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IL-15 Regulates Both Quantitative and Qualitative Features of the Memory CD8 T Cell Pool

Michelle M. Sandau,*† Jacob E. Kohlmeier, † David L. Woodland, † and Stephen C. Jameson*‡

Memory T cells are critical for immunity to various intracellular pathogens. Recent studies have indicated that CD8 secondary memory cells, induced by prime-boost approaches, show enhanced protective function compared with primary memory cells and exhibit phenotypic and functional characteristics that distinguish them from primary memory cells. However, little is known about the cytokine requirements for generation and maintenance of boosted memory CD8 T cells. We studied the role of IL-15 in determining the size and composition of the secondary (2°) memory CD8 T cell pool induced by *Listeria monocytogenes* infection in mice. Following boosting, IL-15–deficient animals failed to generate a subset of CD8 effector memory cells, including a population of IL-7Rαlow cells, which were prominent among secondary memory cells in normal mice. IL-15 deficiency also resulted in changes within the IL-7RαhighCD62Llow subset of 2° memory CD8 T cells, which expressed high levels of CD27 but minimal granzyme B. In addition to these qualitative changes, IL-15 deficiency resulted in reduced cell cycle and impaired Bcl-2 expression by 2° memory CD8 T cells, suggesting a role for IL-15 in supporting both basal proliferation and survival of the pool. Analogous qualitative differences in memory CD8 T cell populations were observed following a primary response to Sendai virus in IL-15−/− animals. Collectively, these findings demonstrate that IL-15 plays an important role in dictating the composition rather than simply the maintenance of the CD8 memory pool.

Immuno-logical memory results in a more rapid and effective immune response to Ags that have been encountered previously (1–3). A number of studies on memory CD8 T cells have shown that they differ from naive cells in their speed and efficiency with which they enter cell cycle and differentiate into effector cells (2, 4–6). In the context of infection, these factors translate into a more rapid and robust clearance of pathogens by memory versus naive CD8 T cells (1, 2, 7).

There are considerable data on the role of cytokines in the homeostasis of naive and memory CD8 T cells, especially with respect to the function of IL-7 and IL-15 (8–10). The receptors for IL-7 and IL-15 both use the common γ-chain (γc), but these cytokines have distinct effects on T cell maintenance. IL-7 signaling is mediated through γc and the unique α-chain IL-7Rα (CD127). IL-7 is critical for the development and survival of naive T cells (11, 12) and is also important for differentiation of memory CD8 T cells following the effector stage (13, 14). Similar to its impact on naive CD8 T cells, IL-7 promotes maintenance of memory cells, in part, through up-regulation of Bcl-2 (15). IL-15 uses γc, IL-2Rβ (CD122), and the unique chain, IL-15Rα. Several reports have demonstrated that IL-15 plays a critical role in maintenance of memory CD8 T cells, as illustrated by the decay of this pool in IL-15 and IL-15Rα−/− deficient animals (16–18). Although IL-15 can enhance expression of prosurvival Bcl-2 family member proteins in naive and memory T cells (19), its best defined role in T cell homeostasis is to promote proliferation of CD8 memory T cells (16, 20, 21). Hence, IL-15–driven basal proliferation of CD8 memory cells allows for the indefinite maintenance of this pool, whereas the memory pool declines in IL-15−/− (and in IL-15Rα−/−) animals (8, 15, 16, 20–22).

Most current studies, however, have focused on the properties of CD8 memory T cells produced following a single round of immunization, whereas vaccination protocols advocate repeated boosting to produce the most effective memory response (2, 7, 23, 24). Interestingly, recent studies have shown differences in the differentiation state and functional potential of primary (1°) versus secondary (2°) (i.e., boosted) memory CD8 T cells (25, 26). These reports indicate that 2° memory CD8 T cells take much longer than 1° memory cells to lose expression of effector molecules (such as granzyme B) and to acquire expression of L-selectin (CD62L) and the ability to make IL-2 upon restimulation (25, 26). Presumably, as a result of their decreased expression of CD62L, boosted memory CD8 cells exhibit differential tissue localization, being less prevalent in lymph nodes (LNs) compared with 1° memory CD8 T cells (25, 26). Finally, the functional capacity of 2° memory CD8 cells was found to be enhanced compared with their 1° memory counterparts, exhibiting increased cytolytic potential and providing more efficient protection against the intracellular pathogen *Listeria monocytogenes* (25). Taken together, these data indicate that there are qualitative as well as quantitative differences in the CD8 memory pool following boosting.

Very little is known about the homeostasis of the 2° memory pool, in particular whether the established role for IL-15 in maintenance of the 1° memory CD8 T cell pool (8, 15, 16, 20–22) also applies to boosted CD8 memory T cells. Indeed, recent studies suggest IL-15 may be less significant in supporting the 2° memory CD8 T cell pool. Secondary memory CD8 T cells were found to be much less sensitive to IL-15–driven in vitro proliferation compared with their
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1° memory counterparts (25). This finding was also correlated with the delayed acquisition of CD62L by 2° memory CD8 T cells, because CD62L was shown to be upregulated only after cell division of the 2° memory pool (25). Moreover, in vivo basal proliferation in both normal and lymphopenic animals was impaired in the 2° versus 1° memory CD8 pool (25). Taken together, these data suggest that 2° memory CD8 T cells may show reduced sensitivity to endogenous levels of IL-15 and hence undergo slower basal proliferation compared with 1° memory CD8 T cell pool (25).

