Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes

See related Commentary on pages 805–806.

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The lineage relationship between short-lived effector T cells and long-lived memory cells is not fully understood. We have described T-GFP mice previously, in which naive and early activated T cells express GFP uniformly, whereas cells that have differentiated into effector cytotoxic T cells selectively lose GFP expression. Here we studied antigen-specific CD8 T cell differentiation using T-GFP mice crossed to the TCR transgenic (Tg) mice P14 (specific for the lymphocytic choriomeningitis virus glycoprotein peptide, gp33-41). After activation with antigenic peptide, P14XT-GFP CD8$^+$ T cells cultured in high-dose IL-2 developed into cells with effector phenotype and function: they were blastoid, lost GFP expression, expressed high levels of activation and effector markers, and were capable of immediate cytotoxic function. In contrast, cells cultured in IL-15 or low-dose IL-2 never developed into full-fledged effector cells. Rather, they resembled memory cells: they were smaller, were GFP$^+$, did not express effector markers, and were incapable of immediate cytotoxicity. However, they mediated rapid-recall responses in vitro. After adoptive transfer, they survived in vivo for at least 10 weeks and mounted a secondary immune response after antigen rechallenge that was as potent as endogenously generated memory cells. In addition to providing a simple means to generate memory cells in virtually unlimited numbers, our results suggest that effector differentiation is not a prerequisite for memory cell generation.

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Introduction

During an immune response, antigen-specific T cells proliferate enormously and develop into effector T cells capable of immediate effector functions, such as cytotoxicity and IFN-γ production (1, 2). Following a successful immune response, activated effector T cells undergo large-scale apoptosis, presumably to maintain homeostasis in T cell numbers (3). However, the process leaves behind an enhanced pool of relatively quiescent antigen-experienced memory T cells that persist over long periods of time and mount a rapid and augmented response upon rechallenge with antigen. The basis for retention of memory T cells amid elimination of a vast majority of activated effector T cells is not well understood. A key issue is whether memory cells can arise only from fully differentiated effector cells or also directly from antigen-activated cells that have bypassed the effector differentiation pathway (4).

Proliferation, differentiation, and survival of T cells is regulated by cytokines that act in conjunction with signals induced by the engagement of the T cell antigen receptor (TCR). Two cytokines, IL-2 and IL-15, both capable of supporting T cell growth after activation, have received particular attention in recent years. Although IL-2 and IL-15 were thought previously to have shared biologic activity in supporting the proliferation and differentiation of activated T cells, recent studies, including those with mice deficient for these cytokines or their receptor α chains, have shown that their functions are not redundant. Specifically, IL-15 appears to be more important for maintenance of NK cells and memory CD8 T cells, whereas IL-2 functions as a regulator of both the induction and death of cytotoxic effector cells (5–11).

We have described previously the T-GFP mouse model in which green flourescent protein (GFP), expressed by all naive T cells, is selectively extinguished once activated CD8$^+$ T cells differentiate into effector cytotoxic T lymphocytes (CTLs) (12). To study the role of IL-2 and IL-15 in an antigen-specific CD8$^+$ T cell response, we crossed T-GFP mice to P14 mice transgenic for a TCR that recognizes the lymphocytic choriomeningitis virus glycoprotein peptide (LCMV–gp33-41) epitope (13). In this system, we found that after TCR engagement, provision of an adequate amount of IL-2 resulted in the generation of effector cells capable of immediate effector function, whereas IL-15 or low

doses of IL-2 never allowed the cells to fully acquire effector phenotype and function, but rather rendered them into memory-like cells.

Methods

Mice. T-GFP transgenic (Tg) mice have been described (1). P14 TCR-Tg mice (13) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and were backcrossed to T-GFP mice to derive P14XT-GFP double Tg mice. Expression of both transgenes was confirmed by flow cytometry by testing for GFP, Vα2, and Vβ8 expression. P14 mice that were backcrossed to the C57 background for over 10 generations were the kind gift of Rafi Ahmed, (Emory University, Atlanta, Georgia, USA). All mice were maintained under viral antigen free/specific pathogen free conditions in microisolator cages and were used when they were 6–10 weeks of age. Under these conditions, more than 90% of splenic $CD8^+$ T cells were naive cells; that is, they were GFP^+, L-$selectin^+$, CD44 low, $CD25^-$, $CD69^-$ (see Figure 2).

Gp33 vectors and peptide. WR strain of vaccinia virus was obtained from American Type Culture Collection (ATCC; Rockville, Maryland, USA). Recombinant vaccinia virus encoding the full-length LCMV glycoprotein was a gift of M.B. Oldstone, Scripps Research Institute, La Jolla, California, USA (14). A recombinant strain of *Listeria* monocytogenes (XFL703) that expresses the LCMV gp33-41 epitope within a secreted dihydrofolate reductase fusion protein was constructed as described previously (15). The LCMV glycoprotein peptide 33-41 (gp33 peptide, KAVYNFATC) was synthesized at BioSource International (Camarillo, California, USA).

