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Cutting Edge: IL-15–Independent Maintenance of Mucosally Generated Memory CD8 T Cells

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Effective vaccines against intracellular pathogens rely on the generation and maintenance of memory CD8 T cells (Tmem). Hitherto, evidence has indicated that CD8 Tmem use the common γ-chain cytokine IL-15 for their steady-state maintenance in the absence of Ag. This evidence, however, has been amassed predominantly from models of acute, systemic infections. Given that the route of infection can have significant impact on the quantity and quality of the resultant Tmem, reliance on limited models of infection may restrict our understanding of long-term CD8 Tmem survival. In this article, we show IL-15–independent generation, maintenance, and function of CD8 Tmem after respiratory infection with influenza virus. Importantly, we demonstrate that alternating between mucosal and systemic deliveries of the identical virus prompts this change in IL-15 dependence, necessitating a re-evaluation of the current model of CD8 Tmem maintenance. The Journal of Immunology, 2011, 186: 6667–6671.

Although there is considerable evidence substantiating the role of γc cytokines in CD8 Tmem development and maintenance, there remain contexts in which to explore these roles. The landmark studies implicating IL-15 in the maintenance of CD8 Tmem were conducted in models of acute, systemic infection where i.v. infection of IL-15−/− mice with vesicular stomatitis (VSV) or lymphocytic choriomeningitis viruses resulted in the reduction of Ag-specific CD8 Tmem, with Tmem attrition exacerbated over time (3–5). Alterations in type or route of infection, however, can impact the CD8 Tmem pool. Qualitatively different CD8 Tmem is generated in response to Ag delivered by either the mucosal or systemic route (6), and these different Tmem populations may require distinct homeostatic signals for their proliferation and survival.

The majority of pathogens that cause human diseases enter the host via a mucosal route, and unlike their systemically derived counterparts, the longevity of mucosal CD8 Tmem is limited (7). Importantly, after influenza infection, protection from challenge with heterosubtypic viruses is highly correlated with the retention of a pool of Tmem in the airways (8, 9). We hypothesized that limited availability of IL-15 or the loss of IL-15 responsiveness by airway-resident Tmem was responsible for this attrition. We demonstrate, however, that loss of IL-15 neither prevents the generation of Tmem nor accelerates the loss of mucosally generated, influenza-specific CD8 Tmem in the airways or the periphery. Moreover, altering the route of infection correspondingly alters the requirement for IL-15 in CD8 T cell homeostasis. Together, our data demonstrate that both IL-15–dependent and independent Tmem pools exist, and CD8 T cells primed in the mucosa require distinct signals for their long-term maintenance.

Materials and Methods

Mice, viruses, and infection

Age- and sex-matched C57BL/6 and IL-15−/− mice were purchased from the National Cancer Institute (Bethesda, MD) and Taconic Farms (Germantown, NY). Drs. S. Mark Tompkins (University of Georgia, Athens, GA) and Leo Lefranc¸ois (University of Connecticut, Farmington, CT) generously provided the influenza viruses (A/HK-x31[H3N2] and A/PR/8 [PR8, H1N1]) and VSV-NJ, respectively. Animals were infected intranasally (i.n.) with 10^3 PFU x31, 5 × 10^3 PFU PR8, or 10^4 PFU VSV or i.v. with 10^8 PFU VSV.

Tissue preparation and flow cytometry

Single-cell suspensions were obtained from spleens, lymph nodes, and peripheral blood and depleted of erythrocytes using Tris-buffered NH4Cl. Airway-resident and lung parenchyma lymphocytes were isolated as previously described (10).
MHC class I tetramers reactive against the influenza nucleoprotein (NP) (H-2D(b)/ASNE/NMETM) and VSV NP (H-2K(b)/RGYQQYGL) were generated by the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University). Tetramer staining was conducted at room temperature 1 h with other indicated mAbs (eBiosciences, San Diego, CA; BD Pharmingen, San Jose, CA; or BioLegend, San Diego, CA). For BrdU staining, 0.8 mg/ml BrdU (Sigma, St. Louis, MO) was added to drinking water. Isolated cells were intracellularly stained with FITC-labeled anti-BrdU (BD Pharmingen) and analyzed on a BD LSR II digital flow cytometer with BD FACSDiva software.

