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Distinct Roles for IL-2 and IL-15 in the Differentiation and Survival of CD8+ Effector and Memory T Cells

Diana M. Mitchell, Eugene V. Ravkov, and Matthew A. Williams

IL-2 provides a memory differentiation signal to CD8+ T cells during the primary response that impacts the ability of the subsequent memory pool to mount a successful recall response. In this study, we find that although primary effector CTL development is modestly decreased in the absence of IL-2, the persistence of short-term and long-term effector memory CD8+ T cells on pathogen clearance is greatly diminished. Furthermore, secondary challenge of CD8+ memory T cells lacking the high-avidity IL-2R results in a failure to repopulate the effector pool. The role of IL-2 in promoting effector differentiation is not shared with the highly related cytokine, IL-15. Although IL-15 supports the survival of effector CD8+ T cells after pathogen clearance, its absence does not impair either primary or secondary effector CTL differentiation, nor does it impact the differentiation of long-term effector memory CD8+ T cells. These findings indicate a unique role for IL-2, but not IL-15, in promoting the differentiation not only of primary effector CD8+ T cells, but also of CD8+ memory T cells capable of secondary effector differentiation. The Journal of Immunology, 2010, 184: 6719–6730.

During the primary phase of the immune response to acute infection, Ag-specific CD8+ T cells receive instructional signals that dictate later stages of differentiation. On Ag recognition, CD8+ T cells undergo massive clonal expansion and acquire effector functions that are critical for the elimination of intracellular pathogens, including cytolytic function and the ability to produce proinflammatory cytokines, such as IFN-γ and TNF-α. After the infection is resolved, most of the effector population dies, leaving behind a long-lived population of memory cells capable of rapid secondary protection on re-exposure to the same or a related pathogen (1, 2).

CD8+ memory T cell precursors can be identified among the effector population at the peak of the response to acute infection based on the expression of cell surface molecules, such as IL-7Rα and KLRG1 (3, 4). Intensive efforts are underway to understand the nature of the differentiation signals that differentially promote the emergence of effector cells that express high levels of KLRG1 and low levels of IL-7Rα (short-lived effector cells [SLECs]), and memory precursor cells that express low levels of KLRG1 and high levels of IL-7Rα. CD4+ T cell-derived "help" is of particular importance in the generation of functional (capable of secondary responses to Ag) CD8+ memory T cells (5–9). Other studies have suggested that memory potential may depend at least in part on asymmetric division at the initiation of the T cell response (10, 11) or differential expression of the transcription factor T-bet driven by exposure to inflammatory cytokines, such as IL-12 (3).

Our recent studies have focused on the role of IL-2 in CD8+ memory T cell differentiation. Like others (12–14), we found that in the absence of IL-2 signals, CD8+ T cells showed only modest impairment in their ability to make robust primary responses after acute infection. However, IL-2 signals during the primary response were required for the ability of the ensuing CD8+ memory cells to generate optimal secondary responses (15). Several other observations indicated that the impact of IL-2 on CD8+ T cells impacted multiple differentiation pathways. For example, memory T cells generated in the absence of IL-2 skewed to a central memory-like phenotype as measured by expression of CD62L and the ability to produce IL-2 on restimulation (15).

Prior studies have suggested that IL-2 and the closely related cytokine IL-15 differentially regulate certain aspects of CD8+ memory T cell differentiation. Although activation in the presence of high levels of IL-2 in vitro preferentially promotes the subsequent in vivo development of effector and effector memory T cells, activation in the presence of IL-15 preferentially promotes central memory differentiation (16, 17). Both of these cytokines have been used or proposed as potential immunotherapeutics. High-dose IL-2 treatment has been used clinically to treat several types of cancer, including renal cell carcinoma and melanoma, with modest effects on a subset of recipients (18–20). The use of IL-15 has been suggested for boosting T and NK cell antitumor responses and as a vaccination adjuvant in various model systems (21–27). Although IL-15 has a well-described role in promoting the homeostasis and survival of CD8+ memory T cells (28), differing mouse models of acute infection demonstrate either no role (29, 30) or a significant role (31) for IL-15 in the generation of effector CTL responses. In all, it remains unclear how and to what extent IL-2 and IL-15 mediate overlapping, differing, or even opposing functions, particularly in the early phases of activation in which T cells enter into their differentiation program (32, 33).

Because CD8+ T cell effector responses were robust even in the absence of IL-2 signals, we hypothesized that related cytokines may compensate for the lack of IL-2 signals during acute infection. IL-15 was an obvious initial candidate. IL-2 and IL-15 belong to a family...
of cytokines using the γc as a component of their receptors. Among this family, IL-2 and IL-15 are particularly related, as they share the β-chain (CD122) and γc (CD132) of their heterotrimERIC receptor. Therefore, IL-2 and IL-15 promote apparently distinct biological outcomes while using similar JAK/STAT and protein tyrosine kinase pathways (34, 35). Because signals through both the IL-2R and the IL-15R are delivered by the β-chain and γc, one possibility is that the biological effects of IL-2 and IL-15 signals in driving effector and memory CTL differentiation overlap. However, IL-2 and IL-15 signals differ in magnitude, timing, and context. Although IL-2 binds its receptor as a soluble molecule, IL-15 is presented in trans by surface-bound IL-15Rα (36, 37), restricting the most potent IL-15 signals to periods of cell-cell contact, such as during the interaction of a T cell with an APC. In support of this idea, dendritic cells are a key source of IL-15 for memory T cell homeostasis and survival (38). Furthermore, expression of the high-affinity IL-2R is largely restricted to the first few days of the response; whereas, IL-15 signals are presumably available to T cells during the initiation of the T cell response as well as during memory maintenance. It is possible therefore that these differences can be invoked to explain the distinct biological impacts of IL-2 and IL-15 on the T cell response. In this scenario, IL-15, rather than sharing a role with IL-2 during the primary response, could have opposing functions, such as have been suggested in the respective roles of IL-2 and IL-15 in driving the differentiation of effector and memory T cells (16, 17).