Peptide stimulation. Splenocytes from P14 or P14XT-GFP mice were incubated with 10 μg/ml gp33-41 peptide for 1 hour, washed, and cultured in RPMI supplemented with 10% FBS. Two days later, the antigen-presenting cells (APCs) were removed by isolating the nonadherent cells and viable T cells were recovered by Histopaque (Sigma Chemical Co., St. Louis, Missouri, USA) gradient centrifugation. The cells were then washed and cultured in fresh tissue-culture flasks in medium supplemented with the indicated concentrations of either rIL-2 or rIL-15 (R&D Systems Inc., Minneapolis, Minnesota, USA). Media supplemented with cytokines were replaced every 2 days. All experiments were done using cells cultured for 7–9 days. At this time, more than 90% of all viable cells were $CD8^+$, $V\alpha2^+$, and $V\beta8^+$ cells, as determined by flow cytometry (not shown).

Flow cytometry. For phenotypic analysis, peptide lines and peritoneal exudate lymphocytes were stained with anti-mouse CD8 Cy5 and phycoerythrin-labeled (PE-labeled) Ab's to mouse CD62L, CD69, CD44, CD25, or CD43 (1B11) (PharMingen, San Diego, California, USA). To detect CCR7 expression, recombinant CCR7 ligand, ELC-Ig chimera was used. ELC coding sequence was PCR amplified from the plasmid pCRII-ELC (16) and cloned into pcDNA 3.1-Fc cassette, which contains the human IgG1 Fc coding sequence, with a mutation that abolishes Fc receptor binding (17). This construct was transfected into 293T cells, and ELC-Ig was purified from the culture supernatants by affinity chromatography on a protein A column followed by fast-protein liquid chromatography (FPLC). To stain for CCR7, cells were incubated with ELC-Ig (1 μg/ml) followed by goat anti-human Ig biotin and streptavidin PE. To detect P-selectin binding, recombinant P-selectin Ig chimera was used as a first step, followed by goat anti-human IgM PE as described (18). All samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View , California, USA).

To measure cell proliferation of peptide-primed cells during cytokine treatment, splenocytes were activated with gp33 for 2 days and treated with cytokines in replicate cultures. At 24-hour intervals for the next 5 days, one set of cultures was treated with 30 μg/ml bromodeoxyuridine (BrdU) for 6 hours, washed, and stained externally with αCD8 Cy5 and intracellularly with FITC-labeled anti-BrdU Ab.

Intracellular IFN-γ, cytotoxicity, and lymphocyte proliferation assays. These assays have been described (12). Briefly, to detect IFN-γ production, 10^6 cells were stimulated with plate-bound αCD3 (2 μg/well) or gp33 peptide–pulsed irradiated syngeneic splenocytes (10^5 cells) for 6–8 hours in the presence of 1 μM brefeldin A, stained with CyChrome-labeled anti-CD8, fixed, permeabilized, and stained with PE-labeled anti-mouse IFN-γ Ab.

To measure peptide-specific cytotoxicity, peptide lines or peritoneal exudate lymphocytes (PELs) were tested for lysis of ^{51}Cr-labeled EL-4 ($H2^b$) target cells unpulsed or pulsed with 10 μg/ml gp33 peptide in a 4-hour chromium-release assay. Background cytotoxicity of nonpeptide-pulsed EL-4 cells and uninfected EL-4 cells (always less than 5%) was subtracted to calculate peptide and viral-specific cytotoxicity.

To detect cell proliferation of memory cells in response to restimulation, splenocytes were cultured in triplicate in 96-well culture plates with gp33 peptide (2 days) followed by IL-15 treatment (5 days), and restimulated with αCD3 or gp33 as described above. Two days after stimulation, the cultures were pulsed with ^3H thymidine (0.5 μCi/well) for 6 hours, harvested, and counted for ^3H incorporation.

Rechallenge of peptide lines. To study recall responses, viable cells from peptide- and cytokine-treated cells (day 7) were restimulated with αCD3- or gp33 peptide–pulsed syngeneic splenocytes and tested for phenotype and function 2 days later.

Adoptive transfer experiments. P14 mice that were backcrossed into the C57/BL6 background for over 10 generations (gift of Rafi Ahmed) were used for these studies. Naive $CD8^+$ T cells (>90% $CD62L^+$, CD44 low) were isolated from splenocytes using a CD8 subset purification column (R&D Systems Inc.). The isolated cells were more than 80% pure (not shown). Peptide-specific cell lines were generated from splenocytes with IL-2 or IL-15, as described above. Cells (10^6) were adoptively transferred into naive C57/BL6 mice through tail vein injec-

