesized that lung T_{rm} were continuously formed from circulating precursors. Systemic antibody mediated depletion has been routinely used to show that, in most tissues, T_{rm} are maintained independently from the circulating memory population(22, 23). However, antibody depletion of the systemic memory CD8+ T cell population also depleted lung T_{rm} (Fig. S8). As an alternative, we utilized a system where depletion is based on cellular interactions (22). Male and female P14 cells were transferred in a 1:1 ratio into female recipients, which were

subsequently infected either IN with PR8-GP33 or EC with Vac-GP33 (Fig. 3b). Male P14 cells underwent initial expansion in numbers like female P14 cells but were rejected from the circulation after 2 weeks (Fig. 3c). As previously reported (22), male P14 in the skin were detectable for several weeks after they were systemically rejected (Figure 3d, e), indicating maintenance of skin T_{rm} without input from circulating memory CD8 T cells. Male P14 were also detectable in the lung parenchyma for several weeks after systemic rejection (Figure 3d, e) suggesting that the parenchyma provides a niche that shields male P14 from deletion. However, the 1:1 ratio is not maintained long-term and male P14 are progressively lost from the lung parenchyma (Figure 3d, e). Although we cannot formally exclude the possibility that anti-male cytotoxic T lymphocytes (CTL) gradually killed male P14 cells, in conjunction with the observed apoptosis of lung T_{rm} (Figure 2d–i), these data strongly suggested that lung T_{rm} were not adequately maintained without input from the circulating memory CD8 T cell population, and that maintenance of the lung T_{rm} pool required continuous recruitment of circulation-derived precursors. Consistent with this hypothesis, blocking the entrance of circulating memory P14 into the lung parenchyma by administration of pertussis toxin (Ptx) 3 weeks after infection significantly ($p < 0.05$) reduced the number of T_{rm} in the lung parenchyma one week later. In contrast, Ptx treatment did not affect the number of systemic memory P14 in the spleen (Figure 3f). These data are consistent with the requirement for a continuous influx of memory P14 cells from the circulation to maintain stable lung T_{rm} numbers over the short-term period.

Continuous formation of lung T_{rm} has been proposed (24, 25) and was thought to be driven by persisting IAV antigen. Antigen has been shown to persist for at least 2 months in the lung after IAV infection (25), making this an attractive explanation for the continuous formation, but also the eventual disappearance of lung T_{rm} (7). To confirm continuous recruitment of circulating memory CD8 T cells to the lung and to assess whether persisting antigen drives conversion of recruited memory CD8 T cells to lung T_{rm} we adoptively transferred spleen derived (CD69–CD103–) memory P14 cells that were generated by X31-GP33 infection into naive, PR8 (no antigen) or PR8-GP33 (antigen) infected mice (Figure 4a). Analysis of the lung 1 week after transfer revealed *de novo* generation of T_{rm} in all groups of mice (Figure 4b). A substantially larger fraction of transferred P14 cells entered the lung parenchyma of PR8 and PR8-GP33 infected mice compared to naive mice (Figure 4c). This suggested that the lung environment still drives additional recruitment of P14 cells into the parenchyma weeks after IAV infection, but this process does not require cognate antigen. Additionally, conversion to a T_{rm} phenotype was higher in PR8 and PR8-GP33 infected compared to naive mice, suggesting the post-IAV lung environment was permissible to *de novo* T_{rm} generation weeks after infection (Figure 4d). However, no difference in P14 conversion to T_{rm} was observed between PR8 and PR8-GP33 infected mice. Thus, the IAV-experienced lung contains cues other than antigen to drive formation of lung T_{rm} from circulating precursors. Cytokines like TGF- β , IL-33 and TNF have been implicated in formation of T_{rm} (10, 26) with IL-33 specially implicated in the function of lung resident innate lymphocyte cells (ILC) (27). As IAV infection increased lung of IL-33 (28) and TNF(29) (Fig. S9), we transferred memory P14 CD8 T cells derived from spleens of PR8-GP33 infected mice into naive recipient mice (Fig. 4e) that were treated or not with blocking (anti-IL-33R) or neutralizing antibodies (anti-TNF) prior to analyses on day 7 (Fig. 4e).

Blocking the IL-33 receptor reduced the number IV–P14 in the lung parenchyma (Figure 4f, g), but did not affect conversion to a T_{rm} phenotype (Figure 4f, h.). On the other hand, neutralization of TNF reduced both the accumulation (Figure 4f, g) of P14 cells in the lung parenchyma and acquisition of T_{rm} phenotype (Figure 4f, h), suggesting a role for this cytokine in formation and maintenance of lung T_{rm} .

