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The mechanisms responsible for the generation and maintenance of T cell memory are unclear. In this study, we tested whether IL-2 is required for generating and maintaining CD8⁺ memory T cells by analyzing the long-term survival, phenotype, and functional characteristics of IL-2-replete (IL-2⁺/⁺) and IL-2-deficient (IL-2⁻/⁻) CD8⁺ TCR-transgenic lymphocytes in an adoptive transfer model. We found that IL-2 is not essential for the in vivo generation, maintenance, or recall response of CD8⁺ memory T cells. However, IL-2 increased the size of the CD8⁺ memory pool if present at the time of initial T cell activation but reduced the size of the pool if present during memory maintenance by inhibiting the proliferation of CD8⁺ memory T cells. Thus, IL-2-based vaccine strategies or immunosuppressive regimens that target IL-2 should take into account the divergent roles of IL-2 in CD8⁺ T cell immunity. The Journal of Immunology, 2000, 165: 3031–3036.

A defining feature of the adaptive immune response is its ability to generate memory lymphocytes (1–3). Upon encounter with a foreign Ag, Ag-specific T lymphocytes proliferate and differentiate into effector cells. The majority of effector cells undergo apoptosis after the Ag is eliminated (4). The few that survive become memory cells that persist for a long period of time, sometimes throughout the life of an animal (5–7). Memory T cells are specific to the Ag they encountered during the primary immune response and react rapidly upon re-encounter with the same Ag.

The mechanisms responsible for the persistence of T cell memory are unclear. It appears that memory T cell populations are maintained through the homeostatic replication of a subgroup of memory cells and through the intrinsic ability of some memory cells to survive in the resting state for an extended duration (8, 9). Although earlier studies suggested that continued antigenic stimulation is required for maintaining T cell memory (9–12), recent evidence indicates that CD4⁺ and CD8⁺ memory T cell populations persist in the absence of specific or cross-reactive Ags presented by MHC molecules (13–15). Therefore, it is possible that Ag-independent factors, such as cytokines, are critical for the survival and proliferation of memory T cells. IL-2 enhances the survival of naive T cells and stimulates the proliferation of primary activated lymphocytes (16, 17). In addition, it has been proposed that IL-2 is required for generating and maintaining memory T cells (18, 19). This hypothesis, however, is challenged by the findings that IL-2 programs T cells for activation-induced apoptosis and that IL-2⁻/⁻ mice are not immunodeficient but instead display exaggerated T cell immunity (4, 17).

In this study, we tested whether IL-2 is required for generating and maintaining CD8⁺ memory T cells by analyzing the long-term survival, phenotype, and functional characteristics of Ag-activated IL-2-replete (IL-2⁺/⁺) and IL-2 gene-knockout (IL-2⁻/⁻) CD8⁺ TCR-transgenic (TCR-tg) lymphocytes (2C) in an adoptive transfer model. The 2C TCR recognizes the L8 MHC class I Ag and can be tagged by a chimeric Ab (1B2) that permits the detection of small numbers of Ag-specific cells in vivo (20). We demonstrate here that CD8⁺ memory T cells can be efficiently generated and maintained in the absence of IL-2. However, IL-2 increased the size of the CD8⁺ memory population if present during T cell activation but reduced its size if present during the maintenance period. These findings indicate that IL-2 has a dual role in the generation and maintenance of CD8⁺ T cell memory.

Materials and Methods

Mice

BALB/c (H-2b), C57BL/6 (H-2d) Rag1⁻/⁻, and C57BL/6 IL-2⁻/⁻ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 2C TCR-tg mice were provided by Dr. Dennis Loh and bred at the Veterans Affairs Medical Center animal facility (Atlanta, GA) (20). IL-2-deficient 2C mice were generated by cross-breeding C57BL/6 IL-2⁻/⁻ mice with C57BL/6 2C mice. Genotyping was performed by PCR amplification of DNA extracted from tail clippings. Transgenic and gene-knockout mice were housed under specific pathogen-free conditions.