In this study, we find that IL-2 plays a central role in the differentiation and survival of effector CTL and short-term effector memory CTL that persist during the first few months postinfection, as well as tissue-resident long-term effector memory CTL. IL-2Rα–deficient CD8+ effector T cells responding to acute infection display robust cytotoxic production but modest decreases in CTL activity. On secondary challenge, IL-2Rα–deficient CD8+ memory T cells display a severe defect in their ability to differentiate into secondary effector CTL, maintaining an IL-7Rαhi CD62Llo phenotype and a cytokine production profile typical of memory CTL, not effector CTL. However, we find little role for IL-15 signals during the primary response, either alone or in combination with IL-2, in promoting effector or effector memory differentiation or programming the recall capacity of CD8+ central memory T cells. Instead, the dominant role of IL-15 was to promote the survival of effector and memory populations after pathogen clearance. Although excess IL-15 signals may serve as an adjuvant or have immunotherapeutic benefit for CD8+ T cell responses, our findings suggest that in settings of acute infection, physiological IL-15 signals to T cells during the primary response do not play a significant role in CD8+ effector and memory T cell differentiation, particularly when compared with IL-2.

Materials and Methods

Mice and infections

The 6- to 8-wk-old C57BL/6 (B6), B6.129S4-H2dR1a19tm1Dw (IL-2Rα–deficient), B6.SJL-PtprcA/DybJ (B6.SJL, Ly5.1+), and B6.PL-Thyl1/+ CyJ (B6.PL, Thy1.1+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6N(Tac-IL15) (IL-15–deficient) mice were purchased from Taconic Farms (Germantown, NY). Wild-type (WT) and IL-2Rα–deficient P14 TCR transgenic mouse colonies were maintained at the University of Utah. All animal experiments were conducted with the approval of the IACUC committee at the University of Utah. Lymphocytic choriomeningitis virus (LCMV) Armstrong 53b was grown in BHK cells and titrated in Vero cells (39). Mice were infected i.p. with 2 × 106 PFUs. Recombinant Listeria monocytogenes expressing the LCMV gp33-41 (generated using described methods) was propagated in BHI broth and plated on agar plates as previously described (40–42). Prior to infection, the bacteria were grown to log phase and concentration determined by measuring the OD at 600 nm (OD of 1 = 1 × 108 CFU/ml). For secondary challenges, mice were injected i.p. with 2 × 108 CFU. Recombinant vaccinia virus expressing LCMV gp (VV-gp) (provided by J.L. Whitton, Scripps, La Jolla, CA) was generated and propagated as described (43). For secondary challenges, mice were injected i.p. with 2 × 107 PFU.

Irradiation chimeras

To generate WT/IL-2Rα–deficient mixed bone marrow (BM) chimeras, recipient mice were given 900 rads using an analytical x-ray irradiator located in the mouse vivarium at the University of Utah. One day later, we harvested BM from the femurs and tibias of WT and IL-2Rα–deficient donors. After RBC lysis, BM cells were incubated with biotinylated anti-CD3 Abs (eBioscience, San Diego, CA), followed by incubation with antibody magnetic beads (Miltenyi Biotec, Auburn, CA). CD3+ cells were depleted by passage through a magnetic column according to the manufacturer’s instructions (Miltenyi Biotec). CD3-depleted WT and IL-2Rα–deficient BM cells were mixed 1:1 and injected i.v. into irradiated hosts. By using different combinations of congenic markers, we readily distinguished WT (Ly5.1+), IL-2Rα–deficient (Thyl.2+) and residual P14 (Thyl.1+) T cells in E/T ratios ranging from 1:8–1:10 wk later. Similar methods were used to generate P14 irradiation chimeras. P14 irradiation chimeras were generated with a 1:1 mix of WT or IL-2Rα–deficient P14 BM with B6 BM. P14 was harvested a minimum of 8 wk postirradiation.

Cell suspensions and adoptive transfers

Splenocytes and lymph node (LN) cells were harvested at the indicated time points and resuspended in RPMI 1640 supplemented with 10% FBS and antibiotics. Liver and lung lymphocytes were harvested by collagenase digestion as previously described (44). Untouched CD8+ P14 T cells were isolated from the spleens and LNs of WT or IL-2Rα–deficient P14 BM chimeras by incubation with a biotinylated Ab mixture, followed by antibody magnetic beads and depletion on a magnetic column, per manufacturer's recommendations (Miltenyi Biotec). In addition, we added biotinylated CD44 Ab (eBiosciences, San Diego, CA) to eliminate CD44hi ”memory phenotype” P14. TCR transgenic T cell purity was assessed by staining with CD44, Vc2, and V8.1 Abs, followed by flow cytometric analysis. WT (Thy1.1+) and IL-2Rα–deficient (Thy1.1+Thy1.2+) P14 were mixed 1:1 and coincubated i.v. into naive B6 (Thy1.2+) mice at the indicated doses 1 d prior to infection.

Peptide restimulation and intracellular cytokine staining

Splenocytes were resuspended in RPMI 1640 containing 10% FBS and supplemented with antibiotics and l-glutamine. Mice were restimulated with 0.1 μM 2-D2–restricted peptide (gp33-41) in the presence of brefeldin A (1 μg/ml GolgiPlug). Cells were stimulated with cell surface Abs, permeabilized and stained with cytokine Abs (specific to IFN-γ, TNF-α, and IL-2) using a kit per manufacturer’s instructions (BD Biosciences, Mountain View, CA).

CTL assays

We used a FACS-based cytotoxicity assay as previously described (45). EL4 cells were incubated with 0.1 μM gp33-41 peptide for 2 h at 37°C. Cells were washed and incubated with FACS-sorted WT or IL-2Rα–deficient P14 CTLs for 2 h at 37°C at E/T ratios ranging from 1:8–1:10 wk later. Specific killing was measured by comparison with killing of unloaded control targets cells.

Tetramer staining and analysis

The H–2Db–restricted gp33-41 monomer was generated, biotinylated, and tetramerized with streptavidin-conjugated allophycocyanin using described methods (46, 47), with modifications as described in protocols available on the National Institutes of Health Tetramer Core Facility Web site (http://tetramer.yerkes.emory.edu/). Staining was performed at 4°C for 1 h in FACS buffer (PBS with 2% FBS and 0.02% sodium azide).