Thus, our data show that the maintenance of IAV-induced lung T_{rm} CD8 T cell population critically depended on the circulatory CD8 T cell memory pool. Continuous seeding of the lung T_{rm} niche by circulating memory CD8 T cells appeared to be antigen-independent, and at least partially driven by local inflammatory cues (e.g. IL-33 and TNF).

IAV-induced circulating memory CD8 T cells lose the capacity to form T_{rm} over time

The capacity of circulating memory CD8 T cells to replenish lung T_{rm} contradicts the observation that T_{rm} are lost approximately 6 months post infection. Therefore, we hypothesized that with time circulating IAV-specific memory CD8 T cells lose their intrinsic capacity to generate T_{rm} . To probe this, we transferred memory P14 cells isolated from spleens of mice infected with PR8-GP33 for 20–30 days (early memory) or >100 days (late memory) into recipient mice infected with PR8 virus 21 days before (Figure 5a). Late memory P14 cells showed a dramatic decrease in accumulation in the lung parenchyma relative to early memory P14 (Figure 5b). Furthermore, late memory P14 displayed poor conversion to a T_{rm} phenotype compared to early memory P14 (Figure 5c). These data suggest that circulating memory CD8 T cells intrinsically lose the capacity to form lung T_{rm} over time, independent of the change in the local inflammatory cues.

To assess the molecular signatures which could potentially explain the difference between early and late circulating memory CD8 T cells in forming lung T_{rm} , we performed genome-wide mRNA expression in early (20–30 day post infection) and late (>100 days post infection) spleen-derived memory P14 generated by IN PR8-GP33 infection. Many genes (2,657; based on 1.25-fold difference, $p < 0.05$) were differentially expressed in late vs. early memory P14, with a similar number being up- (1,326) or down-regulated (1,331) (Fig. S10a). Of note, transcriptome analysis suggested differential expression of 3 transcription factors identified as master regulators of T_{rm} formation: Eomes (1.68x upregulated in late vs. early memory P14), Prdm-1 (Blimp-1) (2.19x downregulated in late vs. early memory P14) and Hobit (1.21x downregulated in late vs. early memory P14) (13, 30). Real-Time quantitative PCR (Q-PCR) analysis performed on mRNA isolated from early and late IAV-induced memory P14 demonstrated increased Eomes ($p < 0.0001$) and decreased Blimp-1 ($p = 0.021$) and Hobit ($p = 0.0033$) mRNA expression in late vs. early splenic memory P14 cells (Fig. 6a). Intracellular protein staining confirmed that a substantially higher percentage of late compared to early memory P14 cells upregulated Eomes (Fig. 6b). Combined, these data show that the transcription factor profile of late memory P14 cells is incompatible with acquisition of T_{rm} phenotype.

Additionally, ingenuity pathway analyses (IPA) revealed substantial alterations in a Leukocyte Migration gene set between late and early memory P14, represented by 158 genes ($p = 5.48 \times 10^{-25}$) (Table S1). Importantly, gene set enrichment analysis (GSEA) showed that leukocyte migration-associated genes were significantly negatively enriched ($FDR < 0.01$;

normalized enrichment score -1.45) in late memory P14 (Fig. S10b), suggesting some compromise in ability to migrate to tissues. To further refine our gene analysis and focus only on T lymphocyte migration, we performed a literature-based selection of genes from the leukocyte migration pathways based on: a) expression by T lymphocytes and b) associated with cell movement/migration/chemotaxis. This approach shortlisted 70 genes, that were further divided to specific categories, based on the function of their protein products (Table S2). Notably, expression of most of these genes ($>75\%$) was down-regulated in late vs. early memory P14 (Fig. S10c). Combined, the gene expression signatures strongly suggested that, with time, memory CD8 T cells downregulate the complex functional network of molecules collectively controlling cell migration to peripheral tissues. In turn, the reduced capacity of late memory CD8 T cells to enter lung tissue likely contributed to the decline in the T_{rm} population.

Our data showed that with time, IAV-induced circulating memory CD8 T cells lose the capacity to form *de novo* lung T_{rm} . We propose that inefficient conversion to T_{rm} , based on an unfavorable transcription factor landscape, together with the decreased recruitment to the lung tissue due to loss of migratory capacity in late circulating memory CD8 T cells underlies the waning of lung T_{rm} .