We sought to further investigate the role of IL-15 in the maintenance, phenotype, and function of 1° and 2° memory CD8 T cells by examining these populations in normal versus IL-15−/− animals. In agreement with others, we found that 2° memory CD8 T cells exhibit reduced basal proliferation compared with 1° memory counterparts (25), but that the presence of IL-15 still impacts the maintenance of the 2° memory CD8 T cell pool. Surprisingly, although IL-15 has not been reported to influence the phenotype or subset distribution of the 1° memory CD8 T cell pool, we found that IL-15 has a qualitative effect on the 2° memory CD8 T cell population: In the absence of IL-15, 2° memory CD8 T cells expression of granzyme B was reduced, and an IL-7Rαlow population (found prominently among 2° memory CD8 T cells pool in wild-type animals) was completely absent. IL-15 deficiency also resulted in impaired basal proliferation and reduced expression of the prosurvival protein Bcl-2. In addition, we found that IL-15 deficiency led to similar changes in the subsets of primary memory CD8 T cells induced by intranasal immunization with Sendai virus. Hence, our findings suggest a novel role for IL-15 in supporting the maintenance, subset composition, and phenotype of the memory CD8 T cell pool.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and B6 IL-15−/− were purchased from Taconic Farms (Germantown, NY). OT-I TCR transgenic (tg) mice (27) were bred to B6.PL (Thy.1.1) mice (The Jackson Laboratory) to generate the OT-I-LPL strain. Enrichment of CD44high OT-I-LPL cells was performed by negative selection purification, as previously described (28), and 1 × 10^6 OT-I cells were adoptively transferred into B6 and IL-15−/− mice via the tail vein 1 d prior to infection. All mice were maintained under specific pathogen-free conditions at the University of Minnesota. All animal handling and experiments were carried out with approval from the University of Minnesota or Trudeau Institute Institutional Animal Care and Use Committees.

Infections

Recombinant L. monocytogenes expressing secreted OVA (LM-OVA) protein 10403s were provided by H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA) and J.T. Harty (University of Iowa, Iowa City, IA). Bacteria were grown in tryptic soy broth with 50 μg/ml streptomycin to an absorbance at 600 nm of ~0.1. For primary infections, 1 × 10^5 to 5 × 10^4 CFU were injected i.v., whereas for secondary infections, ~1 × 10^5 were injected i.v. Boosting was performed at least 4 wk after the primary infection. Throughout these experiments, 1° responses were analyzed at days 31–39 postinfection, while 2° responses were analyzed at days 28–39 postboosting. The number of bacteria injected was confirmed by dilution and growth on tryptic soy agar plates containing streptomycin. For Sendai virus (Enders strain) infections, 8–12-wk-old mice were anesthetized with 2,2,2-trimethoxyethanol (200 mg/kg), and 250% egg infectious doses were delivered intranasally in a volume of 30 μl.

Flow cytometry

Single-cell suspensions from spleen and pooled LNs, bone marrow (BM), and liver were analyzed by flow cytometry. Endogenous OVA-specific CD8 T cells were identified by co-staining with CD8α and H-2Kd-OVA tetramer produced as described previously (29). Tetramers specific for the Sendai virus nucleoprotein epitope (Snp-NP244-253Kb) were produced by the Trudeau Institute Molecular Biology Core Facility. Co-staining with CD8α and Thy.1.1 identified transferred OT-I-LPL cells. Phenotypic analysis was carried out with additional staining with Abs to CD122, CD62L, CD127, CD27, CD43 (1B11) (purchased from BD Biosciences [San Jose, CA] and eBioscience [San Diego, CA]), CXCR3 (purchased from R&D Systems, Minneapolis, MN), and CCL19-Fc (generously provided by J. Cyster, University of California San Francisco, San Francisco, CA). Flow cytometry was performed on a FACSCalibur and LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

CD8 T cell enrichment for secondary adoptive transfers

B6 mice receiving adoptive transfer of 10^6 OT-I T cells were primed and boosted with LM-OVA as described above. At day 5 of the recall response, splenocytes were enriched for CD8 T cells by negative selection, using a mixture of FITC-labeled Abs against CD4, CD19, NK1.1, and I-AK, followed by staining with anti-FITC paramagnetic microbeads (Miltenyi Biotec, Auburn, CA) and removal over magnetic columns (Miltenyi Biotec). OT-I cells in the flow through were enumerated as described above before secondary transfer into uninfected B6 or IL-15−/− hosts and were analyzed 23 d later (4 wk from the booster infection).

Intracellular staining

For intracellular staining, cells were surface stained first, then fixed and permeabilized (BD Pharmingen, San Diego, CA), followed by anti-mouse Bcl-2 or hamster IgG isotype control (BD Pharmingen), or anti-human granzyme B or mouse IgG1 isotype control (Caltag Laboratories, Burlingame, CA).

Intracellular cytokine staining

Splenocytes were stimulated with or without SINFEKPL peptide (250 nM for IFN-γ and TNF-α production 1 μM for IL-2 production), in the presence of brefeldin A, for 4 h at 37°C. Cells were fixed and permeabilized (BD Pharmingen), according to the manufacturer’s protocol, and then stained with the appropriate Abs. An unstimulated sample was generated for each animal, and the frequency of cytokine staining in these samples was subtracted from the frequency in Ag-stimulated samples to quantitate the specific response.

BrdU labeling

Two weeks after the last infection, mice were administered with freshly made BrdU (0.8 mg/ml administered with 2% sucrose) in the drinking water, for 14 d. Cells were harvested and surface stained with CD8α, Thy.1.1, and IL-7Rα. Cells were then fixed, permeabilized, and DNAse treated according to the manufacturer’s protocol. Cells were then stained with anti-BrdU (BD Pharmingen).