In vitro generation of effector CD8⁺ T cells

Splenocytes were isolated from IL-2⁺/⁺ and IL-2⁻/⁻ 2C mice and stimulated in vitro with mitomycin C-treated BALB/c splenocytes in RPMI 1640 medium supplemented with 1 mM l-glutamine, 1% sodium pyruvate, 50 μM 2-ME, 100 μg/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Grand Island, NY), and 10% FCS (Sigma, St. Louis, MO). A total of 50 U/ml recombinant mouse IL-2 (Genzyme, Cambridge, MA) was added to all cultures. After 96 h of mixed lymphocyte culture, live cells were isolated by ficoll density centrifugation using Lympholyte-M (Cedarlane, Hornby, Ontario) and were subsequently enriched for T cells by nonadherence to nylon wool columns (Polysciences, Warrington, PA). IL-2⁺/⁺ and IL-2⁻/⁻ 2C T cell-enriched preparations (a total of ~2.5 × 10⁶ 2C T cells in each) containing 5 × 10⁶ CD8⁺ 1B2 cells (quantitated by two-color flow cytometry as described below) were mixed with 3-fold more (~7.5 × 10⁷) naive T cell-enriched IL-2⁺/⁺ and IL-2⁻/⁻ non-TCR-tg C57BL/6 splenocytes, respectively, and were then adoptively transferred by i.v. injection into Rag1⁻/⁻ mice. Control Rag1⁻/⁻ mice were adoptively transferred with 5 × 10⁶ naive IL-2⁺/⁺ or IL-2⁻/⁻ 2C T cells with 18 U.S.C. Section 1734 solely to indicate this fact. 3

3 Abbreviations used in this paper: tg, transgenic; BrdU, 5-bromo-2-deoxyuridine.
CD8+1B2+ cells mixed with ~7.5 × 10^7 naive T cell-enriched IL-2/+ or IL-2/− non-TCR-tg C57BL/6 splenocytes, respectively. 2C lymphocytes were mixed with non-TCR-tg, syngeneic splenocytes before transfer to simulate physiologic settings in which Ag-specific cells are present at much lower frequency than in TCR-tg mice. Mice were sacrificed at either 1 wk or 10 wk after adoptive transfer and spleen and lymph node cells were harvested to test for the presence of CD8+1B2+ cells. Memory cells were identified according to the following criteria: 1) survival advantage over naive cells; 2) high CD44 and low CD62L expression (CD44highCD62Llow); and 3) ability to mount a recall CTL response. Absence of IL-2 production in Rag1−/− hosts that received IL-2/− non-TCR-tg lymphocytes was confirmed by 35 cycles of RT-PCR amplification of splenic and lymph node mRNA.

In vivo generation of memory T cells

Naive IL-2/+ or IL-2/− CD8 T cell-enriched splenocyte preparations (a total of ~2.5 × 10^7 T cells) that contain 5 × 10^6 CD8+1B2+ cells were mixed with 3-fold more (~7.5 × 10^7) naive T cell-enriched IL-2/+ or IL-2/− non-TCR-tg splenocytes, respectively, and were subsequently adoptively transferred to Rag1−/− mice by i.v. injection. Twenty-four hours later, the Rag1−/− hosts were immunized i.p. with mitomycin C-treated BALB/c splenocytes. At 10 wk, 0.8 mg/ml BrdU was added to the drinking water for 7 days, after which the mice were sacrificed and lymph node cells were harvested. A separate group of Rag1−/− mice harboring IL-2/− CD8 memory lymphocytes was injected with 5 μg recombinant murine IL-2 i.p. twice during the BrdU labeling period. Surface staining of lymph node cells with 1B2 and anti-CD8 was performed as described in the previous sections. Cells were then fixed in 70% ethanol and incubated with 50 μg/ml of anti-BrdU (Becton Dickinson) and three-color flow cytometry analysis was performed. The percentage of BrdU+ cells was determined after gating on the CD8+1B2+ population.