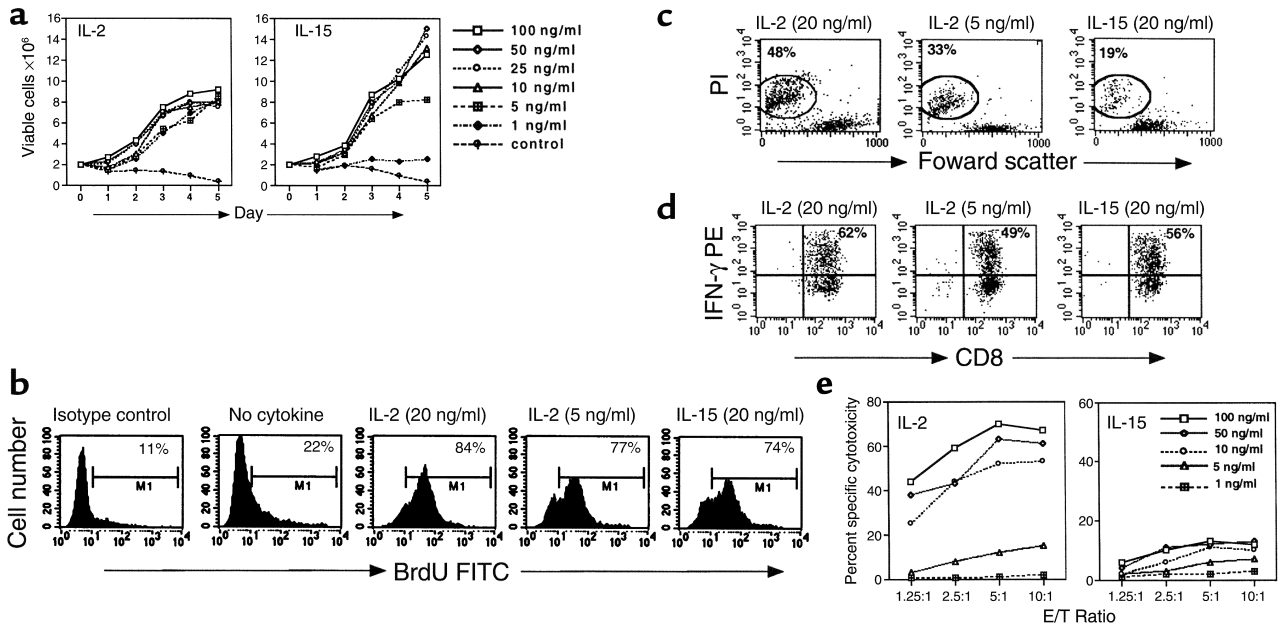

Figure 1

Effect of IL-2 and IL-15 on activated CD8 T cell proliferation and function. (**a** and **b**) All tested concentrations of IL-2 and IL-15 at 5 ng/ml or greater support cell proliferation. Cell proliferation was measured by viable cell counts (**a**) or BrdU incorporation (**b**) as described in Methods. In **b**, histograms of BrdU incorporation by $CD8^+$ gated cells on day 3 of cytokine treatment are shown. Background staining obtained with isotype control is also shown for comparison. Similar results were seen for all treatment groups on each day for all 5 days tested (not shown). (**c**) IL-2 induces increased cell death compared with IL-15. Cell death was measured by flow cytometric analysis of non-gated cells after staining with propidium iodide (PI). Shown are dot plots after 3 days of cytokine treatment. (**d**) Both IL-2– and IL-15–treated cells acquire the ability for IFN-γ production. On day 7 following peptide stimulation and cytokine treatment, the cultures were restimulated with αCD3 for 6 hours, stained externally with αCD8 Cy 5, intracellularly with αIFN-γ Ab, and examined by flow cytometry. Less than 2% of unstimulated cells produced IFN-γ (not shown). (**e**) Peptide primed cells cultured with IL-2 at concentrations of 10 ng/ml or greater exhibit high levels of cytotoxicity, whereas cells cultured with IL-2 at or below 5 ng/ml and all tested concentrations of IL-15 exhibit greatly diminished levels of cytotoxicity. On day 7 after stimulation, cultures were tested for specific killing of gp33 peptide-pulsed EL-4 target cells by chromium-release assay.

tion. To induce the generation of endogenous memory cells, some recipients of naive cells were infected with 10^6 pfu of recombinant vaccinia virus encoding the LCMV glycoprotein (14) 2 days after transfer. All mice were rested for 10 weeks and subsequently infected by intraperitoneal injection with 10^6 pfu of recombinant *Listeria* monocytogenes encoding the gp33 epitope. On day 4 after challenge, PELs were harvested and tested for gp33-specific IFN-γ production and peptide-specific cytotoxicity.

Results

Effect of IL-2 and IL-15 on the proliferation and function of antigen-activated $CD8^+$ T cells. First, we compared the ability of IL-2 and IL-15 to support the proliferation and functional differentiation of antigen-stimulated CD8 T cells obtained from P14 mice. These mice express a transgene-encoded TCR specific for the immunodominant LCMV glycoprotein peptide, gp 33-41 (KAVYNFATM), presented in the context of $H2\text{-}D^b$ class I MHC molecules (13). P14 splenocytes were stimulated with LCMV gp33-41 synthetic peptide (hereafter referred to as gp33 peptide) and cultured for 2 days without cytokines, following which the cells were washed and cultured in varying concentrations of either IL-2 or IL-15. Cell proliferation was measured by BrdU incorporation, and viable cells were counted at 24-hour intervals throughout the 5-day period of cytokine treatment. In the absence of any cytokines, activated cells did not proliferate significantly, but rapidly decreased in numbers (Figure 1, a and b). In contrast, cells cultured either with IL-2 at all concentrations tested (1–100 ng/ml) or with IL-15 at 5 ng/ml or greater, proliferated equally well during the first 3 days of cytokine treatment. At days 4 and 5 the viable cell numbers in IL-2–treated cultures began to plateau, whereas IL-15–treated cultures continued to expand. These differential effects of IL-2 and IL-15 possibly were due to their respective pro- and antiapoptotic effects on CD8 T cells, as evidenced here by propidium iodide staining (Figure 1c) and observed previously in other model systems (8).