Circulating effector memory CD8 T cells are precursors of IAV-induced lung T_{rm}

Interestingly, analyses of the Leukocyte Migration genes revealed that two out of the relatively few mRNAs enriched in late memory P14 cells were those coding for CCR7 and CD62L (Fig. S10c), well known to be required for homing to secondary lymphoid tissue and as canonical markers of central memory (T_{cm}) CD8 T cells (31). Additionally, we observed that the vast majority ($>85\%$) of PR8-GP33 induced circulating memory P14 cells upregulated CD62L by day 90 post-infection. However, the T_{rm} population in the lung was composed of predominantly CD62L $^-$ cells (Fig. 7a). As T_{rm} P14 had a surface phenotype like T_{em} (CD62L $^-$), it was highly likely that they were recruited from the circulating T_{em} pool, although active downregulation of CD62L upon recruitment to the lung could not be ruled out. To discriminate between these possibilities, PR8-GP33 induced splenic memory Thy1.1 P14 cells were isolated on day 65 post-infection (when an optimal ratio of CD62L $^+$ and CD62L $^-$ P14 was observed), separated into enriched CD62L $^+$ and CD62L $^-$ subpopulations and transferred into mice infected IN with PR8 IAV 21 days earlier (Fig. 7b) and their capacity to convert into T_{rm} was assessed 7 days later. Of note, the T_{rm} master regulator, Eomes, was differentially expressed in the donor populations with CD62L $^+$ P14 (blue) being Eomes^{hi}, and CD62L $^-$ (red) expressing less Eomes (Figure. 7c). Importantly, the vast majority (89%) of P14 cells isolated from the lungs of CD62L $^+$ recipients were CD62L $^+$, while most (76%) of the P14 cells in the lungs of CD62L $^-$ recipients were CD62L $^-$ (Fig. 7d). This finding strongly suggested that down-regulation of CD62L after cell entry into the lung was unlikely to account for the observed enrichment of T_{em} -like cells (CD62L $^-$) in the lung. Of note, while P14 cells recovered from the lungs of CD62L $^-$ recipients (red) were found in the blood (IV $^+$) and parenchyma (IV $^-$), almost all P14 cells in the lungs of CD62L $^+$ recipients (blue) were IV $^+$, with only $\sim 1\%$ of cells residing in the parenchyma (Fig. 7e). Importantly, a discernable fraction of IV $^-$ P14 ($\sim 10\%$) formed CD69 $^+$ CD103 $^+$

lung T_{rm} in the CD62L⁻ recipients (Fig. 7f). In contrast, we did not detect any lung T_{rm} P14 in CD62L⁺ recipients (Fig. 7f).

Thus, our results identified circulating T_{em} as the precursors for *de novo* formation of IAV-specific lung T_{rm} . These data suggested the intriguing hypothesis that expansion of the circulating T_{em} population induced by IAV infection could increase numbers of lung T_{rm} . To test this hypothesis, we exposed PR8-GP33 immune or naive P14 recipient mice to systemic infection with recombinant *L. monocytogenes* expressing GP33 (LM-GP33) or an unrelated epitope derived from the *P. berghei* TRAP protein (LM-TRAP) (32, 33) 45 days after the initial PR8-GP33 infection (Fig. 8a). Mice were sacrificed 30 days after the systemic boost (75 days after initial PR8-GP33 infection) and circulating and lung P14 were analyzed (Fig. 8a). As predicted, systemic boosting with LM-GP33 enhanced the fraction of circulating T_{em} P14 cells (~84% were CD62L⁻), in sharp contrast to all the other immunization groups where circulating T_{cm} P14 dominated the response (Fig. 8b, c). As depicted in Fig. 8d, the most dramatic result of systemic boosting with LM-GP33 was an ~120x increase in the frequency of lung P14 memory cells, relative to non-boosted or LM-TRAP infected controls. Of note, naive mice that received the systemic LM-GP33 boost generated a circulating memory P14 population but did not generate a detectable population of P14 lung T_{rm} (Fig. 8d, e). Additionally, boosting IAV immune mice with LM-TRAP did not alter P14 lung T_{rm} numbers. However, in support of our hypothesis, the large increase in circulating T_{em} in PR8-GP33 immune mice boosted with LM-GP33 was associated with an ~10-fold increase in lung P14 T_{rm} at 30 days post boost (Fig. 8d, e). The increase in lung T_{rm} did not result from marked increase in frequencies of CD69+CD103⁺ P14 cells in the lung IV⁻ population, but rather from an increase in the total number of lung IV⁻ P14 cells in the LM-GP33 boosted mice (Fig. 8d, e). Thus, systemic boosting of IAV-induced memory CD8 T cell responses substantially increased the frequency of circulating T_{em} cells, which in turn enhanced the seeding of IAV-experienced memory CD8 T cells in the lung. The sustained ability of the lung environment to convert T_{em} to T_{rm} , in the context of a larger number of lung parenchymal T_{em} , resulted in the substantial increase in number of lung T_{rm} .