Statistical analysis

An unpaired, two-tailed Student’s t test was used to determine significance with Prism software (GraphPad, San Diego, CA). Unless otherwise noted, statistical significance is indicated as follows: *p < 0.05; **p < 0.01; and ***p < 0.001.

Results

Maintenance of the secondary memory CD8 T cell pool requires IL-15

Previous studies indicated that certain features of the 2° memory CD8 T cell pool (such as the low basal proliferation and slow re-acquisition of the CD62Lhigh phenotype) might be explained by insensitivity to IL-15 (25), leading to the hypothesis that physiological levels of IL-15 may be less relevant for maintenance of 2° memory CD8 T cells compared with their 1° memory counterparts. To test this idea, we investigated the characteristics of 2° memory CD8 T cells produced by priming and boosting wild-type B6 mice and IL-15−/− mice with L. monocytogenes (LM-OVA). In these studies, we examined the response of both polyclonal (endogenous) CD8 T cells and of OT-I TCR tg T cells adoptively transferred (at low numbers) into B6 and IL-15−/− hosts. The latter approach was designed to avoid complications related to potential changes in the Ag-specific TCR repertoire in 1° versus 2° responses of B6 versus IL-15−/− strains and also controlled for the potential influence of IL-15 during CD8 T cell development (17–19).

Earlier studies showed that, following priming, CD8 memory cells decline in an IL-15−/− host (16, 20). However, if the hypothesis...
described above were correct, we might expect that the 2° CD8 memory pool may be less influenced by IL-15. Both 1° and 2° memory OVA/Kb-specific CD8 T cells were analyzed in IL-15−/− and B6 mice infected with LM-OVA, at a minimum of 30 d and maximum of 42 d post (re)infection. This timeframe was selected as a means to assay relatively early memory formation, as well as ensuring the presence of primary memory cells in the IL-15−/− host. As expected (16, 20), the number of Ag-specific memory CD8 T cells in the IL-15−/− hosts was lower than in B6 following the 1° infection (Fig. 1A), but at 5 d after boosting, we observed similar numbers of OVA/KbCD8° cells in B6 and IL-15−/− animals, indicating memory cells in both populations were competent to respond and expand to similar total numbers (Fig. 1A). At >30 d following boosting, the Ag-specific memory pool had contracted in both strains, but the frequency of OVA/KbCD8° cells was elevated compared with the primary memory pool, suggesting effective boosting. However, similar to the pattern observed in the 1° response, we found that the number of 2° memory cells was decreased in IL-15−/− compared with B6 animals (Fig. 1A). Similar results were obtained when tracking TCR tg OT-I cells (adoptively transferred at 10° cells before priming) (data not shown), which argues that these results are not due to differences in TCR repertoire or recruitment of new naive responder cells during the secondary response. These data suggested that, even in the secondary response, IL-15 had an impact on the maintenance of the CD8 memory pool.

Next, we sought to test whether IL-15 was required for the generation and/or maintenance of 2° memory cells. Therefore, we used a secondary adoptive transfer of OT-I T cells that were primed and boosted in B6 mice; on day 5 following the boost, the OT-I T cells were retransferred into uninfected B6 or IL-15−/− hosts, then memory cells were enumerated 28 d following the boost. If IL-15 was required for maintaining 2° memory cells, we would expect to see a decline in numbers of OT-I cells recovered from the IL-15−/− hosts. Our data supported this hypothesis, and the number of OT-I 2° memory cells recovered from the IL-15−/− hosts was much lower than the number recovered from the B6 hosts (Fig. 1B). These data further suggest that deprivation of IL-15 after 2° infection results in a dramatic decrease in cell numbers.

IL-15 is thought to contribute to the maintenance of 1° memory CD8 T cells chiefly by induction of slow basal proliferation, which maintains the CD8 memory pool indefinitely: hence, a hallmark feature of IL-15-deficient animals is severely reduced memory CD8 T cell proliferation (8, 10, 15). Because 2° memory CD8 T cells already show a reduced turnover compared with the 1° memory pool (25), it was unclear whether a similar mechanism could account for the impaired maintenance of 2° memory CD8 T cells we observed in IL-15−/− hosts (Fig. 1A, 1B). Thus, we assayed the proliferative capacity of 1° and 2° memory OT-I CD8 T cells in B6 and IL-15−/− animals by assessing their in vivo BrdU incorporation over a 14-d period (Fig. 1C). Basal proliferation within the 1° memory OT-I CD8 T cell pool was reduced in IL-15−/− compared with B6 hosts, as expected (16, 20), and we also observed reduced turnover of 2° memory CD8 T cells compared with 1° memory CD8 T cells in B6 hosts, as reported previously (25) (Fig. 1C). However, the proliferation of 2° memory CD8 T cells was reduced still further in IL-15−/− animals (Fig. 1C). These data suggest that, even though the turnover of 2° memory cells in B6 animals is reduced (compared with 1° memory CD8 T cells), this proliferation still depends, at least in part, on IL-15.