Effector functions were evaluated 7 days after stimulation (5 days after cytokine treatment) by testing for the cells' ability to produce IFN-γ and to mediate peptide-specific cytotoxicity. Both IL-2 and IL-15 supported IFN-γ production equally well (Figure 1d). However, while cells cultured in IL-2 at 10 ng/ml or greater were highly cytotoxic, cells cultured in IL-15 or IL-2 at doses of 5 ng/ml or less exhibited little cytotoxicity (Figure 1e). To determine if cells cultured in IL-15 acquired cytotoxic potential transiently, we also tested cytotoxicity 2 and 4 days after peptide activation. While cytotoxic potential progressively increased for cells cultured in high-dose IL-

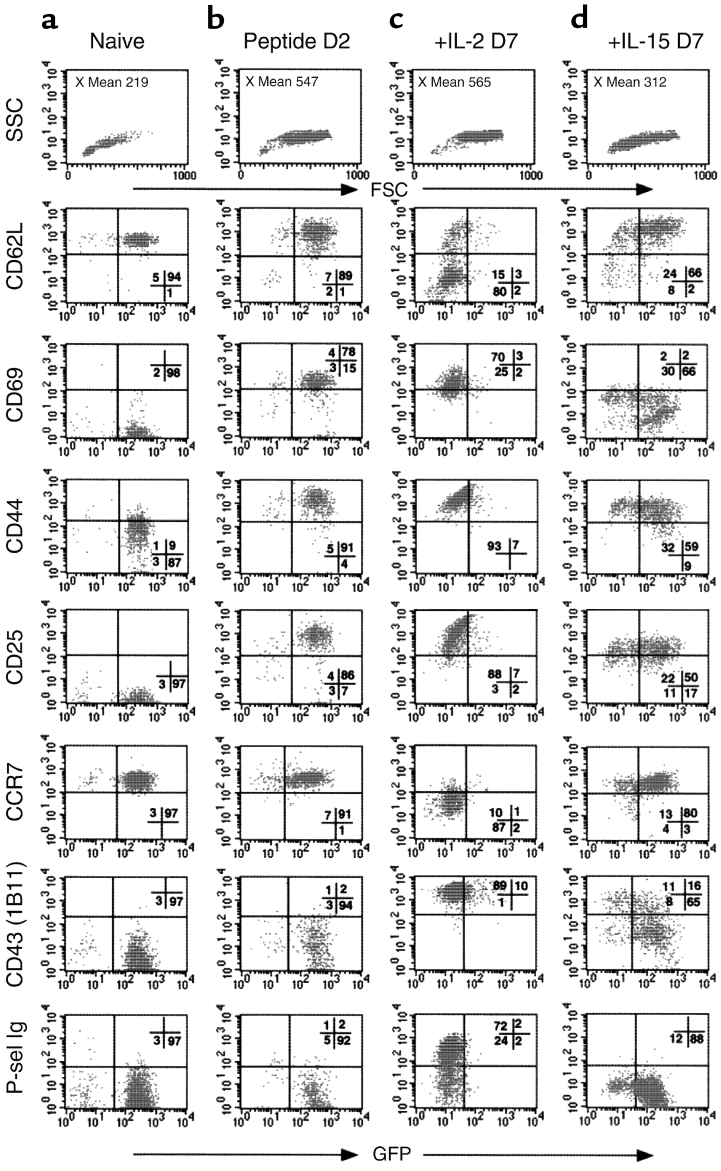

Figure 2
IL-2 and IL-15 induce distinct phenotypic changes in activated CD8+ T cells. Splenocytes from P14XT-GFP double-Tg mice were either examined directly (naive cells) (**a**), or stimulated with gp33 peptide and examined after 2 days (peptide D2) (**b**), or stimulated with peptide for 2 days and then cultured in the presence of 20 ng/ml of either IL-2 (+ IL-2) (**c**), or IL-15 (+IL-15) (**d**) for the next 5 days and analyzed by flow cytometry for size, GFP expression, and expression of surface markers. Representative results from one experiment (of three performed) are shown after gating on CD8+ T cells. Quadrant statistics are shown as percentage of gated cells. Low concentrations of IL-2 (5 ng/ml or less) induced phenotypic changes in primed CD8 T cells similar to IL-15 (not shown). P-sel, P-selectin.

2, IL-15–treated cells did not exhibit comparable levels of cytotoxicity at any time point (not shown). Thus, a threshold dose of IL-2 appears to be necessary for differentiation of antigen-activated CD8 T cells into full-fledged effector cells, whereas any doses of IL-15 above 5 ng/ml and subthreshold doses of IL-2 support proliferation and IFN-γ production, but do not support differentiation into effector CTLs.

IL-2 and IL-15 induce distinct phenotypic changes in antigen-activated CD8+ T cells. Several studies suggest that compared with effector cells, memory cells exhibit reduced levels of cytotoxicity (19–23). Thus, we examined cells cultured in IL-2 and IL-15 for expression of phenotypic markers that are preferentially associated with naive, effector, or memory cells.