Results

IL-2 is not required for the maintenance of CD8+ memory T cells

To study the role of IL-2 in the maintenance of Ag-specific CD8+ T cell memory, IL-2/+ and IL-2/− CD8+ T lymphocytes were activated in vitro in the presence of excess IL-2 and were then transferred to Rag1−/− hosts. Control Rag1−/− mice were adoptively transferred with naive IL-2/+ or IL-2/− CD8+ lymphocytes. Both activated and naive 2C lymphocytes were mixed with naive, non-TCR-tg syngeneic T lymphocytes before adoptive transfer to simulate physiologic settings in which Ag-specific T cells are present at much lower frequency than in TCR-tg mice. Because 2C lymphocytes can be identified by a clonotypic Ab (1B2), this experimental model allowed us to compare the in vivo survival, phenotypic, and functional characteristics of previously activated CD8+ T cells to that of naive cells in the presence or absence of IL-2. In the experiments that follow, similar findings were obtained whether lymph node or spleen cells were analyzed. Therefore, only the results of lymph node analysis are shown.

One week after adoptive transfer, the number of previously activated IL-2/+ or IL-2/− CD8 memory T lymphocytes present in the lymph nodes of Rag1−/− hosts was similar to that of naive IL-2/+ or IL-2/− CD8+ lymphocytes (Fig. 1, A and B). No significant difference in the number of CD8+1B2+ cells was observed between the IL-2/+ and IL-2/− 2C populations (Fig. 1, A and B). However, at 10 wk after adoptive transfer, the number of previously activated IL-2/+ or IL-2/− CD8+ lymphocytes significantly exceeded that of naive IL-2/+ or IL-2/− CD8+ lymphocytes (Fig. 1, A and B), indicating that previously activated lymphocytes have a survival advantage over their naive counterparts. Like the naive 2C TCR-tg (CD8+1B2+) population, the naive non-TCR-tg (CD8+1B2+) population had diminished dramatically by 10 wk (Fig. 1A). Moreover, at 10 wk after adoptive transfer, previously activated IL-2/+ or IL-2/− 2C lymphocytes were present in significantly greater numbers than previously activated IL-2/+ or IL-2/− 2C lymphocytes (Fig. 1, A and B). These findings suggest that long-lived, Ag-specific, CD8+ memory T cells are present in Rag1−/− mice adoptively transferred with previously activated 2C lymphocytes, and that the number of memory cells is increased, rather than diminished, in the absence of IL-2.

To confirm that long-lived, previously activated IL-2/+ or IL-2/− 2C lymphocytes are memory cells, we tested their phenotypic and functional characteristics. As shown in Fig. 1C, previously activated IL-2/+ or IL-2/− 2C lymphocytes, present at 10 wk after adoptive transfer, express high levels of CD44 (CD44high) and low levels of CD62L (CD62Llow), a phenotype...
associated with CD8+ memory T cells in mice. We then asked whether a recall immune response can be elicited in Rag1−/− mice that received previously activated 2C lymphocytes. As shown in Fig. 1D, Ag-specific stimulation induced a rapid and strong CTL response in mice that harbored previously activated IL-2+/+ or IL-2−/− lymphocytes but not in those that harbored naive cells.

FIGURE 1. Long-term survival of CD8+ memory T cells in the absence of IL-2. Naive or in vitro-activated IL-2+/+ and IL-2−/− 2C lymphocytes (5 × 10^6 CD8+1B2+ cells) were mixed with ~7.5 × 10^7 naive non-TCR-tg IL-2+/+ and IL-2+/− T cells, respectively, and were adoptively transferred to syngenic Rag1−/− mice. At 1 or 10 wk after transfer, Rag1−/− hosts were sacrificed and lymph node cells were analyzed by flow cytometry for the presence of CD8+1B2+ cells. A, Flow cytometric analysis showing the percentage of naive or previously activated IL-2+/+ and IL-2+/− CD8+1B2+ cells present at 1 and 10 wk after adoptive transfer. Density plots shown are representative of four experiments. B, Absolute number (mean ± SD, n = 4) of naive or previously activated IL-2+/+ and IL-2+/− CD8+1B2+ cells present at 1 and 10 wk after adoptive transfer. *, p < 0.05 compared with naive cells; †, p < 0.05 compared with IL-2+/+ activated cells. C, Flow cytometric analysis of CD62L and CD44 expression on naive (solid histograms) or previously activated (open histograms) IL-2+/+ and IL-2+/− CD8+1B2+ cells present at 10 wk after adoptive transfer. Histograms shown are representative of four experiments. D, CTL recall response of Rag1−/− hosts at 10 wk after adoptive transfer. Ex vivo CTL activity was measured 3 days after challenging Rag1−/− hosts with BALB/c splenocytes (mean ± SD, n = 4).
These findings prove that neither the long-term maintenance nor the recall response of CD8⁺ memory T cells is dependent on IL-2.