Discussion

Here we show that the maintenance of lung T_{rm} contrasts sharply to well-studied skin T_{rm} population. In concordance with earlier reports (7) we find a gradual loss of lung T_{rm} (defined as IV⁻ CD69+CD103⁺ cells) with time after IAV infection and concurrent loss of CD8 T cell mediated control of heterosubtypic infection. We provide evidence that lung T_{rm} display enhanced apoptotic properties (increased caspase 3/7 level, reduced Bcl-2 expression and decreased mitochondrial membrane potential) when compared to skin T_{rm} . Thus, the reduced Bcl-2 expression and other features of apoptosis in lung T_{rm} appear to result from the tissue environment itself. The lung environment starkly contrasts the skin, both in term of physiology as well as microbiome composition (34), making it rather challenging to identify the reason for the enhanced apoptosis in this tissue. For example, multiple studies have demonstrated a crucial role of TGF- β in formation, but also maintenance of tissue resident memory T cells (10, 13, 26, 35–37). Furthermore, it has been shown recently that integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ produced by skin stromal cells contribute to successful maintenance of T_{rm} in this organ by activating latent TGF- β (37). It is possible that such sources of TGF-

β are absent or only partially effective in the lung, thus limiting TGF- β signaling and survival of cells residing in lung parenchyma.

Alternatively, possible long-term elevated levels of TGF- β in the lung might lead to suppression of T-bet (13, 38, 39), which in combination with low expression of Eomes could result in inefficient IL-15 signaling in lung T_{rm} (40). As cytokines signaling through common γ chain receptors, (e.g. IL-15), decrease T cell apoptosis through induction of Bcl-2, TGF- β induced deficiencies in cytokine signaling could decrease anti-apoptotic Bcl-2 and enhance apoptosis (41, 42).

As a further support for apoptosis rather than migration from the lung as a driving force of T_{rm} disappearance, we showed that blocking of S1P1 mediated migration did not prevent the loss of T_{rm} from the lung parenchyma. However, we cannot completely rule out that T_{rm} CD8 T cells are shed into the airways, contributing to the gradual loss of the population in the lung parenchyma. Recent finding showing that a network of macrophages prevents shedding of CD4 T_{rm} cells from female reproductive tract into the lumen, in a non-S1PR1 but rather cytokine-mediated manner, makes shedding of lung T_{rm} CD8 T cell into the airway another possible scenario (43). Further studies of the mechanisms underlying the transient nature of IAV-induced lung T_{rm} will require detailed analyses of tissue architecture, physiology and microbiology, as they jointly participate in shaping lung-specific environmental cues important for T_{rm} formation and survival.

Interestingly, unlike skin T_{rm} , which are maintained without input from the circulating memory CD8 T cell pool, lung T_{rm} are replenished from CD69–CD103– circulating memory CD8 T cells even in the absence of persisting antigen. These results are in concordance with earlier reports using parabiotic mice (25, 44) or adoptive transfers (5, 6) which show that memory CD8 T cells can migrate into the lung during the steady state and in the absence of antigen. However these earlier studies did not use an IV stain to distinguish CD8 T cells in the lung parenchyma from those in the lung vasculature and did not employ the markers to distinguish whether the lung CD8 T cells were transient effector memory subsets or became genuine T_{rm} .

Together, apoptosis of lung T_{rm} and their replenishment from the circulating memory populations does not explain the gradual loss of lung T_{rm} and protection. However, our results showing that circulating memory CD8 T cells lose the capacity to form lung T_{rm} with time resolves this issue, and supports a model where decreasing cellular input, in combination with the loss of existing populations through apoptosis underlies the relatively slow decline of T_{rm} in the lungs. Mechanistically, we show that with time circulating memory CD8 T cells acquire a transcription factor profile that may not be permissible for conversion to T_{rm} in the lung (upregulated Eomes, downregulated Blimp-1 and Hobit) (13, 30). Additionally, bioinformatic analyses emphasize a loss in general migratory capacity in memory CD8 T cell populations with time, which supports our observation that circulatory memory CD8 T cells eventually lose the capacity to populate lung parenchyma.