Abs and flow cytometry

Fluorescent dye-conjugated Abs were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA), or BD Biosciences (Mountain View, CA) with the following specificities: CD8, Thy1.1, Thy1.2, CD45.1, Vα2, Vβ8.1, CD44, IL-7Rα, KLRG1, CD27, CD62L, CXC3R1, CD122, CD25, Eomesodemin (Eomes), T-bet, granzyme B, CD107a, TNF-α, IL-12, and IFN-γ. Cell surface Ab staining was performed in PBS containing 2% FBS, and intracellular cytokine staining was performed as described previously. For T-bet and granzyme B staining, cells were permeabilized using the same buffers as for
in intracellular cytokine staining (BD Biosciences). For Eomes, cells were permeabilized and stained using the same buffers as those used for the anti-FoxP3 Abs per the manufacturer’s instructions (eBioscience). For CD107a, the Ab was mixed with resuspended cells during peptide restimulation prior to intracellular cytokine staining. Multiparameter (6–7 color) analysis of Ab-stained cells was performed on a FACS-Canto II flow cytometer (BD Biosciences) and results analyzed using FlowJo software (TreeStar, Ashland, OR). Cell sorting was with on a FACSVantage (BD Biosciences) at the University of Utah FACS core facility.

**Microarray**

RNA was isolated using RNEasy kits (Qiagen, Valencia, CA) from FACs-sorted P14 cells (day 8 postinfection). Message was amplified and converted to Cy3 or Cy5-labeled cRNA using a commercially available kit per manufacturer’s instructions (Agilent Technologies, Palo Alto, CA). Four biological duplicates from each group were hybridized to Agilent whole mouse genome microarrays. For each of the four replicates, dual hybridization was performed using RNA obtained from WT and IL-2Rα-deficient P14 isolated from the same animal. Results were normalized and analyzed for differences in log expression values using GeneSifter software (Geospiza, Seattle, WA). Microarray data are publicly available at the online depository Gene Expression Omnibus (accession GSE19598, www.ncbi.nlm.nih.gov/geo/) and conforms to all MIAME guidelines.

**Results**

**Impaired accumulation and survival of CD8+ end-stage effector T cells in the absence of IL-2 signals**

Our previous studies found that in the absence of IL-2 signals, developing CD8+ memory T cells rapidly converted to a CD62Llo phenotype (15). To characterize this finding further, we analyzed the responses of LCMV-specific P14 TCR transgenic T cells either expressing or lacking the IL-2Rα. IL-2Rα-deficiency results in the loss of CD44+CD27- regulatory T cell function and multigang autoimmuneinit, at a young age (48–50). To mitigate nonspecific effects this environment might have on T cell function, we sought to generate IL-2Rα-deficient P14 donors that lacked autoimmune side effects. We generated mixed BM chimeras by transferring a 1:1 mix of WT B6 and either WT or IL-2Rα-deficient P14 BM into lethally irradiated B6 hosts, as previously described (15). Because the WT BM gave rise to a functional regulatory T cell population, the chimeras remained healthy with no signs of autoimmunity. Naïve (CD44lo) WT and IL-2Rα-deficient P14 cells were harvested from the chimeras and cotransferred into naive B6 hosts, followed by LCMV infection. Because of their variable expression of Thy1 alleles, we simultaneously tracked WT P14 (Thy1.1+) and IL-2Rα-deficient P14 (Thy1.1Il2Rα-deficient) responses in B6 hosts (Thy1.2+) at various time points postinfection.

As previously observed (15), WT P14 responders expanded modestly (~2-fold) better than IL-2Rα-deficient P14 responders by the peak of the response. Both populations also formed memory populations that persisted at stable levels throughout the course of our experiments (Fig. 1A). However, a slightly higher fraction of IL-2Rα-deficient P14 cells was lost during the contraction phase, as compared with the peak of the response (Fig. 1B). To determine the cause of this loss, we analyzed the differentiation of SLEC and memory precursor populations at the peak of the response and in the transition to memory. Recent studies have found that SLECS can be differentiated from memory precursor/memory cells based on variable expression of IL-7Rα and KLRG1 (3). At the peak of the primary response (day 8), we observed 2- to 3-fold fewer KLRG1hiIL7Rαlo effector CTL among the IL-2Rα-deficient P14 responders, as compared with WT P14 (Fig. 1C, 1D). WT P14 formed a population of detectable KLRG1lo effector phenotype CTL that slowly faded from the memory pool over the course of 4–6 mo (referred to here as short-term effector memory cells). In contrast, IL-2Rα-deficient short-term effector memory CTL disappeared rapidly, comprising 10- to 20-fold lower levels in the spleen at day 42 postinfection (Fig. 1C, 1D). WT and IL-2Rα-deficient P14 demonstrated no differences in the generation of KLRG1hiIL7Rαlo memory precursors at the peak of the response (day 8 postinfection). Numerical differences in the number of WT and IL-2Rα-deficient P14 could almost entirely be ascribed to deficiencies in the generation of effector phenotype cells. Several other markers also confirmed the rapid disappearance of effector cells in the IL-2Rα-deficient P14 population. Besides these expression patterns of KLRG1 and IL-7Rα, differentiated effector populations were CD27lo, CD62Llo, CXCR3lo, and CD43lo. Effector cells bearing these characteristics also disappeared rapidly in the absence of IL-2 signals in both the spleen and liver (Supplemental Figs. 1, 2).

**Lack of IL-2 signals adversely impacts the function of effector CTLs**

Because we observed modest but significant and reproducible differences in the number of CD8+ effector CTL at the peak of the response in the absence of IL-2 signals, we tested the hypothesis that IL-2 might also be important for optimal effector function. Previous studies in which CTL received little or no IL-2 signals were disrupted during in vitro activation, followed by in vivo adoptive transfer suggested that IL-2 might play an important role in the development of effector function (16). We focused on a time point at which the high-affinity IL-2R was expressed at high levels (day 5) as well as the peak of the effector response (day 8), at which time the high-affinity IL-2R was no longer expressed (Fig. 2A).