IL-2 is not required for in vivo generation of CD8⁺ memory T cells

To test whether IL-2 is essential for generating CD8⁺ T cell memory, we adoptively transferred naive IL-2⁺/⁺ or IL-2⁻/⁻ 2C lymphocytes to Rag1⁻⁻/⁻ hosts. As in the previous model, naive 2C lymphocytes were mixed with naive, non-TCR-tg, syngeneic T lymphocytes before adoptive transfer. The adoptively transferred mice were then challenged with either PBS or L²⁺⁺-, bearing allogeneic splenocytes. Ten weeks later, lymph node cells were analyzed by flow cytometry. A significantly larger number of naive CD8⁺ 1B²⁺ cells was detected in allostimulated than in naive mice (Fig. 2). The number of long-lived CD8⁺ 1B²⁺ cells was comparable in mice that harbored either IL-2⁺/⁺ or IL-2⁻/⁻ 2C lymphocytes. Moreover, long-lived CD8⁺ 1B²⁺ cells present in allostimulated mice, but not those present in naive mice, exhibited a CD44⁺CD62L⁻ low phenotype (data not shown). These findings indicate that CD8⁺ memory T cells can be generated in vivo in the absence of IL-2. Similar findings were obtained when spleen cells were anlayzed (data not shown).

The effects of exogenous IL-2 on the in vivo generation and maintenance of CD8⁺ memory T cells

We have observed in this study that the size of the CD8⁺ T cell memory population is increased if 2C lymphocytes are activated in vitro in the presence of IL-2 and then maintained in vivo in its absence (Fig. 1). However, when 2C lymphocytes were activated and maintained in vivo in the absence of IL-2, the size of the resulting CD8⁺ T cell memory population was similar to that observed if IL-2 was present (Fig. 2). These findings suggest that IL-2 has divergent effects on the generation and maintenance of memory T cells. To study the effect of IL-2 on memory generation, IL-2⁻/⁻ 2C lymphocytes were activated in vivo in the presence or absence of exogenous IL-2 and were then maintained in a second Rag1⁻⁻/⁻ host without further IL-2 administration. To study the effect of IL-2 on memory maintenance, IL-2⁻/⁻ 2C lymphocytes were activated in vivo in the absence of exogenous IL-2 and were then maintained in a second Rag1⁻⁻/⁻ host that received twice weekly injections of either PBS or recombinant IL-2. In both experiments, the number of CD8⁺ 1B²⁺ was quantitated 10 wk after lymphocyte activation. As shown in Fig. 3A, administering IL-2 to mice at the time of IL-2⁻/⁻ 2C lymphocyte activation resulted in a significantly larger number of long-lived CD8⁺ 1B²⁺ (38% increase over mice injected with PBS), indicating that IL-2 potentiates the generation of CD8⁺ T cell memory. In contrast, repeated administration of IL-2 to mice harboring previously activated IL-2⁻/⁻ 2C lymphocytes resulted in a significantly smaller number of long-lived CD8⁺ 1B²⁺ cells (30% decrease compared with mice injected with PBS) (Fig. 3B), suggesting that IL-2 interferes with the maintenance of CD8⁺ T cell memory. Unlike its effects on memory T cells, IL-2 administration did not alter the number of naive CD8⁺ 1B²⁺ T cells (Fig. 3, A and B).