Until recently, there was no cell surface marker that could unambiguously differentiate between effector and memory CD8+ T cells. We have described recently T-GFP mice in which loss of GFP expression characterizes effector CTLs (12). Subsequently, Sallusto et al. have reported that a subset of memory T cells (called central memory cells) are characterized by lack of immediate effector function and retention of L-selectin and the chemokine receptor CCR-7 (24). Moreover, Harrington et al. have reported that the CD43 epitope recognized by the mAb, 1B11 is expressed on effector, but not memory CD8 T cells (23). Thus, we crossed P14 mice with T-GFP mice and examined the double-Tg CD8 T cells before and after peptide and cytokine treatment for expression of these markers as well as the common T cell activation markers CD69, CD25, and CD44.

Freshly isolated CD8+ T cells from the double-Tg mice expressed surface markers characteristic of naive T cells; that is, they did not express CD69, CD44, CD25, or the CD43 epitope 1B11, and were uniformly L-selectin– and GFP-positive (Figure 2a). When the P14XT-GFP splenocytes were stimulated in vitro with the gp33 peptide, CD8+ T cells developed a blastoid morphology and expressed high levels of all activation markers except the CD43 epitope 1B11, but retained GFP and L-selectin expression during the first 2 days of stimulation (Figure 2b).

To study the effect of cytokines, the peptide-activated cells were washed after 2 days of stimulation and cultured in high doses (20 ng/ml) of IL-2 or IL-15 for an additional 5 days before testing the phenotype. While cells cultured in IL-2 maintained the large blastoid size, IL-15–treated cells were smaller, as determined by their forward scatter profile (Figure 2c). Flow cytometric analysis also revealed that the cells cultured in IL-2 had uniformly lost GFP, L-selectin, and CCR-7 expression. However, they were strongly positive for the CD43 1B11 epitope. In contrast, cells maintained in IL-15 mostly retained GFP, L-selectin, and CCR-7 expression, but did not express the 1B11 epitope.

Appearance of the CD43 1B11 binding epitope on T cells requires modification of *O*-glycans by core 2 *N*-acetylglucosaminyltransferase (C2 GlcNAcT). C2 GlcNAcT activity is induced by T cell activation, but is

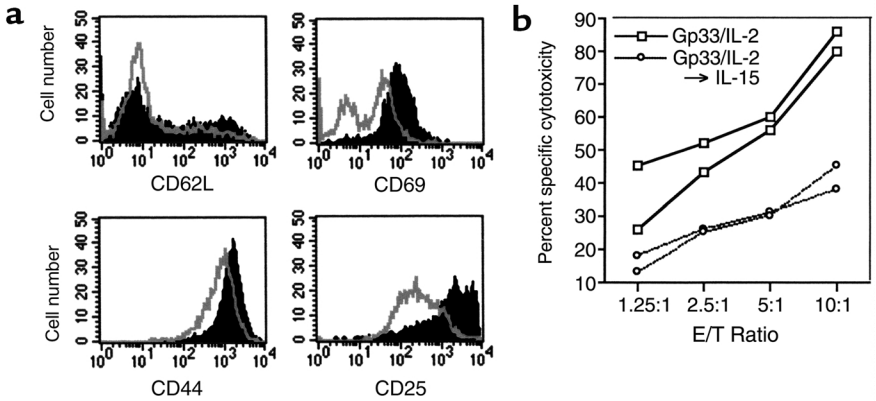

Figure 3
IL-15 reduces activation and cytotoxic function of fully differentiated effector CTLs. P14XT-GFP Tg splenocytes were stimulated with the gp33 peptide and cultured in 20 ng/ml IL-2 for 7 days. The cells were then washed and maintained in either IL-2 or IL-15 (20 ng/ml) for the next 5 days before testing for activation phenotype (**a**) and peptide-specific cytotoxicity (**b**). Overlay histograms of cells cultured in IL-2 (squares) and IL-15 (circles) are shown after gating for CD8 expression in **a**. Data for two sets of independent cell lines are shown in **b**.

downregulated in memory T cells, which return to expressing a core 1 *O*-glycan with increased sialylation (25). Because modification by C2 GlcNAcT is also required for P-selectin glycoprotein ligand–1 (PSGL-1) to bind P-selectin (26), we tested P-selectin binding activity after cytokine treatment using recombinant P-selectin Ig chimera (18). Strong P-selectin binding was seen only in the IL-2–treated cells. Naive cells, 2-day peptide-activated cells, and the IL-15–treated cells all failed to bind P-selectin.

Among the activation markers, the IL-2–treated cells maintained high levels of CD69, CD44, and CD25, while the IL-15–treated cells showed intermediate levels of expression, most notably for CD69 and CD25.

Taken together, the IL-2–stimulated peptide-primed $CD8^+$ T cells lost GFP, L-selectin, and CCR7 expression, but expressed high levels of CD69, CD25, CD44, andCD43 1B11 epitope and functional PSGL-1. In contrast, the IL-15–treated cells retained GFP, L-selectin, CCR7, and CD44 expression, but downregulated CD69 and CD25 and did not bind 1B11 or P-selectin. These phenotypic differences suggest that while high-dose IL-2 differentiates activated $CD8^+$ T cells into effector cells, IL-15 may drive them toward a memory phenotype that is reminiscent of central memory cells (24). Cells cultured in low-dose (5 ng/ml) IL-2 developed a phenotype similar to IL-15–treated cells (not shown).