Initially, we measured intracellular expression of the transcription factors T-bet and Eomes. These related T-box transcription factors have been implicated in the differentiation of effector T cells and in the acquisition of effector T cell function, such as IFN-γ production and CTL activity, as well as in the differentiation of functionally and phenotypically normal CD8+ memory T cells (3, 45, 51, 52). We found no differences in expression of T-bet at either day 5 or day 8 postinfection. Conversely, IL-2Rα-deficient P14 demonstrated a reproducible 2-fold increase in the amount of Eomes at day 8 postinfection (Fig. 2A). These differences were not due to differences in the composition of each population, as direct comparison of short-term effector and memory precursor effector populations revealed the same 2-fold disparity in Eomes expression (data not shown). Although these differences are modest, they remain of interest given that similar differences in Eomes expression in mice with a single functional allele impacts CD8+ T cell differentiation and effector function (51). Nevertheless, these results are inconsistent with an obligate role for IL-2 in the acquisition of effector function, given that Eomes expression was higher in the absence of IL-2. Furthermore, they suggest that IL-2 influences Eomes expression indirectly, as no expression differences were seen at day 5 when WT P14 responders were still actively receiving IL-2 signals (Fig. 2A).

IL-2Rα-deficient CTL expressed granzyme B and degranulated and produced cytokines on restimulation (Fig. 2A, 2B, Supplemental Fig. 3). However, IL-2Rα-deficient CTL demonstrated a modest decrease in CTL activity at day 8 postinfection (Fig. 2C). To assess effector CTL development at this time point, we analyzed RNA expression by WT and IL-2Rα-deficient P14 CTL by microarray. We observed significant upregulation of effector molecules involved in cytolyis and effector differentiation in WT CTL, including granzymes and perforin, as well as an increase in T-bet (Table I). Although Blimp-1 was not significantly upregulated in WT cells, Bcl-6 was significantly upregulated in IL-2Rα-deficient CTL (Table II). A variety of NKR s, likely indicators of CTL differentiation (3), also demonstrate increased expression in...
WT CTL. IL-2Rα−deficient CTL expressed higher levels of IL-2 and TNF-α as well as receptors that mediate trafficking to and within secondary lymphoid organs (CCR7 and CXCR5) (Table II). These findings were predicted by cell surface staining and again indicate a skewing away from a differentiated effector phenotype. Although we observed differences in expression of several pro- and antiapoptotic mediators, no clear pattern emerged to explain the failure of IL-2Rα−deficient effector CTL to survive after Ag clearance. However, WT P14 CTL demonstrated enhanced expression of a variety of cell cycle participants, indicating that IL-2 may drive cell division later in the primary response (Supplemental Table I). This finding corresponds to previous observations...
by others (13, 53) and our own finding that WT responders demonstrate enhanced expansion between days 5 and 8 postinfection (15). Overall, although many of the gene expression differences are individually modest, they collectively support a role for IL-2 in driving the differentiation and enhancing the function of primary CTL. One possible interpretation of these results is that differences in cytolytic function (Fig. 2C) and expression of CTL differentiation markers and cytolytic molecules (Table I) reflect differences in the generation of short-lived effector CTL in the absence of IL-2 signals (Fig. 1C, 1D), and future studies will be needed to directly compare the differentiation status of purified SLEC and memory precursor effector cell populations in the presence or absence of IL-2 signals.

Poor differentiation of secondary effector T cells in the absence of IL-2

Because CD8+ memory T cells generated in the absence of IL-2 signals mount poor secondary responses, we assessed their ability to become secondary effector cells. To assess polyclonal endogenous recall responses, we generated mixed BM chimeras using a 1:1 mix of BM from WT and IL-2Rα-deficient donors. At 8–10 wk posttransplant, mice were challenged with LCMV. As measured by MHC class I tetramers (Fig. 3A) and the frequency of IFN-γ–producing cells after peptide restimulation, even in the absence of IL-2 signals CD8+ T cells generated robust primary responses and long-lived memory populations similar to that of WT responders. Mice were rechallenged with either a recombinant Listeria monocytogenes expressing the LCMV gp33 (Lm-gp33) or a recombinant VV-gp at 150 d postinfection. As previously observed (15), IL-2Rα-deficient CD8+ memory T cells demonstrated a significant deficit in their ability to generate secondary responses, as compared with WT. Furthermore, poor recall responses were not due to competition with WT memory CTL. Similar differences were seen when FACS-purified WT and IL-2Rα-deficient memory P14 were transferred into separate naive B6 hosts prior to rechallenge (Supplemental Fig. 4). A closer analysis of the tetramer-binding cells at the peak (day 5) of the secondary response revealed that IL-2 signals were required for the generation of secondary effector CTL (KLRG1hiIL-7Rbhi) (Fig. 3B). Furthermore, secondary responders induced in the absence of IL-2 remained CD62Lhi (Fig. 3B), CD27hi, and CXCR3hi (Supplemental Fig. 5), all characteristics of memory cells, not effector cells. These defects were present but modest at the peak of the primary response and greatly exacerbated on secondary challenge (Fig. 3B). To assess function, we restimulated splenocytes ex vivo both pre- and postrechallenge. Prior to rechallenge, both WT and IL-2Rα-deficient memory populations primarily consisted of cells capable of simultaneously producing IFN-γ and TNF-α (double producers), or IFN-γ, TNF-α, and IL-2 (triple producers), with few cells only able to produce IFN-γ (single producers). After rechallenge and the development of secondary effectors, the cytokine-producing profile of WT responders shifted dramatically toward single or double producers, with few triple producers. Conversely, the cytokine-producing profile of IL-2Rα-deficient secondary responders was largely unchanged, again reflecting an inability to generate large numbers of secondary effector CTL (Fig. 3C).