Plaque and in vivo CTL assays
For plaque assays, lungs from x31-immune wild type (WT) and IL-15−/− mice challenged with PR8 7 d previously were processed and incubated with a monolayer of Madin Darby canine kidney cells as previously described (10). Monolayers were washed, overlaid with MEM containing 1.2% Avicel, challenged with PR8 7 d previously were processed and incubated with a monolayer of Madin Darby canine kidney cells as previously described (10). Monolayers were washed, overlaid with MEM containing 1.2% Avicel, monolayers were washed, overlaid with MEM containing 1.2% Avicel, monolayers were washed, overlaid with MEM containing 1.2% Avicel, monolayers were washed, overlaid with MEM containing 1.2% Avicel, and 72 h postinfection (p.i.), monolayers were fixed and stained with crystal violet.

For in vivo CTL assays, half the splenic targets were pulsed with 10 μM influenza NP peptide (ProImmune, Bradenton, FL) for 1 h at 37˚C and the remainder unpulsed, labeled with 10 and 1 μM CFSE, respectively, and 2 × 105 targets were injected 50:50 into recipient mice. Fifteen hours later, percent target killing was calculated as follows: 100 − (% of NP-pulsed targets in infected recipients/% unpulsed targets in infected recipients) × 100.

Statistics
Unpaired two-tailed Student t test was applied using Prism software (GraphPad, San Diego, CA). The p values <0.05 were considered statistically significant and are indicated in Fig. 3.

Results and Discussion
IL-15 is dispensable for the generation and maintenance of influenza-specific CD8 Tmem
After infection with influenza virus, a population of Ag-specific CD8 T cells is activated, migrates to the lung airways, and differentiates into CD8 Tmem where, if maintained in adequate number, it confers protection to heterologous infections (7–9). Unfortunately, in the months after infection, this population declines, despite the fact that a reservoir of splenic CD8 Tmem continues to migrate to the lung airways to maintain steady-state numbers (11). Because CD8 Tmem in other models of acute viral infection require IL-15 for their long-term survival (3–5), we hypothesized that the limited longevity of the protective CD8 Tmem in the lung airways after infection is due to limited availability of IL-15 at this site.

To first determine whether IL-15 is required for the generation of influenza-specific CD8 Tmem, we infected C57BL/6 WT and IL-15−/− mice with x31 influenza and monitored the CD8 T cell response to influenza NP via MHC class I tetramers in the blood over time. The frequency of NP-specific CD8 T cells in the circulation of IL-15−/− mice remained similar to WT animals 40 d p.i. (data not shown). Thus, in contrast with studies with VSV (3), we found no defect in influenza-specific CD8 Tmem in the absence of IL-15. Other studies, however, have found IL-15 to be essential only for the maintenance of CD8 Tmem (4, 5). Therefore, it was possible that after full differentiation and trafficking to specific sites, influenza-specific Tmem, would gradually become more dependent on IL-15 for survival. To test this, we collected lymphocytes from the bronchoalveolar lavage, lung parenchyma, spleen, and lung-draining mediastinal lymph nodes of WT and IL-15−/− mice at an early memory time point (day 31) and later (day 115) p.i. Although the overall frequency of NP-specific Tmem decreased between days 31 and 115 p.i. in WT and IL-15−/− animals, both groups harbored a similar frequency of NP-specific CD8 T cells in all tissues (Fig. 1A, 1C). Because the majority of CD8 T cells in the lung and lung airways are influenza specific after infection, it is possible that IL-15 could have an important homeostatic effect on CD8 Tmem in these sites without any obvious changes in the overall frequencies of these cells in IL-15−/− mice. However, comparisons of the numerical fold loss of influenza-specific CD8 Tmem days 30 and 120 p.i. also did not reveal any differences in the attrition of memory cells between WT and IL−−/− mice over time (Fig. 1B, 1D).