Finally, we identify the circulating T_{em} pool as the main source of precursors for lung T_{rm} . These results suggest that the progressive shift of systemic memory CD8 T cells towards

T_{cm} , and concurrent loss of T_{em} populations, underlies the slow decay of IAV-induced lung T_{rm} . Consistent with this notion, we show that systemic booster vaccination, through expansion of the circulating T_{em} population, can increase the numbers of lung T_{rm} CD8 T cells. Interestingly, despite the >100x increase in frequency of lung CD8 T cells numbers of T_{rm} increased only ~10-fold, suggesting that optimization of the booster approach may be possible. For example, IN boosting with replication deficient viruses expressing IAV antigens or concurrent induction of mild inflammation in the lung during boosting may further enhance lung T_{rm} . Additionally, further work is needed to determine the durability of the boosted lung T_{rm} .

In conclusion, we identify several key features of IAV-induced lung T_{rm} CD8 T cells that differentiate them from T_{rm} populations in other microenvironments. Additionally, we provide an explanation for the gradual decline of this population in the lung with time. Properties of IAV-induced lung T_{rm} CD8 T cells most relevant for their transient nature are increased susceptibility to apoptosis and close dependence on circulating T_{em} pool for their maintenance. Importantly, expansion of the T_{em} population leads to an increase in numbers of lung T_{rm} CD8 T cells, which may inform novel strategies to enhance the longevity of T_{rm} pool and immunity to IAV infection.

Materials and methods

Study Design

The main aim of the study was to explain the gradual waning of IAV-induced lung T_{rm} , which correlates with loss of heterosubtypic protection. For this purpose we adopted a mouse model of IAV infection. The initial phase of the study confirmed the appropriateness of our model and developed approaches to evaluate the biological status and longevity of IAV-induced lung T_{rm} . Subsequent studies addressed the biology underlying recruitment and conversion of circulating memory CD8 T cells into lung T_{rm} , the waning of this ability with time and the capacity of systemic booster immunization to restore lung T_{rm} . All experiments were performed at least twice. The study involved sublethal infections with IAV or Vac or euthanasia prior to excessive body weight loss after IAV challenge, thus, no predetermined outcomes such as weight loss were used in the study and no outliers were excluded from the data analyses.

Mice

C57Bl/6 mice were originally derived from the National Cancer Institute (Fredericksburg, MD) and a colony is maintained *in house*. P14 transgenic mice (on a C57Bl/6 background) were acquired from Jackson Laboratories (Bar Harbor, ME). All animal studies and procedures were approved by the University of Iowa Animal Care and Use Committee, under PHS assurance, Office of Laboratory Animal Welfare guidelines.

Viral and bacterial infections

Influenza A/PR/08/34 H1N1 (PR8) and recombinant PR8 or X31 expressing GP33 (45, 46) were grown in chicken eggs. Allantoic fluid was diluted in PBS and mice received a sub-lethal dose (2×10^4 TCID₅₀) while lightly anesthetized. Epicutaneous infection with

Vaccinia virus expressing GP33 (Vac-GP33, a generous gift by Dr. Wherry at U Penn) was performed by applying 5×10^6 PFU of the virus on the center of the ear pinna, followed by poking 25 times with 27-gauge needle. For intranasal infection with Vac-GP33 mice were inoculated with 10^7 PFU of the virus. LCMV Armstrong infections were performed by intraperitoneal (IP) injection of 2×10^5 PFU of the virus. Systemic booster/mock booster immunizations were performed by IV injection of 10^7 CFU of recombinant *L. monocytogenes* expressing GP33 (LM-GP33) or a *P. berghei* TRAP-derived epitope (LM-TRAP (32).

Influenza protection and lung virus titers

C57Bl/6 mice were infected with X31 IAV (a reassortant IAV with 6 internal genes of PR8 and the hemagglutinin and neuraminidase of H3N2 A/Aichi/2/68). Heterosubtypic protection by early memory CD8 T cells was assessed 30 days post infection. Half of the mice were inoculated with CD8-depletion antibody, clone 2.43 (400 µg IP and 100 µg IN), while the rest of the animals were treated with equal amounts of Rat IgG control antibody. Two days post depletion, X31-immune mice and naive control animals were IN challenged with heterosubtypic PR8 (H1N1) virus. Lungs were harvested and virus titers assessed three days post challenge. Protection by late memory CD8 T cells was assessed in the same way, with PR8 challenge performed >100 days post initial X31 infection.