IL-2 promotes the differentiation of CD8+ long-lived effector memory T cells

Our prior studies confirmed that CD8+ memory T cells generated in the absence of IL-2 signals quickly converted to a CD62Lhi central memory phenotype (15). However, because in the current study, we observed in the absence of IL-2 signals a rapid loss of KLRG1hiIL-7Rbhi effector cells that are also CD62Llo, we considered the possibility that our prior observations simply reflected a loss of this population and not a role for IL-2 in the generation of bona fide tissue-residing long-lived effector memory T cells. To test this possibility we assessed CD62L expression by IL-7Raihi memory T cells as a measure of true effector memory T cell differentiation and survival. We found that even this population demonstrated a rapid loss of CD62Llo effector memory T cells in the spleen after acute LCMV infection in the absence of IL-2 signals (Fig. 4A). Furthermore, WT tissue-residing memory P14 in the liver demonstrated a selective advantage over time as compared with IL-2Rα–deficient memory P14. This survival advantage was intermediate in the spleen and not observed in the lymph nodes (Fig. 4B, 4C). We therefore concluded that IL-2 played a central role in the differentiation of effector memory T cells in both the spleen and peripheral sites of infection.

In all, these data indicate that IL-2 influences a wide spectrum of effector differentiation, from end-stage effector cells at the peak of the response to both short-lived and long-lived effector memory CTL in secondary lymphoid tissues and peripheral sites of infection. Numerically, the differentiation of central memory T cells appears to be independent of IL-2, as we observe roughly similar numbers of central memory phenotype WT and IL-2Rα–deficient CTL at early memory points (data not shown). Because IL-2Rα–deficient memory CTL at early memory time points are largely central memory phenotype already, the overall number of central memory cells remains stable throughout memory maintenance. On the other
hand, at early memory time points the WT memory population is largely composed of short-term and long-term effector memory cells. Over time, the memory population remains stable but eventually converts to a central memory phenotype, whether due to conversion of existing effector memory cells to central memory cells (54) or replacement of effector memory cells with central memory cells due to homeostatic mechanisms (55). Although the end result at late memory time points is a 3-fold difference in the number of central memory cells (Fig. 1), our results suggest that IL-2 mainly plays a role in effector and effector memory CTL differentiation and that the defect in central memory cells is more precisely one of secondary effector differentiation.

IL-2 and IL-15 jointly promote the emergence and survival of effector CTL

Although IL-2 plays an important role in CD8⁺ secondary effector T cell differentiation after secondary challenge, we remained puzzled by our observation that IL-2 played a much more modest role in driving robust expansion during the primary response. One likely explanation is that during in vivo infection, other growth and inflammatory factors compensate for the absence of IL-2. Of the potential candidates, we focused on IL-15. Because IL-15 is highly related to IL-2 and shares a similar signaling apparatus, we hypothesized that IL-15 signals during the primary response could cooperate with IL-2 and compensate for the lack of IL-2 signals in the differentiation of CD8⁺ effector and memory T cells.

To probe a joint role for IL-2 and IL-15 signals to CD8⁺ T cells, we adoptively cotransferred WT and IL-2Rα–deficient P14 cells into either WT or IL-15–deficient hosts. Because mouse CD8⁺ T cells are not an in vivo source of IL-15 in mice, we were able to assess the response of P14 T cells in the absence of either IL-2 or IL-15 signals, or both. At the peak of the primary response (day 8 postinfection), the absence of either IL-2 or IL-15 alone resulted in a similar decrease (∼2–3-fold) in the number of end-stage effector cells (KLRG1hiIL-7Rαlo or CD62Llo) among WT or IL-2Rα-deficient (KO) responders at the peak of the primary (day 8 postinfection with LCMV) or secondary (day 5 postchallenge with Lm-gp33) response. The bar graph indicates the ratio of WT and IL-2Rα–deficient tetramer-binding responders at day 8 after primary LCMV infection (WT), day 150 after primary infection (mem), and 5 d after rechallenge with either Lm-gp33 or VV-gp (A). Error bars indicate the SEM (n = 3–4/group), and differences in fold expansion are statistically significant (p < 0.05). B, Representative flow plots, gated on tetramer binding CD8⁺ T cells, indicate the frequency of effector phenotype (KLRG1hiIL-7Rαlo or CD62Llo) cells among WT or IL-2Rα-deficient (KO) responders at the peak of the primary (day 8 postinfection with LCMV) or secondary (day 5 postchallenge with Lm-gp33) response. C, The bar graph indicates the ratio of WT and IL-2Rα–deficient tetramer-binding effector phenotype (KLRG1hiIL-7Rαlo) responders at day 8 after primary LCMV infection and 5 d after rechallenge (at day 150) with either Lm-gp33 or VV-gp. Error bars indicate the SEM (n = 3–4/group; p < 0.05 when comparing ratios after the primary or secondary responses). D, After 4 h restimulation with peptide, we assessed the ability of WT and IL-2Rα–deficient responders to make cytokines at either day 150 postinfection with LCMV or day 5 postchallenge with Lm-gp33. The pie charts indicate the average frequency of responders within the IFN-γ–producing population capable of making TNF-α and/or IL-2 (n = 3). Results are representative of two separate experiments. KO, knockout.
and lymph nodes (the results are representative of four separate experiments. The ratio increase with LCMV in the indicated tissues. Error bars indicate the SEM (postinfection).

Tumors were ablated by injection of 2000 cells into the left flank of B6 mice. We then transferred 500 WT or IL-2Rα-deficient P14 cells into B6 or IL-15–deficient mice and infected with LCMV. At 42 d postinfection, we harvested the spleen and transferred into infection-matched B6 or IL-15-deficient secondary hosts. We subsequently analyzed the persistence of WT or IL-2Rα-deficient end-stage effector cells that lacked IL-15 signals during the primary response only, during the contraction phase only (3, 51, 56), and we considered as an alternative that IL-15 was required only for the survival of these cells, not their differentiation.