Surprisingly, not only did the IL-15 deficiency fail to exacerbate the loss of CD8 Tmem from the airways, the frequency of splenic CD8 Tmem recovered was unaltered, even though 40–60% of these cells express CD122, which is required to receive survival signals from transpresented IL-15 (12) (data not shown). In addition, frequencies of CD8 Tmem specific for other influenza epitopes were similar in all tissues at day 35 p.i. (Supplemental Fig. 1). Thus, although IL-15 contributes to the migration of Ag-specific CD8 T cells at the effector stage of the immune response to influenza (10), it does not contribute to development and homeostatic maintenance of these CD8 T cells once differentiated into memory at either the site of infection or in the periphery.

The homeostatic proliferation of influenza NP-specific CD8 Tmem in WT and IL-15−/− mice is equivalent
IL-15 is important for the homeostatic proliferation of CD8 Tmem (13), whereas other γc cytokines such as IL-7 are more important for providing prosurvival signals to CD8 Tmem (14). To determine whether IL-15 is required for the homeostatic proliferation of Tmem derived from a mucosal infection, we examined the incorporation of BrdU by NP-tetramer−/− CD8 T mem cells isolated from WT and IL-15−/− mice. On both days 30 and 120 p.i. the percentage of replicating NP-specific CD8 Tmem was similar in all tissues of WT and IL−−/− mice (Fig. 1E–G). Thus, in contrast with models of systemic viral infection where CD8 Tmem turnover was severely impaired over time, CD8 Tmem homeostasis after respiratory infection persists independently of IL-15 signaling.

The differentiation of influenza-specific CD8 Tmem subsets is unaltered in IL-15−/− mice
In the linear model of Tmem development, transitioning CD8 T eff can elect one of two fates delineated by the distinct expression of the killer-lectin–like receptor G-1 (KLRG-1) and the IL-7 Rα-chain (CD127), which denote KLRG-1hiCD127lo short-lived effector cells (SLECs) and KLRG-1hiCD127hi memory precursor effector cells (MPECs) (15). IL-15 has been shown to be particularly important for the survival of SLECs during the contraction phase of the CD8 T cell response (16). Consistent with these findings, frequencies of NP-specific MPECs were slightly elevated in the tissues of IL−−/− animals at day 32 p.i. (data not shown). However, by day 115 postinfection infection, frequencies of MPECs were equivalent in IL−−/− and WT mice (Fig. 2A, 2D), indicating that even though SLECs were rapidly lost in the absence of IL−15 signaling, MPECs were preserved as they transition into a memory population.
Moreover, CD8 T$_{\text{mem}}$ are phenotypically heterogeneous and may be subclassified as either CD62L$^{\text{lo}}$ extralymphoid tissue-homing effector memory (Tem) or CD62L$^{\text{hi}}$ lymphoid-homing central memory (Tcm) (17). To test whether an IL-15 deficiency differentially affected a specific T$_{\text{mem}}$ subset, we monitored the expression of CD62L on influenza-specific CD8 T cells in both WT and IL-15$^{-/-}$ mice over time. The kinetics of CD62L expression on the NP-specific CD8 T cells in the blood (data not shown) and tissues of both IL-15$^{-/-}$ and WT animals was similar at day 115 p.i. (Fig. 2B), demonstrating that the sustained ratio of influenza-specific T$_{\text{cm}}$ and T$_{\text{em}}$ generated in the presence or absence of IL-15