The T cell memory pool is maintained by the continuous but slow division of memory cells (8, 9, 14). Therefore, we asked whether IL-2 interferes with memory maintenance by inhibiting the proliferation of CD8⁺ memory T cells. To answer this question, BrdU was added for 7 days to the drinking water of Rag1⁻⁻/⁻ mice harboring either IL-2⁺/⁺ or IL-2⁻/⁻ 2C memory lymphocytes, and BrdU uptake by Ag-specific (CD8⁺ 1B²⁺) cells was quantitated by flow analysis. As shown in Fig. 3C, the percentage of BrdU⁺ cells was 2-fold higher in IL-2⁻/⁻ than IL-2⁺/⁺ memory cells. Importantly, administering recombinant IL-2 to mice harboring IL-2⁻/⁻ 2C memory lymphocytes reduced BrdU uptake significantly (Fig. 3C). These data indicate that IL-2 interferes with memory maintenance by decreasing the proliferation rate of the CD8⁺ memory T cell population.

Discussion

The goal of this study was to investigate the role of IL-2 in the generation and maintenance of CD8⁺ memory T cells. To do so, we followed the fate of activated, Ag-specific, IL-2⁺/⁺ and IL-2⁻/⁻ TCR-tg CD8⁺ T cells (2C) in a lymphocyte-deficient host. We found that long-lived populations of previously activated 2C lymphocytes, which have phenotypic and functional characteristics of memory cells, can be efficiently generated and maintained in the absence of IL-2. However, IL-2 increased the size of the CD8⁺ memory pool if present at the time of initial T cell activation but...
The dual role of IL-2 in the generation and maintenance of CD8⁺ T cell memory is consistent with its divergent effects on naive/primary activated T cells and repeatedly activated T cells. IL-2 promotes the survival of naïve T cells and enhances their clonal expansion upon primary antigenic stimulation (22, 23). In contrast, IL-2 promotes the Ag-induced apoptosis of repeatedly activated T cells and thus contributes to the clonal contraction of effector lymphocytes (24–28). Our finding that IL-2 enhances memory generation if present during initial T cell activation is consistent with the hypothesis that the size of the memory pool is directly proportional to the magnitude of T cell clonal expansion during the primary immune response (1). However, we identified in this study an inhibitory effect of IL-2 on the proliferation of CD8⁺ memory T cells which leads to a smaller memory pool. Although it is possible that IL-2 decreases BrdU uptake by memory T cells by promoting activation-induced cell death (AICD), this is an unlikely event because memory T cells are thought to be resistant to AICD (2). Furthermore, our finding that IL-2 suppresses rather than stimulates memory T cell proliferation is consistent with a recent report showing that in vivo inhibition of IL-2 increases the cycling of memory-phenotype CD8⁺ T cells in mice (29). Therefore, the results suggest that IL-2 could serve as a vaccine adjuvant if used at the time of primary antigenic stimulation but will lead to a paradoxical decline in the memory population if used during the posteffector phase of the immune response. Conversely, when designing immunosuppressive or tolerance-inducing strategies, one should take into account the possibility that long-term IL-2 blockade could amplify the pool of unwanted allospecific memory T cells. It is important to note, however, that the net effect of endogenous IL-2 on the size of the CD8⁺ memory pool in an immunized animal is negligible (Fig. 2), possibly because the enhancing effect of IL-2 on memory generation is offset by its inhibitory effect on memory maintenance.

Our demonstration that IL-2 is not required for the maintenance of CD8⁺ T cell memory contradicts the results of Ke et al. (18), who observed that the number of CD8⁺ memory T cells declines significantly in the absence of IL-2. Unlike our study, Ke et al. employed an in vivo model based on the adoptive transfer of an IL-2-dependent CD8⁺ T cell line. The requirements for survival and proliferation of IL-2-dependent T cell lines differ from those of primary T cells because prolonged in vitro culture with IL-2 may modulate the expression of cytokine receptors and survival genes in T lymphocytes. Saparov et al. (19), in contrast, observed that IL-2 expression during primary T cell activation correlates with the magnitude of the secondary immune response elicited 1 wk after primary activation, leading the authors to suggest that IL-2 promotes effector/memory T cell generation. Our findings extend this observation by demonstrating that IL-2 enhances CD8⁺ memory T cell generation in an established model of immunologic memory. However, our results identify a paradoxical effect of IL-2, specifically that it interferes with the maintenance of long-lived CD8⁺ memory T cells.