**FIGURE 1.** IL-15 influences the maintenance and basal proliferation of 2° memory CD8 T cells. *A*, B6 and IL-15−/− mice were primed and (31 d later) boosted with LM-OVA, as indicated. The number of endogenous splenic OVA/Kb-specific CD8 T cells was enumerated by multiplying the percentage of OVA/KbCD8° cells present by the total number of cells recovered. Statistically significant differences (⁎p < 0.05; ⁎⁎p < 0.01) between B6 and IL-15−/− groups are indicated. B, A total of 10° OT-I T cells were adoptively transferred into B6 mice, which were then primed and boosted with LM-OVA. At day 5 of boosting, bulk CD8 T cells were transferred into B6 and IL-15−/− hosts, and recovery of donor OT-I cells in the spleens of these secondary hosts determined 23 d later (i.e., 28 d after boosting). C, B6 and IL-15−/− animals were adoptively transferred with 10° OT-I T cells and primed/boosted as indicated. In the experiment shown, 1° infected mice were analyzed 39 d after priming, and 2° infected mice were analyzed 28 d after boosting. The mice were placed on BrdU drinking water for 14 d, and then the percentage of OT-I cells showing BrdU incorporation was determined. Statistical differences (⁎⁎p < 0.01) are indicated. A and C are representative data from at least three experiments, whereas B is representative of two experiments.

**IL-15 deficiency alters the phenotype of the 2° memory CD8 T cell pool**

Although these data suggested IL-15 may influence maintenance similarly in both 1° and 2° memory CD8 T cell homeostasis, other data suggest 2° memory CD8 T cells differ from their 1° memory counterparts by multiple phenotypic and functional properties (26). Hence, we next sought to determine whether IL-15 influences the composition of the 2° memory CD8 population.

A feature of the 2° CD8 T cell response is that there is a delay in contraction of the activated population and a prolonged representation of cells that display low levels of CD62L expression compared with the 1° memory pool (25, 26, 30). Also, it has been suggested that this phenotype is linked to the reduced proliferative
response of 2′ memory CD8 T cells in response to IL-15 (25). Such a model would predict that the presence or absence of IL-15 in the host would not impact the 2′ memory CD62L phenotype. Indeed, when we assessed CD62L expression on either endogenous OVA/Kb tetramer+ (data not shown) or OT-I CD8 T cells ±30 d after boosting with LM-OVA in various tissues (spleen, LN, BM, and liver), we found that the prominent CD62Llow phenotype of the splenic 2′ memory CD8 pool was unaffected by IL-15 deficiency (Fig. 2A). In addition, we found that the IL-15 status of the host did not influence the relative frequencies of CD62Lhigh and CD62Llow cells in BM and liver (data not shown). CD62L expression was high on 2′ memory CD8 T cells recovered from the LN of boosted animals from either host (data not shown), as expected. Such data argued that the presence or absence of IL-15 did not impact re-expression of CD62L in 2′ memory cells. Likewise, we observed a similar (high) frequency of CD62Lhigh memory cells in the 1′ memory OT-I population in both B6 and IL-15−/− hosts (data not shown), in keeping with previous reports that the presence of IL-15 in vivo does not influence memory CD8 T cell phenotype (8, 15).

However, the CD62Llow subset, which persists long term in 2′ CD8 memory pools in normal mice, have been reported to exhibit distinct phenotypic features, including low expression of low expression of CCR7 (31), which is reminiscent of effector (26). Low expression of both CD27 and CD62L has been correlated with low expression of CD127 (a critical signaling component of the receptor for IL-15) and CD44, were similar between the 2′ memory from wild-type and IL-15−/− hosts (data not shown). Taken together, these data indicate that, although some characteristics of 2′ memory CD8 population (such as prominent representation of CD62Llow cells) were maintained in IL-15−/− animals, other features (such as low expression of CD27 and CCR7) were altered in the absence of IL-15, thus suggesting IL-15 supports production or maintenance of a subset of TEM cells.

Absence of CD127low memory CD8 T cells in IL-15−/− animals

In addition to the features discussed above, it has been reported that the boosted CD8 T cell memory pool includes a sizeable and relatively long-lived population of CD127low cells (26). This contrasts with 1′ responses, where there is compelling evidence that IL-7 is important for long-term survival of the 1′ memory CD8 T cell pool (13, 14). Because there may be overlap between the CD8 T cell responses induced by IL-15 and IL-7 receptors (10, 15), we were particularly interested in whether the prominent pool of CD127low 2′ memory CD8 T cells would be IL-15 dependent.

Hence, we analyzed 1′ and 2′ memory OT-I CD8 T cells in B6 and IL-15−/− hosts. The vast majority of 1′ memory CD8 T cells was CD127high in both B6 and IL-15−/− animals, yet, as expected based on previous studies (26), at least 25% of 2′ memory CD8 T cells in B6 hosts were CD127low cells (Fig. 3A, third panel), and virtually all these cells were CD62Llow (Fig. 3B). In contrast to this finding, however, the 2′ memory CD8 T cells recovered from the IL-15−/− animals were uniformly CD127high (Fig. 3A, third panel, 3B). This pattern of expression was also observed for 2′ memory OT-I cells recovered from the BM and liver of both B6 and IL-15−/−, whereas in the LN, 2′ memory CD8 T cells were CD62Llow and CD127high in both B6 and IL-15−/− mice (data not shown). In addition, these cells lack expression of activation markers, such as CD69 (data not shown), indicating they were not recently activated effector cells. These data suggest that the generation and/or maintenance of CD127low 2′ memory CD8 T cells was compromised in IL-15−/− animals.