**FIGURE 2.** Neither the maintenance nor the function of specific influenza NP-reactive CD8 T$_{\text{mem}}$ subsets is altered in IL-15$^{-/-}$ mice. NP-Tet$^+$ CD8 T cells from the indicated tissues were isolated and analyzed for tetramer reactivity and CD11a expression. Representative flow plots for the individual tissues from WT and IL-15$^{-/-}$ mice are shown. Mean percent NP-Tet$^+$ of total CD8$^+$ T cells in WT (shaded bars) and IL-15$^{-/-}$ (open bars) ± SEM on days 31 ($n = 3$–4 mice/group; data represent 3 independent experiments) and 115 p.i. ($n = 5$ mice/group). At days 31 (B), and 120 (D) p.i., NP-Tet$^+$ CD8 T cells were quantified in the lung and lung airways. Fold loss from peak was calculated as follows: number of NP-Tet$^+$ CD8 T cells at day 31 or 120/average number of NP-Tet$^+$ CD8 T cells at day 10. E, Representative flow plots for intracellular anti-BrdU staining in NP-Tet$^+$ CD8 T cells from lungs of WT and IL-15$^{-/-}$ mice 15 d after BrdU infusion in drinking water. The mean percent BrdU$^+$ among NP-Tet$^+$ CD8 T cells are plotted for WT (shaded) and IL-15$^{-/-}$ (open) mice ± SEM on days 30 (F) and 120 p.i. (G) ($n = 3$ mice/group).

**FIGURE 1.** IL-15 is not required for the homeostatic maintenance of influenza-specific CD8 T$_{\text{mem}}$. At days 31 (A) and 115 (C) post-x31 infection, CD8$^+$ T cells were isolated from the specified tissues and analyzed for tetramer reactivity and CD11a expression. Representative flow plots for the individual tissues from WT and IL-15$^{-/-}$ mice are shown. The mean percent NP-Tet$^+$ of total CD8$^+$ T cells are plotted for WT (shaded bars) and IL-15$^{-/-}$ (open bars) ± SEM on days 31 ($n = 3$–4 mice/group; data represent 3 independent experiments) and 115 p.i. ($n = 5$ mice/group). At days 10, 31 (B), and 120 (D) p.i., NP-Tet$^+$ CD8 T cells were quantified in the lung and lung airways. Fold loss from peak was calculated as follows: number of NP-Tet$^+$ CD8 T cells at day 31 or 120/average number of NP-Tet$^+$ CD8 T cells at day 10. E, Representative flow plots for intracellular anti-BrdU staining in NP-Tet$^+$ CD8 T cells from lungs of WT and IL-15$^{-/-}$ mice 15 d after BrdU infusion in drinking water. The mean percent BrdU$^+$ among NP-Tet$^+$ CD8 T cells are plotted for WT (shaded) and IL-15$^{-/-}$ (open) mice ± SEM on days 30 (F) and 120 p.i. (G) ($n = 3$ mice/group).
is equivalent. Recently, work in respiratory models of infection demonstrated that CD27CD43CD8 Tmem mediate superior recall responses in the lung (18). Therefore, we assayed the frequency of CD27CD43 CD8 Tmem in WT and IL-15−/− mice at day 115 p.i. and determined that this phenotype of CD8 Tmem was similar in both groups of animals (Fig. 2C). Thus, although the possibility exists that an IL-15−independent subset of CD8 Tmem expands to compensate for the loss of an IL-15−dependent CD8 Tmem subset, using three different Tmem subset phenotypes, we could observe no requirement for IL-15 in maintaining distinct CD8 Tmem populations after influenza infection.

True immunological memory is defined by the ability of Ag-specific CD8 Tmem to rapidly control an infection after a secondary encounter. Although similar frequencies of influenza-specific CD8 Tmem were maintained in WT and IL-15−/− mice, an IL-15 deficiency could functionally impair the memory population as observed in other studies (19). To test this possibility, x31-immune WT and IL-15−/− or naive WT mice were challenged with a lethal dose of PR8, the heterosubtypic H1N1 influenza virus that shares a conserved NP protein with x31. Day 7 postchallenge virus in the lung was quantified by plaque assay. Whereas naive animals averaged 750 PFU/mg lung tissue, the lungs of both WT and IL-15−/− memory mice were completely devoid of virus (Fig. 2D). The functionality of NP-specific CD8 Tmem generated in the absence of IL-15 was also tested via an in vivo CTL assay. Equal numbers of naive splenocytes pulsed either with or without influenza NP-peptide and differentially labeled with CFSE were injected into WT and IL-15−/− mice 45 d p.i. with x31. Fifteen hours posttransfer, spleens of recipient animals were harvested and the percentage of specific killing was determined. Both WT and IL-15−/− influenza-immune recipient mice killed ~55% of the peptide-pulsed targets (Fig. 2F). Together, these data indicate that the functional quality of the CD8 Tmem is preserved independent of IL-15.