Cytokines other than IL-2 may be critical for generating and sustaining T cell memory. Exogenous IL-15 is a potent and selective stimulator of memory-phenotype CD8⁺ T cells in vivo (29, 30). Moreover, memory-phenotype CD8⁺ T cells are selectively reduced in number in IL-15Rα or IL-15 gene-knockout mice (31, 32). Unlike IL-2, IL-15 is ubiquitously expressed by stromal cells in multiple tissues making it a likely cytokine to maintain the

![Graphs showing the effect of IL-2 on CD8⁺ T cell memory](Image)

**FIGURE 3.** IL-2 modulates the generation and maintenance of CD8⁺ memory T cells. A, Rag1⁻/⁻ mice harboring IL-2⁻/⁻ 2C lymphocytes received either recombinant murine IL-2 (IL-2⁻/⁻ (+IL-2)) or PBS (IL-2⁻/⁻) during in vivo T cell activation. Four days later, lymph node cells were adoptively transferred to a second Rag1⁻/⁻ host and CD8⁺1B2⁺ cells were quantitated by flow analysis at 10 wk (mean ± SD, n = 3). The effect of exogenous IL-2 on mice harboring naïve IL-2⁻/⁻ 2C lymphocytes is shown for comparison. *, p < 0.05. B, Rag1⁻/⁻ mice harboring previously activated IL-2⁻/⁻ 2C lymphocytes were given either recombinant murine IL-2 or PBS twice weekly. Ten weeks later, CD8⁺1B2⁺ cells were quantitated by flow analysis (mean ± SD, n = 3). The effect of exogenous IL-2 on mice harboring naïve IL-2⁻/⁻ 2C lymphocytes is shown for comparison. *, p < 0.05. C, Ten weeks after in vivo T cell activation, BrdU was added to the drinking water of Rag1⁻/⁻ mice harboring either IL-2⁻/⁻ or IL-2⁻/⁻ 2C memory lymphocytes. Seven days later, BrdU uptake was measured by flow analysis after gating on the CD8⁺1B2⁺ population. A separate group of Rag1⁻/⁻ mice harboring IL-2⁻/⁻ 2C memory lymphocytes was injected with recombinant murine IL-2 during the BrdU labeling period (IL-2⁻/⁻ (+IL-2)). Histograms shown are representative of three experiments.

Reduced size of the pool if present during memory maintenance by inhibiting the proliferation of CD8⁺ memory T cells.

We employed in this study two adoptive transfer models to investigate CD8⁺ T cell memory. The first model was designed to examine the role of IL-2 in memory maintenance; therefore, IL-2⁻/⁻ or IL-2⁻/⁻ 2C lymphocytes were activated in vitro in the presence of excess IL-2 and then transferred to Rag1⁻/⁻ hosts to follow their long-term survival. Rag1⁻/⁻ mice do not produce IL-2 (confirmed by RT-PCR in our study) because they lack lymphocytes. Therefore, the only potential source of IL-2 in these mice is the adoptively transferred lymphocyte population. The second model was designed to examine the role of IL-2 in memory generation; therefore, naïve IL-2⁻/⁻ or IL-2⁻/⁻ 2C lymphocytes were transferred to Rag1⁻/⁻ hosts, activated in vivo, and their survival followed over an extended period of time. We confirmed in our experiments that long-lived, previously activated 2C lymphocytes are CD8⁺ memory T cells by demonstrating that they have a significant survival advantage over adoptively transferred naïve lymphocytes, are CD44⁺CD62L⁻, and exhibit a rapid CTL response upon antigenic restimulation. The adoptive transfer of TCR-tg lymphocytes to lymphocyte-deficient hosts is an established approach to studying CD4⁺ and CD8⁺ T cell memory in vivo (2, 3) as it allows for the quantitation and analysis of Ag-specific T cells in a manner which is not possible in non-TCR-tg systems.
memory T cell pool in the absence of ongoing inflammation or immunity (31).

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References