Fully differentiated effector CTLs revert to a memory phenotype and function under the influence of IL-15. We also tested if IL-15 could convert differentiated effector cells into memory-like cells. For these studies, effector cells were generated in the presence of 20 ng/ml IL-2, and on day 7 the cells were washed and cultured with 20 ng/ml of either IL-2 or IL-15 for 5 more days before testing their phenotype and function. The 7-day-old effector cells cultured in either IL-2 or IL-15 remained L-selectin and GFP negative. However, effector cells that were switched to IL-15 downregulated CD69 and CD25 and had reduced levels of cytotoxicity compared with parallel cultures treated continuously with IL-2 (Figure 3). These results suggest that IL-15 can convert $CD8^+$ T cells that had previously differentiated into effector CTLs to cells of memory phenotype and function, even though they differed from cells that were only exposed to IL-15 with regard to expression of homing molecules and GFP.

Rapid induction of effector functions in $CD8^+$ T cells cultured in IL-15 upon rechallenge. A hallmark of memory $CD8^+$ T cells is their ability to proliferate and to acquire cytolytic activity rapidly after restimulation. Thus, we tested the proliferation, phenotype, and function of peptide-primed P14XT-GFP cells treated with IL-15 for 7 days and, subsequently, restimulated with αCD3 or gp33 for 2 days. Compared with unstimulated cells, the restimulated cells proliferated more vigorously (Figure 4a), rapidly lost GFP and L-selectin expression and upregulated CD69, CD25, and CD44 expression (Figure 4b, right and middle panels). This phenotype of restimulated cultures closely resembled that seen in parallel cultures continuously maintained in IL-2 (Figure 4b, left panel). Moreover, the restimulated cells had acquired the capacity to mediate a high degree of peptide-specific cytotoxicity compared with unstimulated cells within 2 days of stimulation (Figure 4c). Similar results were obtained when peptide lines generated with low doses of IL-2 (5 and 1 ng/ml) were restimulated (not shown). In contrast, antigenic stimulation of naive $CD8^+$ cells did not induce CTL activity by 2 days of stimulation (not shown). Thus, cells generated under the influence of IL-15 or low doses of IL-2 are capable of rapid-recall responses in vitro.

Antigen-primed CD8 T cells treated with IL-15 and low-dose IL-2 are capable of long-term survival and mount rapid-recall responses in vivo. Cells differentiated with IL-2 and IL-15 were compared with each other and to in vivo–generated memory cells for their ability to mediate recall responses in adoptive transfer experiments. P14 mice that were backcrossed into the C57/BL6 background for over 10 generations (gift of Rafi Ahmed) were used as T cell donors. Groups of C57/BL6 mice were injected intravenously with 10^6 $CD8^+$ T cells obtained from naive P14 mice or with peptide primed cells maintained in 20 ng/ml or 5 ng/ml IL-2, or 20 ng/ml IL-15, or kept without cell injection as a control. To generate memory cells in vivo, 2 days after transfer one group of mice injected with naive cells were infected with recombinant vaccinia virus encoding the LCMV glycoprotein (14). All mice were rested for 10 weeks and were then challenged intraperitoneally with recombinant *Listeria* monocytogenes encoding the LCMV gp33 epitope. On day 4 after challenge, PELs were harvested and tested for peptide-specific IFN-γ production and cytotoxicity (Figure 5).

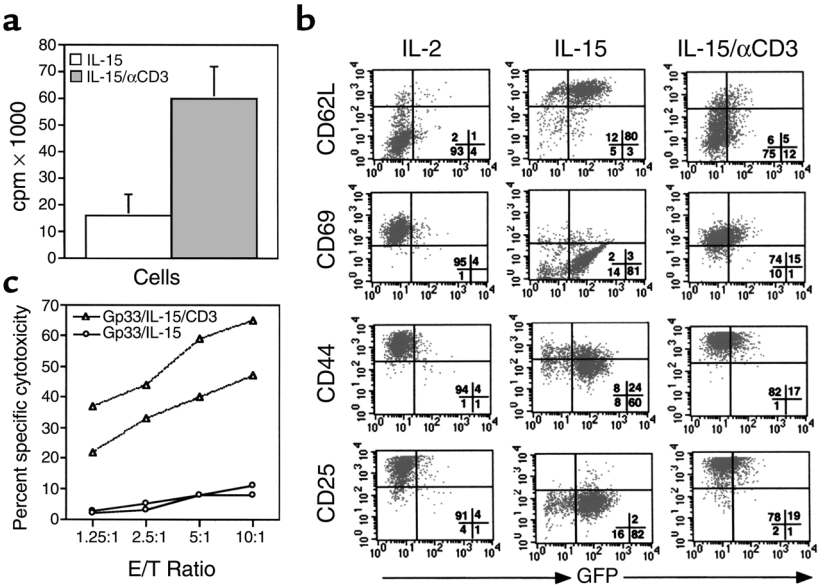

Figure 4
TCR stimulation induces cells maintained in IL-15 to proliferate vigorously and to rapidly acquire effector phenotype and function. Tg splenocytes were stimulated with gp33 peptide, maintained in IL-15 for 7 days and then were restimulated with αCD3 for 48 hours or maintained in IL-15 without restimulation. Subsequently, both populations were tested for proliferation by thymidine incorporation (**a**), activation status (**b**), and peptide-specific cytotoxicity (**c**). In **b** the phenotype of cells maintained in parallel with a high dose of IL-2 (20 ng/ml) is also shown for comparison. Peptide-stimulated cells maintained in low doses of IL-2 (5 ng/ml or less), which resembled IL-15–treated cells in phenotype and function, also proliferated and rapidly acquired effector phenotype and cytotoxic function after restimulation (not shown).