It was possible that responding CD8 T cells in the IL-15−/− animals maintained CD127 expression even during restimulation. At day 5 following 2′ infection, CD127 expression was low on the majority of OT-I cells from both WT and IL-15−/− hosts, but we consistently observed a higher frequency of CD127+ cells in the IL-15−/− animals at this time point (“recall” Fig. 3A). Such data suggest that CD127 is not downregulated as efficiently and/or is re-expressed more quickly in the IL-15−/− deficient hosts during the recall response. Such data indicated that the cells observed during the recall expansion phase could already be altered in the IL-15−/− hosts. Hence, this complicated our attempts to determine the role for IL-15 at the stage past recall expansion and leading into generation of secondary memory cells. To address this issue, we performed retransfer experiments in which OT-I cells were primed and boosted in B6 hosts, recovered at day 5 of the recall response, and then transferred into B6 or IL-15−/− hosts. If the presence IL-15 during priming or early boosting was sufficient to program the appearance of CD127low 2′ memory CD8 T cells, then we would expect to see CD127low cells appear in both B6 and IL-15−/− secondary hosts. In contrast, if the CD127low cells required IL-15 for their maintenance, we would only see them emerge in the secondary B6 recipient. Our results supported the second model, because we observed the adoptively transferred OT-I 2′ effector cells was uniformly high for CD127 in IL-15−/− hosts, but included both CD127high and CD127low subsets in B6 hosts (Fig. 3A, fourth panel). Hence, the presence of IL-15 after recall proliferation was essential for generation of the normal contingent of memory stage subsets.

Since most CD127low cells were found in the CD62Llow subset (Fig. 3B), it was possible that the aberrant expression of other TEM
markers discussed in Fig. 2B related simply to the selective absence of the CD127\textsuperscript{low} pool. Indeed, closer analysis revealed that even within the CD127\textsuperscript{high} subset of CD62L\textsuperscript{low} cells, there was increased expression of CD27 in the IL-15\textsuperscript{−/−} animals (Fig. 3C), although CCR7 staining was similar in IL-15\textsuperscript{−/−} and B6 hosts (data not shown). The expression of these markers (CD27 and CCR7) on the CD62L\textsuperscript{high},CD127\textsuperscript{high} subset was similar in B6 and IL-15\textsuperscript{−/−} animals, suggesting that the IL-15 deficiency had little effect on the phenotype of the central memory T cell-like pool but had a marked impact on the TEM pool.

Hence, IL-15 deficiency has numerous effects on the composition of the 2\textsuperscript{nd} memory CD8 T cell pool, including the loss of CD127\textsuperscript{low} cells and changes in the phenotype of the CD62L\textsuperscript{low},CD127\textsuperscript{high} subset, and use of the adoptive transfer approach revealed that IL-15 can exert these effects following the recall expansion phase.

**Production of CD127\textsuperscript{low} primary memory cells following Sendai virus also requires IL-15**

In our studies, a CD127\textsuperscript{low} subset was prominent in the secondary memory CD8 T cell pool but was rare among primary memory CD8 T cells induced in B6 mice following LM-OVA immunization (Fig. 3A). A similar observation was reported by others analyzing the response to *L. monocytogenes* infection (26). Hence, it was difficult to determine whether the requirement for IL-15 in generating CD127\textsuperscript{low} memory stage CD8 T cells was unique to the secondary memory response or could also apply to primary memory cells. Interestingly, previous studies on the primary immune response to respiratory infection with Sendai virus observed a detectable population of CD127\textsuperscript{low} cells that persisted for months following intranasal infection (34). The basis for this difference in the primary response to *L. monocytogenes* versus Sendai infections is unclear, but use of the Sendai system gave us the opportunity to test the role of IL-15 in generating CD127\textsuperscript{low} cells during the primary response. B6 and IL-15\textsuperscript{−/−} mice were subjected to respiratory infection with Sendai virus and Ag-specific CD8 T cell populations studied 65 d later. Ag-specific (Sen-NP\textsubscript{324–332}K\textsuperscript{b} tetramer binding) primary memory CD8 T cells were detected in both hosts at this time point (Fig. 4A). Sendai-specific CD8 T cells in IL-15\textsuperscript{−/−} animals were only slightly reduced in frequency compared with B6 animals (average ~40% lower percentage; data not shown), which was a less severe defect than we had observed in the *L. monocytogenes* response (Fig. 1). Yet there were substantial changes in representation of Sendai-specific memory CD8 T cell subsets in the two mouse strains. Although a CD127\textsuperscript{low} population was readily observed among Ag-specific primary memory CD8 T cells in B6 animals (Fig. 4A), this population was virtually absent in IL-15\textsuperscript{−/−} hosts.

In the Sendai model, CD127\textsuperscript{low} cells lack expression of CXCR3 and CD27 (Fig. 4B) (34). We observed a corresponding decrease in representation of CXCR3\textsuperscript{low} and CD27\textsuperscript{low} populations in the Sendai-specific memory CD8 T cell pool in IL-15\textsuperscript{−/−} compared with B6 animals (Fig. 4). Thus, for either 1\textsuperscript{st} or 2\textsuperscript{nd} memory CD8 T cells (studied following Sendai virus or *L. monocytogenes* infection, respectively), appearance of a CD127\textsuperscript{low} population required the presence of IL-15. Our data suggest that perturbations of CD8 memory subsets owing to IL-15 deficiency can be observed in both primary and secondary memory populations following infection with distinct pathogens.