Intravascular staining and tissue preparation

Mice were intravenously injected with 2µg anti-CD45.2-APC (clone 104, Biolegend) in PBS. After 3 minutes mice were euthanized and whole lung or skin (ear) were isolated. Lung and skin were cut into small pieces and incubated 1 hour in a mixture of collagenase (125 U/ml) and DNase (0.1 mg/ml) at 37°C. Single cell suspensions were obtained by forcing the organs through a 70 µm mesh screen. Erythrocytes were lysed using Vitalyze (BioE, St. Paul, MN) and leukocytes were purified with 35% Percoll (GE Healthcare) in HBSS.

Statistical analysis

Comparison between 2 study groups was statistically evaluated by unpaired t-test, while comparisons between more than 2 groups were evaluated using multiple comparisons one- or two-way ANOVA, as specified in figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Reduced longevity of IAV-induced lung T_{RM} compared to Vac-induced skin T_{RM}. C57Bl/6 mice (Thy1.2) were seeded with naive P14 transgenic T cells (Thy1.1) and infected with a sub-lethal dose of Vac-GP33 ID (a, b) or PR8-GP33 IN (c, d). At the indicated days post infection mice were injected with fluorescently labeled anti-CD45.2 monoclonal antibody 3 min prior to euthanasia and tissue harvest. a) Representative flow plots of P14 cells from the ear (skin) identified at various time points after EC infection with Vac-GP33 (top panels), IV stain (middle panels) and CD69 and CD103 expression on IV- P14 (bottom panels). b) Total number of IV- P14 and CD69+CD103+ P14 cells in the ear parenchyma. 3 mice/time point. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. c) Representative flow plots of P14 cells from the lung at various time points after PR8-GP33 infection (top panels), IV stain (middle panels) and CD69 and CD103 expression on IV- P14 (bottom panels). d) Total number of memory P14 and CD69+CD103+ P14 in the lung parenchyma. n=3–4 mice/time point. Representative of 3 independent experiments. Error bars represent mean $\pm$ SEM. e) Representative flow plots of NP366 tetramer staining

of lung cells at various time points after sub-lethal IAV PR8 infection (top panels), IV stain (middle panels) and CD69 and CD103 expression on IV– NP366-specific CD8 T cells (bottom panels). f) Total number of memory IV– NP366+ and NP366+CD69+CD103+ CD8 T cells in the lung parenchyma. n=3 mice/time point. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. g) Representative CD69 and CD103 expression of IV– NP366 tetramer stained lung cells 30 and 125 days after IAV X31 infection. h) C57Bl/6 mice were infected with H3N2 X31 IAV and 30 or 125 days later were exposed to heterologous challenge with H1N1 PR8. D30 challenge was performed with and without CD8 T cell depletion. Lung PR8 virus titers measured in naive and X31-infected mice. n=4–5 mice/group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Multiple comparison one-way ANOVA. **p<0.01; ****p<0.0001.

Figure 2.

Apoptosis rather than migration from the lung loss of CFSE0-labeled IAV-induced lung T_{rm} . C57Bl/6 mice (Thy1.2) were seeded with naive P14 transgenic CD8 T cells (Thy1.1) and infected with PR8-GP33 IN (a–i), LCMV IP (a, b) or VacV-GP33 ID (d, f). 30 days post infection mice received intranasal CFSE and IV– P14 cells were analyzed at the indicated time points (a–c). a) Representative flow plots of CFSE labeling of IV– P14 cells at selected time points and b) cumulative analysis of the percentage of CFSE-labeled cells over time. $n=3-5$ mice/time point. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Unpaired t-test; *** $p<0.001$. c) After intranasal CFSE labeling mice received daily injection of FTY720 or vehicle control. Cumulative percentage of IV– CFSE + P14 at selected time points after CFSE staining. $n=3$ mice/time point. Representative of 2 independent experiments. Error bars are mean $\pm$ SEM. d) FlicA (caspase 3/7) staining of IV+ lung/blood (red) and IV– lung/skin (black) P14 cells 30 days after IN PR8-GP33 or EC Vac-GP33 infection. Representative profiles (left), cumulative data (right). $n=4$ mice/group. Representative of 2 independent experiments. Error bars are mean $\pm$ SEM. Multiple comparison one-way ANOVA; $p=0.0003$. e) Representative plots (left) and cumulative results (right) of FlicA (caspase 3/7) staining in different subsets of D30 IV– memory P14 cells isolated from lungs of IAV PR8-GP33 infected mice. $n=4$ mice/group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Multiple comparison one way ANOVA; $p=0.001$. f) Bcl-2 expression assessed 30 days post infection with PR8-GP33 or Vac-GP33 in IV+/blood (red) and IV– (black) P14 cells from lung or skin. Representative plots (left), cumulative data (right). $n=4$ mice/group. Representative of 2 independent