To distinguish a potential differentiation role for IL-15 during the primary response from its known survival role thereafter, we limited, through adoptive transfer, the availability of IL-15 signals to WT or IL-2Rα-deficient P14 responders to the primary phase (days 0–8) or the contraction phase (days 8–42) of the T cell response. We cotransferred WT and IL-2Rα-deficient P14 into B6 mice and infected with LCMV. At day 8 postinfection, WT and IL-2Rα–deficient P14 CTL were harvested from the spleen and transferred into infection-matched B6 or IL-15-deficient secondary hosts. We subsequently analyzed the persistence of WT or IL-2Rα–deficient end-stage effector cells that lacked IL-15 signals during the primary response only, during the contraction phase only, or both. As before, we found that the absence of IL-15 during both the primary response and the contraction phase severely curtailed the persistence of KLRG1hi IL-7Rαlo effector cells and that the effect was exacerbated in the additional absence of IL-2 (Fig. 6). Similar results were observed when IL-15 signals were absent during the contraction phase only (Fig. 6). In contrast, the absence of IL-15 during the primary response alone resulted in levels of effector CTL levels similar to those seen after transfer of WT and IL-2Rα–deficient P14 into WT B6 hosts (Fig. 6). These findings conclusively demonstrate that in contrast to IL-2 the primary role for IL-15 in this setting is the survival of KLRG1hi effector phenotype CTL, not their differentiation.

**IL-15 is not required for secondary CD8+ T cell expansion and effector differentiation**

We next assessed whether IL-15 shared an overlapping role with IL-2 in the differentiation of CD8+ memory T cells capable of secondary responses. We cotransferred 500 WT and IL-2Rα-deficient P14 into B6 or IL-15-deficient mice and infected with LCMV as previously described. At 42 d postinfection, mice were rechallenged with Lm-gp33. WT P14 memory cells expanded robustly by day 5 postrechallenge regardless of the presence or absence of IL-15 signals (Fig. 7A). They also differentiated into secondary effector CTL as determined by expression of IL-7Rα and KLRG1 (Fig. 7B) and their cytokine production profile (Fig. 7C). As observed previously, IL-2Rα-deficient memory cells responded poorly to secondary challenge and failed to acquire phenotypic or functional (cytokine-producing) characteristics indicative of secondary effector differentiation. However, this phenotype was not exacerbated in the absence of IL-15, again indicating that the functional role of IL-2 and IL-15 in CTL memory differentiation and survival were nonoverlapping (Fig. 7A–C).
**Discussion**

Our understanding of the role of IL-2 during in vivo immune responses has undergone changes and revisions over the years. Although it was originally thought to be required for T cell expansion, we find robust CD8+ T cell expansion even in the complete absence of high-affinity IL-2 signals. However, our findings suggest that IL-2 plays a unique and important role as a fate determination and differentiation signal for activated T cells in vivo. Here again, the role of IL-2 is complex. Although IL-2 plays a role in promoting the emergence and function of effector CTLs during the primary response, its impact is particularly pronounced in the rapid disappearance of this population during the contraction phase. Expression of the high-affinity IL-2R generally corresponds with bursts of IL-2 production in vivo, with the notable exception of the regulatory T cell subset. We observe little to no expression of IL-2Ra at day 8 postinfection or beyond, indicating that the high-affinity IL-2R signal is confined to the primary T cell response and expansion phase. We therefore conclude that IL-2 signals during priming influence the generation of effector CTLs during the primary response and the persistence of short-term effector memory CTLs once the virus is cleared. These findings are consistent with previous results demonstrating that IL-2 signals during the first week of infection promote the subsequent survival of IL-7Ra<sup>hi</sup> and CD62L<sup>lo</sup> responders during the contraction phase (15). Two recent reports have also demonstrated a role for IL-2 in driving effector CTL differentiation. These studies found that effector CTL differentiation was influenced by the concentration of IL-2 after activation (57) or by the length of time activated CTLs were able to incorporate high-affinity IL-2 signals (58). Together with our current report, these studies demonstrate a nonredundant role for IL-2 in enhancing effector CTL differentiation, survival, and function. Our report further demonstrates that in the complete absence of high-affinity IL-2 signals, secondary effector CTL differentiation is dramatically impaired. One intriguing possibility is that although strong IL-2 signals enhance effector differentiation, some IL-2 signals are required for memory precursor effector cells to maintain, perhaps through epigenetic changes, their ability to access the effector differentiation transcriptional program. Thus, memory cells generated in the absence of IL-2 signals would be largely unable to enter an effector differentiation pathway on reactivation. Future studies are needed to identify epigenetic changes as well as changes in transcriptional activity that are influenced by IL-2 signals in differentiating CTL in vivo.

**FIGURE 5.** IL-2 and IL-15 jointly promote the persistence of short-lived, but not long-lived, effector memory CTLs. We transferred 5 × 10<sup>3</sup> WT and IL-2Ra<sup>-</sup>-deficient P14 into B6 and IL-15<sup>-</sup>-deficient hosts and infected with LCMV. A, The bar graphs display the number of effector phenotype (KLRG1<sup>hi</sup>IL-7R<sup>a</sup>lo) WT and IL-2Ra<sup>-</sup>-deficient P14 responders in B6 or IL-15<sup>-</sup>-deficient hosts at days 8 or 42 postinfection. Error bars indicate SEM (n = 3). The decrease of P14 cells in the absence of both cytokines, as compared with each cytokine alone, is statistically significant at both time points (p < 0.05). B, Representative flow plots indicate the relative frequencies of effector (KLRG1<sup>hi</sup>IL-7R<sup>a</sup>lo) and memory (KLRG1<sup>lo</sup>IL-7R<sup>a</sup>hi) phenotype cells among WT and IL-2Ra<sup>-</sup>-deficient P14 responders in B6 or IL-15<sup>-</sup>-deficient hosts at the indicated time points postinfection. C, The bar graph indicates the relative frequency of CD62L<sup>hi</sup> cells among WT and IL-2Ra<sup>-</sup>-deficient memory phenotype P14 responders (KLRG1<sup>lo</sup>IL-7R<sup>a</sup>hi) in B6 or IL-15<sup>-</sup>-deficient hosts at the indicated time points postinfection. Error bars represent the SEM (n = 3). Results are representative of three separate experiments.
memory cells (15). We find in this study that despite their rapid divisions, almost no secondary effector CTLs emerge in the absence of IL-2 signals. Although there is some evidence of effector differentiation, including upregulation of KLRG1, secondary effector cells rapidly disappear from the response, suggesting that IL-2 provides a differentiation signal to activated T cells that enables or potentiates entry into an effector/effector memory lineage. Prior studies have shown that secondary effector and memory T cells skew more strongly to the effector and/or effector memory lineage, maintaining low levels of CD62L over long periods as compared with primary memory T cells (59).