**IL-15 deficiency compromises granzyme B expression in the 2\textsuperscript{nd} memory CD8 T cell pool**

In addition to phenotypic differences, 1\textsuperscript{st} and 2\textsuperscript{nd} memory CD8 T cells exhibit significant changes in functional properties. Compared with 1\textsuperscript{st} memory cells, the boosted memory CD8 T pool shows a diminished capacity to produce IL-2, a similar ability to make IFN-γ and TNF-α, and an increased cytolytic capability upon Ag encounter. This latter feature is exemplified by the ex vivo expression of granzyme B in 2\textsuperscript{nd} (but not 1\textsuperscript{st}) memory CD8 T cells (25, 26), a feature that may correlate with improved control of pathogens by 2\textsuperscript{nd} memory cells (25).

We observed no significant differences in the capacity of splenic 2\textsuperscript{nd} memory cells to produce IFN-γ and TNF-α whether the cells were generated in B6 or IL-15\textsuperscript{−/−} environments, and there was little evidence for enhanced cytokine production by 2\textsuperscript{nd} versus 1\textsuperscript{st} memory pools (Fig. 5A, 5B). Also, the ability of 2\textsuperscript{nd} memory cells to simultaneously produce both TNF-α and IFN-γ was not impaired in the IL-15\textsuperscript{−/−} host (data not shown). Similarly, we tested the ability of the cells to produce IL-2. In contrast to previous reports.
(25, 26), we found little difference in the ability of the 2° memory cells to produce IL-2 in comparison with 1° memory cells (Fig. 5C). In addition, the lack of IL-15 did not impact the ability of the cells to produce IL-2 (Fig. 5C). The basis for the difference between our findings and those of published reports (25, 26) is unclear but may relate to the length of time after boosting that memory function was assessed. In any case, these data suggest that the presence or absence of IL-15 has a minimal impact on the capacity of 2° (or 1°) memory CD8 T cells to produce IFN-γ, TNF-α, and IL-2 after restimulation.

Next, we examined the ex vivo expression of granzyme B and determined that 1° memory cells express minimal granzyme B, regardless of whether they are recovered from B6 or IL-15−/− hosts (Fig. 6A). However, we observed an increase in granzyme B expression in 2° memory cells in the B6 host when compared with 1° memory cells (Fig. 6A), as reported previously (25, 26). Interestingly, granzyme B expression was considerably diminished in 2° memory cells from IL-15−/− hosts (Fig. 6). In contrast to previous studies (25), we observed that the majority of granzyme expressing cells were in the CD62Llow pool (Fig. 6B, top row). This raised the question of whether the reduced granzyme B expression simply correlated with the absence of the CD127low subset in IL-15−/− animals. However, we observed a difference in granzyme B expression even within the CD127high (Fig. 6B, bottom row) suggesting that, similar to the phenotypic differences discussed above, the IL-15 deficit impacts the differentiation state of the surviving cells. We observed similar vigorous cytolytic activity of both B6 and IL-15−/−-derived populations of 2° memory CD8 T cells using the 51Cr release assay (data not shown): this assay chiefly depends on the effector cell expression of perforin rather than granzymes (35) but does suggest cytolytic potential is normal (or quickly acquired, in vitro) by 2° memory CD8 T cells from IL-15−/− animals.

Taken together, these data suggested that basal expression of granzyme B, but not potential for cytokine production or cytolysis, is influenced by IL-15.

Maintenance of CD127low memory cells through an IL-15-dependent mechanism

Although our earlier data (Fig. 1) indicated that IL-15 enhanced proliferation in the 2° memory pool, the fact that the composition of the 2° memory pool differed in B6 and IL-15−/− animals demanded reassessment of the function of IL-15 in 2° memory CD8 T cell maintenance. In particular, the presence of CD127low cells in the 2° memory pool from B6 but not IL-15−/− animals suggested a key role for IL-15 in their maintenance. Potentially, the differences between basal proliferation of 2° memory cells in B6 and IL-15−/−
animals might arise by IL-15–driven proliferation of the CD127low subset. To test this, we divided the OT-I cells into either CD127high or CD127low subsets and determined the percentage of those cells that incorporated BrdU over a 14-d labeling period.

In contrast with this model, we observed that the CD127low subset exhibits relatively poor basal proliferation (Fig. 7A). Instead, the majority of cells undergoing basal proliferation in the B6 hosts are CD127high (Fig. 7A). In addition, the proliferation of the corresponding CD127high population in the IL-15−/− host was slightly reduced and similar to the data in Fig. 1C.

We showed earlier that total numbers of 2’ memory CD8 T cells were consistently lower in IL-15−/− versus B6 hosts (Fig. 1A, 1B). Although this is partially explained by the lack of CD127low cells, the number of CD127high cells was also reduced in IL-15−/− hosts compared with B6 hosts (Fig. 3D). Furthermore, although 2’ memory CD8 T cells showed lower basal proliferation in IL-15−/− rather than B6 hosts (Figs. 1C, 7A), the magnitude of this difference appeared to be insufficient to account for the decreased absolute numbers of 2’ memory CD8 T cells in IL-15−/− hosts (Fig. 1A, 1B).

Therefore, we considered whether IL-15 also enhances survival of 2’ memory CD8 T cells. Bcl-2 is an important cell survival protein that is increased as a result of IL-7R signaling and is upregulated in memory CD8 T cells stimulated with IL-15 (19). Hence, we analyzed intracellular Bcl-2 levels in 1’ and 2’ memory CD8 T cells recovered from B6 and IL-15−/− hosts. Although there were minimal differences between expression levels of Bcl-2 in 1’ versus 2’ memory cells from the individual hosts, there was greater expression of Bcl-2 in memory cells recovered from B6 hosts compared with IL-15−/− hosts (Fig. 7B). We also determined Bcl-2 expression by CD127low and CD127high cells in 2’ memory cells recovered from B6 hosts. The 2’ memory CD127high CD8 T cells in B6 hosts expressed higher levels of Bcl-2 compared with the CD127low pool (Fig. 7C, 7D). Similar gating of 2’ memory CD8 from IL-15−/− hosts reveals that the CD127high cells, which persist in the IL-15−/−, express less Bcl-2 than the corresponding population in the B6 host (Fig. 7C, 7D). Taken together, these data would suggest that optimal expression of Bcl-2 requires recognition of both IL-7 and IL-15. Furthermore, these data indicate that IL-15 preserves CD127low TEm 2’ memory cells through survival rather than diminished IL-15–driven proliferation.