experiments. Error bars represent mean $\pm$ SEM. Unpaired t-test; **p=0.004. g) Representative plots (left) and cumulative results (right) of Bcl-2 staining in different subsets of D30 IV– memory P14 cells isolated from lungs of IAV PR8-GP33 infected mice. Reference is Bcl-2 expression by IV+ P14 cells in the lung (red). n=4 mice/group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Multiple comparison one-way ANOVA; p<0.0001. h) Decrease in mitochondrial membrane potential (Ψ mito) assessed 30 days post infection with PR8-GP33 by Mito Flow staining in IV+ (red) and IV– (black) P14 cells from the lung. Representative plots (left), cumulative data (right). n=3 mice/group. Representative of 3 independent experiments. Error bars represent mean $\pm$ SEM. Unpaired t-test; ***p=0.0002. i) Representative plots (left) and cumulative results (right) of Mito Flow staining in different subsets of D30 IV– memory P14 cells isolated from lungs of IAV PR8-GP33 infected mice. n=3 mice/group. Representative of 3 independent experiments. Error bars represent mean $\pm$ SEM. Multiple comparison one-way ANOVA; p<0.0001.

Figure 3.

Maintenance of IAV-induced lung T_{H1} depends on circulating memory CD8 T cells.

a) Representative example of CD69 (top panels) and CD103 (bottom panels) expression on CFSE⁺ and CFSE⁻ IV- P14 cells in the lung at the indicated time points post intranasal CFSE labeling. b) Female mice were seeded with male and female naive P14 cells in a 1:1 mixture and were infected IN with PR8-GP33 or EC with Vac-GP33. c) Percentage of female and male P14 cells in blood after infection with IAV PR8-GP33. n=3–7 mice/group. Representative of 3 independent experiments. Error bars represent mean± SEM. d) Representative flow plots depicting the proportion of female (Thy1.1/1.1) and male (Thy1.1/1.2) P14 cells in spleen, lung or skin at various days after Vac-GP33 (left panels) or PR8-GP33 (right panels) infection. e) Cumulative data depicting ratio of male:female P14 in indicated organs at indicated times post infection. n=3–4 mice/group. Representative of 3 independent experiments. Error bars: mean ± SEM. One-way ANOVA; *p<0.05, **p<0.01, ***p<0.0001 compared to spleen; ##p<0.01, ####p<0.0001 compared to lung. f)

C57Bl/6 mice were seeded with congenically labeled naive P14 (Thy1.1) cells and infected with a non-lethal dose of X31-GP33. After 3 weeks mice received 1µg of Ptx every other day for 1 week. Number of CD69+CD103+ P14 in the lung parenchyma and total number of P14 in the spleen after PBS or Ptx treatment. n=3 mice/group. Representative of 2 independent experiments. Error bars represent mean +/- SEM. Unpaired t-test; ***p=0.0009.

Figure 4.

Circulating memory CD8 T cells are recruited and converted to T_{rm} in the IAV-experienced lung in antigen independent fashion.

a) Memory P14 (Thy1.1) cells were isolated from spleens of X31-GP33 infected mice and transferred into naive mice or mice previously infected with PR8 or PR8-GP33 (Thy1.2). b) Representative flow plots of P14 staining (top panels), IV staining (middle panels) and CD69 and CD103 staining in IV- P14 population (bottom panels) in the lung. c) Cumulative % of IV- P14 and d) % of CD69+CD103+ P14 in the lung parenchyma. n=6 mice/group. Representative of 2 independent experiments. Error bars: mean $\pm$ SEM. Multiple comparison one-way ANOVA; *p<0.05, **p<0.01, ***p<0.001. e) Memory P14 (Thy1.1) cells were transferred into mice previously infected with PR8. Mice were intranasally treated with 100 μ g Rat IgG, anti-ST2 or anti-TNF on Day 0, 2 and 4 post transfer. f) Representative flow plots of P14 staining (top panels), IV staining (middle panels) and CD69 and CD103 staining in IV- populations (bottom panels) in the lung. g) Cumulative % of IV- P14 and h) % of IV- CD69+CD103+ P14 in the lung. n=8-9 mice/group. Cumulative results from 2 independent experiments. Error bars represent mean $\pm$ SEM. Multiple comparison one-way ANOVA; *p<0.05, **p<0.01.