Control mice that were not adoptively transferred did not exhibit peptide-specific IFN-γ production or cytotoxicity. Thus, endogenous gp33 response was not significant on day 4 after infection, and we could attribute the response seen in other experimental groups to be due to the adoptively transferred donor cells. Mice transferred with naive TCR Tg cells showed small but detectable levels of IFN-γ production and cytotoxicity. In contrast, mice that were transferred with IL-15 or low-dose IL-2–treated cells exhibited a vigorous response in both IFN-γ production and cytotoxicity that was equivalent in magnitude to the response seen with in vivo–generated memory cells. Mice that received high-dose IL-2–treated cells showed an intermediate response. Thus, in vitro–activated CD8 T cells treated with IL-15 or low-dose IL-2 show the long-term survival and rapid-recall responses in vivo that are characteristic features of bona fide memory T cells.

Discussion

A number of recent studies suggest that effector and memory cells with differing functional and migratory capabilities arise as an inevitable consequence of antigen encounter (27–29). However, it is not clear what factors other than antigenic stimulation are involved in their generation. Our results show that cytokines can drive the murine antigen-activated $CD8^+$ T cells to differentiate alternately into effector or memory cells. Our results also show that memory cells can arise directly from activated cells without having to go through the effector phase of differentiation. The peptide-activated $CD8^+$ T cells differentiated under the influence of IL-15 or low concentrations of IL-2 proliferated well, but did not develop the effector CTL phenotype and function. Rather, they exhibited several characteristics of bona fide memory cells: they were small; retained L-selectin, GFP and CCR7 expression; decreased expression of activation markers; did not express core-2 *O*-glycans; were directly capable of IFN-γ secretion, but not cytotoxicity; were capable of proliferation and rapid-recall responses in vitro; survived for at least 10 weeks in vivo; and efficiently mounted a secondary recall response. In contrast, peptide-activated cells cultured in the presence of high concentrations of IL-2 exhibited all the characteristics of effector cells. They were large cells with blastoid morphology; lost L-selectin, CCR7, and GFP expression; expressed high levels of activation and effector markers, including core 2 *O*-glycans, as evidenced by the expression of CD43 1B11 epitope and P-selectin binding; and exhibited high levels of peptide-specific cytotoxicity and IFN-γ release. Moreover, the peptide-activated cells generated under the influence of high doses of IL-2 and IL-15 exhibited the migratory properties expected of effector and memory T cells, respectively; while the IL-15–treated cells were capable of migration into peripheral lymph nodes, the IL-2–treated cells were selectively excluded from lymph nodes, but homed preferentially to sites of inflammation (W. Weninger et al., unpublished observations).

The precise lineage by which naive CD8 T cells differentiate into memory cells is unclear and remains controversial (4). Different models have been proposed for the generation of memory cells; the linear differentiation model that predicts that memory cells are the progeny of effector CTLs that escape activation-induced cell death (30, 31). The other view is that memory cells arise from naive cells when they receive weak antigenic stimulation toward the end of an immune response (21, 31). A reverse linear-differentiation model has also been recently suggested in which the so-called central memory cells arise first, and they give rise to effector cells (24, 32). Although the role of cytokine milieu has been acknowledged, no study so far has systematically examined the effect of cytokines in the generation of effector and memory CD8 T cells. Our results clearly show that both effector and memory cells can be generated directly from antigen-activated naive T cells by differential

cytokine treatment. Our results also indicate that memory cells can arise independently of the strength of TCR stimulus or the number of cell divisions. The fact that effector cells that were generated with high-dose IL-2 were also capable of mounting a secondary response after adoptive transfer, albeit to a lesser extent than IL-15–treated cells, suggests that memory cells can also arise from differentiated effector cells. The controversy that exists in the literature can be explained on the basis of the recently recognized existence of different subsets of memory CD8 T cells (24). Moreover, a recent study has shown that a subset of memory cells can be sequestered in peripheral tissues long after the resolution of infection (33). Thus, the cytokine milieu can conceivably determine the conversion of antigen-activated cells into memory cells either before (central memory) or after (effector memory) they have differentiated into effector cells. Our findings that fully differentiated effector cells can be converted to cells with memory phenotype and function by switching them to IL-15 (Figure 3) also supports such a hypothesis.

What are the mechanisms by which IL-2 and IL-15 induce the dramatic differences we have observed? These differences are somewhat surprising considering that both cytokines are thought to signal through the common IL-2/IL-15 receptor βγ chains. However, even the same cytokine, IL-2, can have opposing effects of either promoting T cell survival by inducing the expression of the Bcl-2 family of antiapoptotic molecules (34, 35) or of inducing programmed cell death, possibly by activating the fas-fasL pathway (36). To explain how these opposing signals might be delivered, a threshold model has been proposed for IL-2 signaling, where the level of receptor engagement may determine which signals are actually delivered (37). This is in accordance with our findings that IL-2, at low doses, induced memory cell differentiation as opposed to IL-2 at high concentrations, which induced effector differentiation.