Discussion

Because various pathogens (such as HIV and malaria) are poorly protected by Ab responses, there has been renewed interest in developing vaccines that elicit efficient T cell responses. As with humoral immunity, boosting can augment cellular immune response (2, 7, 23, 24). However, recent studies have revealed that boosting the CD8 T cell response not only increases the number of Ag-specific T cells but also results in qualitative changes in the memory CD8 T cell pool, which impact the phenotype, trafficking, and function of 2’ memory cells compared with their...
The data are representative from at least three experiments.

were analyzed at day 30 and the 2° memory cells at day 39 postinfection. In contrast, 2° memory CD8 T cells exhibit reduced basal turnover (and impaired lymphopenia-driven proliferation) in vivo and show inefficient IL-15 induced proliferation in vitro (25). Hence, it was possible that IL-15 becomes a less relevant cytokine for maintenance of the 2° memory CD8 T cell pool. Instead, our findings indicate that IL-15 has a significant impact on the maintenance, composition, and function of CD8 T cells at the 2° memory stage.

Specifically, we find that IL-15 impacts the early memory CD8 T pool in three distinct ways. First, IL-15 deficiency leads to decreased frequency of 2° memory CD8 T cells. This is probably due to a combination of decreased basal proliferation and impaired survival of 2° memory CD8 T cell pool in IL-15−/− animals. Although we and others (25) found that 2° memory CD8 T cells show reduced turnover compared with their 1° memory counterparts, this proliferative rate was further reduced in IL-15−/− hosts (even when analysis was focused on the CD127high subset found in both strains Figs. 1C, 7A). This is somewhat surprising given that the in vitro response of 2° memory T cells to IL-15 was impaired (25); however, this assay may have underestimated the cells’ sensitivity to the cytokine. Because the expression of the anti-apoptotic protein Bcl-2 has been linked to IL-15 signals (19), we then explored the survival role of IL-15. Although we observed a reduction in Bcl-2 in IL-15−/− deprived 1° memory cells, this was even more marked for the 2° memory CD8 T cell pool (Fig. 7). We speculate other survival proteins of the Bcl-2 family may promote maintenance of the CD127low population in the presence of IL-15; however, this possibility remains unexplored. Taken together, these data suggest that IL-15 plays an important role in directing expression of prosurvival factors as well as supporting basal proliferation of the 2° memory pool.

Second, IL-15 is evidently critical for the differentiation and/or survival of the CD127low subset of CD8 T cells that persist into the memory phase. This population is found for many weeks following primary infection with some pathogens [e.g., Sendai (34) and Fig. 4] and following secondary responses to other microbes [e.g., LM (26) and Figs. 3, 6, 7]. However, cells of this phenotype do decline gradually over time as the memory population stabilizes, and this population overlaps with the short-lived effector pool produced following the primary immune response, which has a similar phenotype and is also dependent on IL-15 for maintenance (37). Indeed, a valid interpretation of our data are that lack of IL-15 leads to quicker or more effective contraction of the effector pool, leading to faster appearance of the stable memory pool. However, this CD127low subset is much more prominent and long lived in the 2° immune response to L. monocytogenes compared with the 1° response, and previous studies suggested the secondary CD8 T cell responses may be less sensitive to IL-15 (25). In contrast, our data demonstrate that CD127low CD8 T cells produced during recall responses are highly dependent on IL-15.

Given the potential overlap between IL-7 and IL-15 signals, it is reasonable to conclude that cells lacking CD127 will become dependent on IL-15 for their survival, and our data are in keeping with that model applying in both primary (Sendai infection) and secondary (L. monocytogenes infection) immune responses. However, an alternative hypothesis is that IL-15 itself induces downregulation of CD127. Naive T cells have been shown to

for 1° memory CD8 T cells, IL-7 plays an important role in dictating differentiation and survival of 1° memory CD8 T cells, whereas IL-15 is critical for their basal proliferation and supporting long-term maintenance of the pool (1, 10, 36). Accordingly, there is a severe decline in production/maintenance of 1° memory CD8 T cells if they are deprived of IL-7, whereas deprivation of IL-15 results in a severe reduction of 1° memory CD8 T cell basal proliferation.

1° memory progenitors (25, 26). These changes in the frequency, subset distribution, and phenotype of 2° memory subsets raise the question of whether the homeostasis of this population is regulated in the same way as 1° memory cells. Current models suggest that,
downregulate expression of CD127 in response to various cytokines, including IL-15 (38). Furthermore, recent studies show that treatment with multivalent IL-15/IL-15Rα complex can promote appearance of CD127low cells during the contraction phase of an immune response (39). It is worth reiterating that the CD127low subset is usually less prominent in the 1° memory CD8 T cell pool; therefore, despite access to physiological levels of IL-15, the majority of 1° memory cells re-express IL-7Ra, which seems critical for their differentiation from the effector stage (13, 14). What regulates the capacity of some 2° memory cells to “wean” themselves off IL-7 and onto IL-15 as a survival cytokine is currently unclear. However, we also observed an impact of IL-15 deficiency on primary CD8 memory subsets, following respiratory infection with Sendai virus. Hikono et al. (34) demonstrated phenotypic and functional heterogeneity among the memory populations produced by this infection, including the appearance of a CD127low memory CD8 T cell population, which persists for months (but not long term). The majority of cells in this CD127low subset was also found to lack expression of CD27 and CXCR3.