We propose that IL-2 provides a differentiation signal that enables entry into and survival within the effector “program.” This may include epigenetic imprinting during the primary response that enable or potentiate effector differentiation on subsequent encounters with Ag. Because CD8+ memory T cells are prone to become highly differentiated secondary effector/effector memory cells on secondary activation, the absence of an IL-2–driven effector differentiation signal during the primary response may specifically and adversely impact the generation of highly differentiated secondary effector CTL. In this sense, IL-2 may enable or potentiate entry into an effector/effector memory lineage after secondary activation, the absence of IL-2 signals. Although there is some evidence of effector differentiation, including upregulation of KLRG1, secondary effector cells rapidly disappear from the response, suggesting that IL-2 provides a differentiation signal to activated T cells that enables or potentiates entry into an effector/effector memory lineage. Prior studies have shown that secondary effector and memory T cells skew more strongly to the effector and/or effector memory lineage, maintaining low levels of CD62L over long periods as compared with primary memory T cells (59).

We propose that IL-2 provides a differentiation signal that enables entry into and survival within the effector “program.” This may include epigenetic imprinting during the primary response that enable or potentiate effector differentiation on subsequent encounters with Ag. Because CD8+ memory T cells are prone to become highly differentiated secondary effector/effector memory cells on secondary activation, the absence of an IL-2–driven effector differentiation signal during the primary response may specifically and adversely impact the generation of highly differentiated secondary effector CTL. In this sense, IL-2 may be most appropriately described as an effector differentiation factor rather than a memory differentiation factor. Although it may not be required for the selection of memory populations during the primary response, its role in driving effector differentiation is a key step in conferring the ability of memory T cells that do emerge to differentiate into effector cells on secondary engagement with Ag.

The molecular and transcriptional nature of the IL-2–driven effector differentiation program remains unknown. Several transcription factors have been implicated in the differentiation of CD8+ effector T cells. Of particular interest are the T-box transcription factors T-bet and Eomes. T-bet and Eomes drive effector differentiation and are regulated in response to inflammatory signals, such as IL-12 or type I IFNs (3, 60). Eomes impacts the differentiation and survival of CD8+ effector T cells by influencing the expression of effector molecules, such as IFN-γ and CD122 (51). Another molecule of interest is the transcriptional repressor Blimp-1. Although past studies have focused on the role of Blimp-1 in plasma cell differentiation, recent studies suggest an important role for this molecule in CD8+ effector differentiation (61, 62) during acute viral infection and CD8+ T cell exhaustion (63) during chronic viral infection. Blimp-1 induction during in vitro T cell activation is dependent on IL-2 and forms a feedback loop to inhibit IL-2 production (64). In addition, similar to responding CD8 T cells that do not receive IL-2 signals, Blimp-1–deficient CD8 T cells also show a defect in effector and effector memory differentiation after acute infection (61, 62), as well as a reduced ability of Blimp-1–deficient memory cells to respond to rechallenge (61).

The levels of T-bet and Eomes protein expression, as well as Blimp-1 mRNA expression, were not reduced in the absence of IL-2 signals. Although we do not find an obligate role for IL-2 in inducing expression of any of these transcription factors during the in vivo response to viral infection, we do not rule out a role for IL-2 in controlling, directly or indirectly, their transcription factor activity posttranscriptionally or posttranslationally. Because Blimp-1, which has recently been shown to promote effector and memory CTL differentiation (61, 62), is a potent repressor of Bcl-6 expression in T cells (65), our finding that Bcl-6 expression is reduced in the absence of IL-2 signals leaves open the possibility that its activity is posttranscriptionally or posttranslationally reduced in the absence of IL-2 signals, despite no changes in mRNA expression. Although expression of the transcription factors T-bet, Eomes, and Blimp-1 are associated with effector CTL differentiation, less
is known about how these transcription factors function and are regulated. For example, there may be activating and/or repressive binding partners and/or modifications affecting their activity. Thus, although our data show that IL-2 is not obligatory for induction of expression of these factors, there are several ways in which IL-2 signals could result in alterations to transcriptional activity and, ultimately, the ability of a responding CD8+ T cell to undergo effector differentiation. Furthermore, even modest differences (<2-fold) in expression and/or activity of some of these transcription factors may have a profound impact on T cell differentiation and function. Future studies will be needed to precisely elucidate the combined role of these transcription factors and others [such as the Blimp-1 repressor Bcl-6 (66, 67)] in the differentiation of CD8+ primary and secondary effector and effector memory T cells, along with the impact of inflammatory mediators and cytokines, such as IL-2, on their activity.

In the absence of IL-2 signals, effector CTLs undergo massive expansion. It is possible that the milieu of growth and inflammatory factors available during the infectious burst can compensate for the lack of IL-2 signals. This hypothesis is supported by the fact that IL-2 is one member of a family of cytokines linked by their receptor usage. Because the signaling apparatus used by IL-2 and IL-15 is largely shared, it is presently unclear what the differences are in the signals that account for their distinct biological effects. Although we find a role for IL-15 in the survival of CD8+ effector T cells after Ag clearance; it does not play a role in the differentiation of this population during primary activation, or is it required for the differentiation of functionally competent CD8+ memory T cells. Instead, our findings suggest that IL-15 plays a fundamentally distinct role from that of IL-2, promoting no the differentiation but survival of CD8+ memory T cells.