**Route of infection alters the requirements for IL-15 by CD8 Tmem**

Although the frequency of VSV and lymphocytic choriomeningitis virus-specific CD8 Tmem decayed over time in IL-15−/− mice (3–5), we failed to observe any accelerated loss of influenza-specific CD8 Tmem in any site. A major difference between these studies is the route of infection that can alter CD8 T cell responses (6). Thus, to directly test the hypothesis that the route of infection results in a differential requirement of Ag-specific CD8 Tmem cells for IL-15, WT, and IL-15−/− mice were systemically (i.v.) or mucosally (i.n.) infected with VSV. On day 30 p.i., lymphocytes were examined for reactivity with the VSV NP-tetramer. As observed previously (4), the frequency of NP-specific CD8 Tmem cells in IL-15−/− mice was reduced by ~50% after systemic infection (Fig. 3). In contrast, however, WT and IL-15−/− animals mucosally infected with the identical virus contained an equivalent frequency of NP-specific CD8 Tmem in all sites examined, despite a lower response magnitude overall (Fig. 3). Thus, altering the route of infection with the same virus concordantly altered the requirement of IL-15 for the development of Ag-specific CD8 Tmem cells.

**CD8 Tmem generated in IL-15−/− mice is fully functional**

We were surprised to find an equal preservation of CD8 Tmem in the secondary lymphoid tissues of IL-15−/− mice infected i.n. with either influenza or VSV. One might speculate that altering infection route favors the development of either Tcm or Tem, which could be differentially dependent on IL-15 for survival. Comparing systemic with intranasal VSV infection, the ratio of splenic NP-specific Tcm to Tem, shifted from 20:80 to 40:60 (data not shown). Although the overall alteration in Tmem subsets could be because of differences in Ag load (20), IL-15−/− mice generated ratios of Tmem subsets equivalent to WT regardless of the route of infection. Although IL-7 can redundantly substitute for IL-15 (2, 21), we did not observe any differences in CD127 expression on Tmem, and we also failed to observe any differences in CD122 expression on circulating Tmem in the absence of IL-15 (data not shown). Therefore, IL-15−independent Tmem generated after mucosal infection are not the result of alterations in the development of a particular Tmem subset or CD122 expression, but are more likely due to a unique priming environment that bestows a homeostatic program on the Tmem that is IL-15 independent.

In addition, the harsh regulatory environment of the lung airways might render this site incapable of sustaining CD8 Tmem. Mucosal immune responses require extensive regulation of immune activation to prevent immunopathology. Thus, the mucosal environment stringently regulates the type, level, and duration of cytokines and chemokines elicited by mucosal infections to activate, recruit, and ultimately sustain (perhaps at set numerical thresholds) the appropriate lymphocytes at these sites. Our experiments contrasting the differential requirement for IL-15 in systemic versus mucosally administered VSV illustrate this phenomenon. Although systemically there may be
a need to maintain large numbers of VSV-specific CD8 T*mem, the regulatory mechanisms in place in the lung could be sufficient to inhibit the maintenance of memory cells beyond a certain threshold, regardless of IL-15 availability. Thus, although adjuvancing IL-15 could prolong CD8 T*mem responses to systemic infections, such regimes may have limited benefit in sustaining differentiated respiratory-derived CD8 T*mem.

Disclosures
The authors have no financial conflicts of interest.

References