We observed that the Sendai virus-specific memory CD8 T cell pool in IL-15−/− mice selectively lacked CD127low, CXCR3low, and CD27low subsets (Fig. 4). Hence, these data indicate that IL-15 preserves CD127low TEM memory cells in addition to changing the CD8 memory subset composition in response to divergent experimental systems.

Third, the lack of IL-15 resulted in altered phenotypic and functional changes in the cells that persist into the 2° memory pool. A subset of CD127high CD62Llow 2° memory CD8 T cells was found in both B6 and IL-15−/− hosts. However, while in IL-15−/− sufficient mice this subset contained CD27low and granzyme B⁺ cells, in the IL-15−/− animals, these cells were CD27high and granzyme B⁻. A CD27low and granzyme B⁺ memory CD8 T pool has been previously observed following the primary respiratory response to Sendai virus (34). CD27 is a member of the TNF receptor superfamly and can undergo functionally relevant interactions with CD70. CD27 signals on CD8 T cells has been shown to promote the generation and survival of memory cells (40–42); therefore, the retained expression of CD27 on cells in the IL-15−/− may be a compensatory mechanism of survival. Alternatively, the presence of CD27 on the IL-15−/− 2° TEM subset may allow these cells to participate in additional responses denied to their B6 counterparts. The lack of granzyme B in the IL-15−/− 2° memory CD8 T cells is similar to previous studies that showed reduced expression of granzyme B in IL-15−/− effector cells (43). Although granzyme B is an important component of cytolytic granules, granzyme B−/− CD8 T cells are typically competent for cytolysis, an assay that chiefly depends on the ability of the effector cells to secrete perforin (35), and we observed similar cytolytic potential of 2° memory CD8 T cells from both B6 and IL-15−/− animals (data not shown). On the other hand, granzyme B−/−/− animals have a severely reduced ability to control enctromelia (44) and show a delayed control of lymphocytic choriomeningitis virus (45). Thus, the lack of steady-state granzyme B in IL-15−/− 2° memory CD8 T cells may potentially influence their ability to rapidly control certain pathogens. Regardless, these data together argue that IL-15 influences the expression of functionally relevant markers (CD27 and granzyme B) in the 2° memory pool. This may be direct in the case of granzyme B, which appears to be induced by IL-15Rα signals (43). A recent study reported that treatment with IL-15/IL-15Rα complex following priming lead to enhanced representation of CD127low cells but no significant changes in expression of CD27 or CXCR3 (among other markers) following the treatment (39). In contrast, we observed that IL-15 deficiency lead to changes in expression of both CD27 (Figs. 2, 3, 4) and CXCR3 (Fig. 4). This may reflect the distinct phases of the immune response studied, because Rubenstein et al. studied the contraction phase of the response (through to day 17 postinfection), whereas our studies focused on the memory phase.

In these studies, we chiefly examined responses of adoptively transferred OT-I T cells following priming and boosting with LM-OVA and focused on relatively early stages of the memory phase (typically ~4 wk postimmunization). This was partly driven by the low numbers and poor maintenance of Ag-specific memory CD8 T cells generated in IL-15−/− animals. However, we observed similar general features of 2° memory CD8 T cells in B6 and IL-15−/− hosts when endogenous OVA/K⁺−specific CD8 T cells were tracked as well as when heterologous prime/boost approaches were used (using LM-OVA for priming and boosting with vaccinia-OVA; data not shown). Furthermore, the characteristic features of 2° memory CD8 T cells discussed above have been reported in different mouse strains, responding to various viral and bacterial pathogens, and appears to hold true for responses by both the endogenous polyclonal CD8 T cell pool and for TCR tg T cells (25, 26). Hence, although there are certainly limitations with use of adoptive transfer approaches (30), the differences between 1° and 2° memory CD8 T cell responses appear to transcend these concerns.

IL-15−/− mice clear 1° and 2° L. monocytogenes infection similarly to B6 mice (our unpublished observations), and this was part of our rationale for using L. monocytogenes in this study. However IL-15−/− animals have been reported to have defects in clearance of vaccinia virus (17) as well as defects in the response to heat-killed Propionibacterium acnes (46). Our data on the multiple effects of IL-15 deprivation on the 2° memory CD8 T cell pool suggest that boosting does not compensate for the lack of IL-15, and it will be interesting to see whether defective immune control of these pathogens by IL-15−/− animals is compounded still further in secondary immune responses.

In summary, our data suggest IL-15 impacts both the maintenance and, more surprisingly, the composition and functional potential of the 2° memory CD8 T cell pool. Hence, despite reported decreased sensitivity of 2° memory CD8 T cells for IL-15, this cytokine still plays a prominent role in dictating the boosted memory CD8 T cells. Hence, its proposed inclusion as a component of vaccines for cell-mediated immunity (47) may be justified, even when a prime boost strategy is used. It will be interesting to determine whether the role of other homeostatic cytokines (e.g., IL-7) changes in the 2° memory CD8 T cell pool.

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Disclosures

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