Because our prior studies found a role for IL-2 in promoting secondary CTL responses in both LCMV and Listeria infectious model systems (15), we concluded that IL-2 played a broad role in memory CTL differentiation in disparate model systems. Although the role of IL-2 in promoting secondary responses after LCMV infection have also been observed by another group (68), one recent study, while also reporting a role for IL-2 in driving effector CTL differentiation after Listeria infection, found that the recall capacity of the resulting memory cells was IL-2 independent (53). It remains possible therefore that the role of IL-2 may depend on the pathogenic stimulus. Clearly, the impact of IL-2 in driving the differentiation of effector and memory CTL requires further study. One complicating factor may be the presence of redundancy within the immune system. Several other cytokines, such as IL-7 and IL-21, also use the γc as a component of their receptors, and these cytokines send a common TCR-independent proliferative signal via the STAT5 and STAT3 transcription factors (69). It is not clear, however, whether the programming of functional memory cells by IL-2 represents a unique signal from IL-2 that IL-7, IL-21, or other γc family member cytokines are unable to deliver, or if it represents a common signal, perhaps mediated by STAT5, that any member of the family could redundantly deliver during T cell activation and differentiation, if it was present in sufficient amounts and its receptor expressed. Future experiments should focus on both redundancy of family member function as well the nature of the pathogenic stimulus in interpreting the in vivo function of IL-2. Because of its role in effector differentiation, future studies will benefit from analyzing the role of IL-2 in models of localized tissue infection.

Our results suggest that IL-15 signals do not overlap with IL-2 signals, but this may reflect differences in receptor expression and cytokine availability. Because IL-15 is expressed in trans (37), T cells must be in close proximity to potential IL-15 producers. It is unclear the extent to which IL-15 signals are available to differentiating CTLs during the primary response, but IL-15 presented by dendritic cells has been shown to induce homeostatic proliferation of memory T cells (38). It is possible that the levels of IL-15 available to T cells differ depending on the nature of the pathogenic stimulus, and our results do not rule out a potential role for IL-15 during primary T cell differentiation if present at high enough concentrations in other infectious model systems. IL-2, in contrast, is secreted at high levels on activation and is probably available to differentiating CTLs throughout the primary expansion phase. One intriguing candidate member of this family with similar induction kinetics is IL-21, which, like IL-2, is largely expressed by CD4+ T cells, is induced on activation and promotes enhanced cytotoxicity by CD8+ T cells (70). Furthermore, IL-21 has been shown to induce both Blimp-1 and Bcl-6 expression during B cell activation and differentiation, suggesting the intriguing possibility that IL-21 may play an effector/memory differentiation role for T cells (71). Recent studies found that like IL-2 (68), IL-21 signals to T cells were not required for primary CTL expansion but did promote their ability to control chronic infection (72–74).

IL-2 is used as an immunotherapy in situations in which the inflammatory burst is comparatively minimal, such as for antitumor immune responses (75). IL-2 also appears to play a significant role in maintaining effector/memory responses during chronic infections (68), indicating that the long-term ability to respond to Ag may require IL-2. It is in this way, perhaps, that in vivo responses reflect the need for IL-2 in promoting the establishment and maintenance of T cell lines in vitro. We anticipate that a more detailed understanding of the role of IL-2 in the differentiation and function of Ag-activated T cells will greatly enhance our understanding of memory T cell biology. In particular, defining its role will aid in a variety of therapeutic strategies aimed at manipulating the T cell response. These include vaccination, immunotherapeutic or immunomodulatory strategies aimed at boosting the immune response, tumor eradication by immune cells, and strategies for which immunosuppression is desirable, such as for prevention of autoimmune responses or transplant rejection.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Short-lived effector/short-lived effector memory differentiation and survival in the spleen is impaired in the absence of IL-2. Representative flow plots display cell surface expression of the indicated molecules by WT and IL-2Rα-deficient (ko) P14 in the spleen at various time points post-infection with LCMV.
**Supplemental Figure 2.** Short-lived effector/short-lived effector memory differentiation and survival in the liver is impaired in the absence of IL-2. Representative flow plots display cell surface expression of the indicated molecules by WT and IL-2Rα-deficient (ko) P14 in the liver at various time points post-infection with LCMV.
Supplemental Figure 3. IL-2Rα-deficient CTLs degranulate efficiently upon restimulation. Representative flow plots indicate CD107a surface expression as a measure of degranulation during a 4 hour restimulation of WT and IL-2Rα-deficient (ko) P14 CTL at day 8 post-infection with LCMV.
**Supplemental Table I.** Microarray analysis of WT and IL-2Rα-deficient (KO) P14 8 days post-infection with LCMV.

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**Supplemental Figure 4.** IL-2Rα-deficient CD8+ memory T cells fail to generate robust recall responses upon secondary challenge in the absence of competing WT memory cells. WT and IL-2Rα-deficient P14 (5 x 10^2 of each) were co-transferred into B6 mice, followed by infection with LCMV one day later. WT and IL-2Rα-deficient memory P14 cells (>300 days post-infection) were FACS-purified from the spleen and transferred separately (1 x 10^4 of each) into naïve B6 hosts. Secondary hosts were challenged with LCMV and recall responses assessed in the spleen 7 days later. Results are representative of 3-4 mice/group and error bars display the SEM.
**Supplemental Figure 5.** IL-2Rα-deficient CD8+ memory T cells fail to differentiate into secondary effector CTL following secondary challenge. Representative flow plots display cell surface expression of CXCR3 and CD27 by WT or IL-2Rα-deficient Db/GP33-41 tetramer-binding cells 8 days after primary challenge with LCMV or 5 days after secondary challenge of day 150 LCMV immune mice with Lm-gp33.
Supplemental Figure 6. IL-15-dependent persistence of short-lived effector memory CTLs is not due to differences in CD122 expression or in the rate of cell division. WT and IL-2Rα-deficient P14 (5 x 10^3 of each) were co-transferred into B6 or IL-15-deficient mice, followed by infection with LCMV one day later. a) Representative flow plots indicate CD122 expression by P14 CTL at the indicated days post-infection. b) Graphs depict the frequency of P14 CTL expressing the cell cycle indicator Ki-67 at days 5 and 9 post-infection. Error bars are the SEM (n=3-4 mice/time point).