Figure 5.

IAV-induced circulating memory CD8 T cells lose the capacity to form lung T_{rm} with time. a) Memory P14 (Thy1.1) cells were isolated from spleens of mice infected with PR8-GP33 20 days (early memory) or >100 days before (late memory). Isolated cells were transferred into mice (Thy1.2) previously infected with PR8. b) Representative flow plots (above) and cumulative data (below) of IV staining of P14 cell from the lung, 7 days post transfer. c) Representative flow plots (above) and cumulative data (below) of CD69 and CD103 staining of IV- P14 cells in the lung. $n=3-5$ /mice group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Unpaired t-test; * $p<0.05$ ** $p<0.01$.

Figure 6.

Late IAV-induced memory CD8 T cells display transcription factor profile unfavorable for T_{rm} formation.

a) Relative mRNA expression of Eomes, Prdm-1 (Blimp-1) and Hobit by Q-PCR using RNA isolated from spleen-derived memory P14 cells 20 days (early memory) or >100 days (late memory) after IN PR8-GP33 infection. n=5 RNA sample pools/group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Unpaired t-test; *p=0.021, **p=0.003, ****p<0.0001. b) Eomes intracellular Flow cytometry staining of early and late spleen-derived memory P14 cells (white) compared to naive P14 cells (gray).

Figure 7.

Lung T_{rm} are recruited from circulating T_{em} .

a) CD62L expression by memory P14 cells (Day 90) isolated from peripheral blood or by IV –CD103+CD69+ P14 isolated from the lung. b) Memory P14 (Thy1.1) cells were isolated from spleens of mice infected with PR8-GP33 65 days earlier. Cells were separated into CD62L+ and CD62L– subpopulations as described, and 3×10^6 separated P14 were transferred into Thy1.2 recipients infected with PR8 21 days earlier. Recipient mice were sacrificed 7 days after the transfer and lung P14 T_{rm} were evaluated. c) Eomes expression by CD62L+ (blue) and CD62L– (red) P14 cells measured prior to separation. d) CD62L expression by P14 cells isolated from lungs of CD62L+ recipients (blue) and CD62L– recipients (red). e) Representative plots (left) and cumulative results (right) of intravascular staining of P14 cells isolated from lungs of CD62L– (red) and CD62L+ (blue) recipients. f) Representative plots (left) and cumulative results (right) of CD69 and CD103 staining of IV – P14 cells isolated from lungs of CD62L– (red) and CD62L+ (blue) recipients. n=4 mice/

group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM.
Unpaired t-test; **p=0.0086, ***p=0.0008.

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Figure 8.

Systemic vaccination boosts IAV-induced T_{H1} pool by expanding circulating T_{H1} .

a) C57Bl/6 mice were seeded with naive P14 cells (Thy1.1) and IN infected with IAV PR8-GP33. Some mice received IV booster immunization with recombinant *L. monocytogenes* expressing GP33 (LM-GP33) or mock booster immunization with *L. monocytogenes* expressing a *P. berghei*-derived TRAP epitope (LM-TRAP) 45 days after initial IAV infection. At the same time a separate group of C57Bl/6 mice was seeded with naive P14 CD8 T cells and IV infected with LM-GP33. Analysis was performed 30 days after the (mock) booster. b) Representative plots and c) cumulative results of CD62L expression measured in splenic P14 cells of mice from all 4 immunization groups. $n=4$ mice/group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Multiple comparison one-way ANOVA. $p<0.0001$. d) Representative flow plots of P14 cells (top panels), IV staining (middle panels) and CD69 and CD103 staining of IV- P14 cells (bottom panels) in the lung. e) Cumulative numbers of CD69+CD103+ P14 in the lung parenchyma. $n=4$ mice/group. Representative of 2 independent experiments. Error bars represent mean $\pm$ SEM. Multiple comparison one-way ANOVA. $p=0.005$.