The effect of IL-15 may also be explained by this threshold effect, because although activated T cells make abundant IL-2 Rα, they exhibit few IL-15 binding sites (38) and thus the availability of IL-15Rα might limit signaling through the βγ complex. However, it is also possible that IL-15Rα engagement induces different physical interactions of the βγ complex than IL-2Rα engagement (39) and may thus transduce signals that are quantitatively or qualitatively different from those initiated by IL-2Rα ligation. Moreover, a recent study found that upon IL-15 treatment, the IL-15Rα chain binds the tumor necrosis factor receptor–associated factor 2 (TRAF2) (40). In fact, it has been proposed that IL-15Rα chain deflects TRAF2 away from TNFR1 and thus prevents assembly of downstream apoptotic molecules on TNFR1 (40). A key biologic difference between IL-2 and IL-15 is their differential effects on apoptosis. As noted earlier, IL-2 can either induce or inhibit apoptosis in activated T cells. On the other hand, IL-15 inhibits apoptosis in activated T cells induced by a variety of stimuli, including cytokine deprivation and fas-mediated cell death (35, 41).

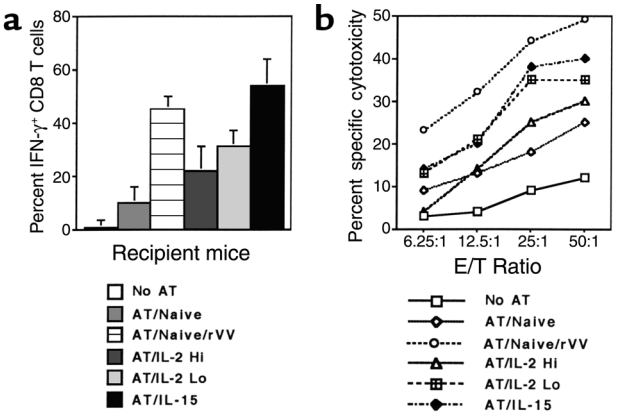

Figure 5
Function of activated CD8+ T cells differentiated with IL-2 and IL-15 after adoptive transfer. P14 Tg splenocytes were stimulated with peptide for 2 days, washed, and cultured with a high (20 ng/ml; AT/IL-2 Hi) or low (5 ng/ml; AT/IL-2 Lo) dose of IL-2 or IL-15 (20 ng/ml; AT/IL-15) for 5 more days and infused intravenously into groups of C57/BL6 mice (10^6 cells per mouse; n = 3 mice per group). Mice were also infused with naive CD8+ T cells from P14 Tg mice (AT/Naive) or with PBS (No AT) as controls. To generate memory cells in vivo, some AT/Naive mice were infected with recombinant vaccinia virus encoding LCMV glycoprotein 2 days after transfer (AT/Naive/rVV). All mice were rested for 10 weeks, after which they were challenged with recombinant *Listeria* monocytogenes encoding the gp33 epitope; on day 4 after infection, PELs were tested for peptide-specific IFN-γ production (**a**) and cytotoxicity (**b**). Mean ± SD of all three experiments are shown in **a**, and results from one representative experiment (of three performed with similar results) are shown in **b**.

Our results suggest that while both IL-2 and IL-15 support the proliferation and survival of activated CD8+ T cells in vitro, only high doses of IL-2 are able to induce an effector phenotype and function. Thus, conditions that promote prolonged cell survival in the absence of a sufficiently strong effector-differentiation signal might transform the activated CD8+ T cells into long-lived memory cells. IL-15 or low doses of IL-2 may provide just such subthreshold survival signals. In preliminary studies, we have found that IL-7, another cytokine that prevents T cell apoptosis (42), can also induce phenotypic changes similar to IL-15 (data not shown). Consistent with this observation, both IL-15 and IL-7 have been shown to be important in the survival and differentiation of CD8+ memory T cells in vivo (43).

Whatever the mechanisms involved, our results suggest that a simple change of cytokine milieu is enough to derive effector or memory CD8+ T cells in vitro. IL-2 and IL-15 are especially suited to serve this role in vivo. IL-2 is produced exclusively by activated T cells, whereas IL-15 can be produced by a variety of nonlymphoid cells, including macrophages, epithelial cells, and fibroblasts (6). These sources of IL-15 could be sequestrated in tissue sites and provide an environment for antigen-primed T cells to become memory cells. For example, in lymphoid organs such as the spleen, adoptively transferred effector CD8+ T cells were found to be predominantly confined to the red pulp, whereas memory cells could enter the periarteriolar lymphoid sheath in the white

pulp, presumably due to differences in CCR7 expression (44). Such anatomic compartmentalization could conceivably provide differential cytokine milieus to generate effector or memory T cells.

Due to a lack of specific markers, it has so far not been possible to unambiguously differentiate effector and memory subsets of CD8 T cells in vivo. Even if markers were available, memory cells are likely to represent a small fraction of responding cells, making comparative mechanistic studies of effector and memory cells difficult. Our P14XT-GFP mice provide a tool to generate unlimited numbers of relatively homogenous effector and memory T cells. Pure populations of such cells can easily be sorted based on GFP expression for molecular studies. Furthermore, our method provides a way to generate memory cells that can survive in vivo for long periods of time. This process might be useful for the development of new vaccination strategies.

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