IL-2 Induces a Competitive Survival Advantage in T Lymphocytes

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IL-2 Induces a Competitive Survival Advantage in T Lymphocytes

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The acquisition of long-term survival potential by activated T lymphocytes is essential to ensure the successful development of a memory population in the competitive environment of the lymphoid system. The factors that grant competitiveness for survival to primed T cells are poorly defined. We examined the role of IL-2 signals during priming of CD4+ T cells in the induction of a long-lasting survival program. We show that Ag-induced cycling of CD4+ IL-2−/− T cells is independent of IL-2 in vitro. However, IL-2−/− T cells failed to accumulate in large numbers and develop in effector cells when primed in the absence of IL-2. More importantly, Ag-activated IL-2−/− T cells were unable to survive for prolonged periods of time after adoptive transfer in unmanipulated, syngeneic mice. IL-2−/− T cells exposed to IL-2 signals during priming, however, acquired a robust and long-lasting survival advantage over cells that cycled in the absence of IL-2. Interestingly, this IL-2-induced survival program was required for long-term persistence of primed IL-2−/− T cells in an intact lymphoid compartment, but was unnecessary in a lymphopenic environment. Therefore, IL-2 enhances competitiveness for survival in CD4+ T cells, thereby facilitating the development of a memory population. The Journal of Immunology, 2004, 172: 5973–5979.

The development of an adaptive immune response requires that Ag-stimulated lymphocytes survive and expand in the presence of a large number of unstimulated (“by-stander”) cells. Some of the progeny of the expanded population then differentiate into effector cells. The prototypic growth factor for T lymphocytes is thought to be IL-2 (1). A large number of studies have shown that IL-2 promotes T cell survival, proliferation, and differentiation into effector cells (2–6). The growth-promoting action of this cytokine is the basis for its therapeutic use to boost immune responses, e.g., in patients with AIDS and in cancer-bearing individuals. However, IL-2 also functions to limit immune responses by stimulating the development and functions of regulatory T cells (7–10) and by promoting Fas-mediated apoptotic responses by stimulating the development and functions of regulatory T cells (7–10) and by promoting Fas-mediated apoptotic death of CD4+ T cells (11–13). Further unraveling the complex physiologic activities of IL-2 on T cells may lead to enhanced insight about how to manipulate these activities for successful therapeutic application of the cytokine.

Homeostasis in the immune system implies that at steady state lymphocytes live at an equilibrium of new cell generation, proliferative expansion, and death (14). Fundamental to the maintenance of homeostasis is the competition between lymphocytes for activating ligands (presumably self-peptide/self-MHC complexes for T cells) and growth and survival factors (particularly IL-7) (15–20). If an Ag-stimulated lymphocyte population is to expand in a “full” lymphoid compartment, the Ag-stimulated cells must have a competitive survival and growth advantage over unstimulated cells. In the absence of competition, lymphocytes undergo rapid proliferation, for instance when the cells are transferred into lymphocyte-depleted recipients (21–23). However, the factors that confer a competitive advantage to activated lymphocytes are essentially unknown. One idea is that the strength of TCR signaling determines the subsequent “fitness” for survival of activated T cells, at least in the short-term, presumably by enhancing survival molecules and cytokine responsiveness (24).

We hypothesized that IL-2 signals during priming were required for the acquisition of long-term survival potential by activated CD4+ T cells. Therefore, we stimulated CD4+ T cells in the absence and presence of IL-2 and followed their subsequent survival over the course of several weeks in vivo. We demonstrated that an obligatory and nonredundant function of IL-2 during priming is to imprint in T cells a long-lived competitive survival advantage, while IL-2 is not required for T cell cycling. We discuss the likely mechanisms and physiologic implications of the survival-promoting action of IL-2.

Materials and Methods

Mice

Male BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, MA) and Rag2−/− BALB/c mice from Taconic Farms (Germantown, NY). Age-matched animals were used at 6–8 wk of age as recipients for adoptive transfer experiments. DO.11.10 transgenic mice, which express a TCR that recognizes the hen egg albumin peptide OVA252–263 in the context of the MHC class II molecule I-Aβ, were provided by Dr. K. Murphy (Washington University, St. Louis, MO). DO.11.10 × IL-2−/− mice were generated in our laboratory by crossing DO.11.10 mice with IL-2−/− mice backcrossed for >10 generations onto the BALB/c background (The Jackson Laboratory, Bar Harbor, ME). All mice were bred and maintained in accordance with the guidelines of the Laboratory Animal Resource Center of the University of California San Francisco. The mutant mouse strains were typed as previously described (13).

T cell purification and in vitro priming

CD4+ T cells were prepared from the lymph nodes and spleens of wild-type and IL-2−/−. DO.11.10 mice using CD4+ Dynabeads (Dynal, Oslo, Norway). Briefly, 2.5–5 × 106 CD4+ T cells were primed with 2.5 × 106 mitomycin C-treated BALB/c splenocytes and 1 μg/ml OVA253–263 in 24-well plates for 4 days in RPMI 1640 supplemented with 1 mM L-glutamine.
penicillin, streptomycin, nonessential amino acids, sodium pyruvate, HEPES (all from Life Technologies, Grand Island, NY), 5 × 10⁻⁵ M 2-ME, and 10% FBS (Sigma-Aldrich, St. Louis, MO). In some experiments, the indicated concentrations of murine IL-2 (R&D Systems, Minneapolis, MN) were added to the cultures.

**CFSE labeling and flow cytometry**

To follow cell division during priming, CD4⁺ T cells were labeled with 1 µM CFSE (Molecular Probes, Eugene, OR) for 12 min at 37°C in serum-free medium. On days 2, 3, and 4, cultures were harvested, FcRs were fixed and permeabilized using the Cytofix/Cytoperm solution according to the manufacturer’s instructions (BD PharMingen). Intracellular staining for Bcl-2 staining, 2 × 10⁵ cells were surface stained with KJ1-26/PE and anti-CD4/CyC as described above and fixed and permeabilized using the Cytofix/Cytoperm solution according to the manufacturer’s instructions (BD PharMingen). Intracellular staining for Bcl-2 was done with a FITC-conjugated hamster anti-mouse Bcl-2 Ab (clone 3F11) or a FITC-conjugated isotype control hamster IgG Ab (BD PharMingen) diluted in Perm/Wash buffer.

**Proliferation and cytokine assays**

For proliferation assays, 2.5 × 10⁶ naïve or primed CD4⁺ T cells were cultured with 2.5 × 10⁵ mitomycin C-treated BALB/c splenocytes and serial dilutions of OVA233-241 in 96-well plates for 2 or 3 days and pulsed for the last 8 h with 1 µCi [³H]thymidine (NEN, Boston, MA). Incorporation of [³H]thymidine was measured by liquid scintillation in a Trilux scintillation counter (Wallac, San Francisco, CA). Results are shown as mean of triplicate cultures ± SD. For cytokine assays, identical 96-well plates were set up and supernatants were collected at the indicated time points. IL-4 and IFN-γ concentrations from duplicate wells were determined by ELISA according to the manufacturer’s instructions (BD PharMingen).

Adoptive transfers and assay for T cell survival

Primed T cells were harvested on day 4 and dead cells removed by centrifugation over a Lympholyte-M density gradient (Cedarlane Laboratories, Hornby, Ontario, Canada) for 20 min at 2000 rpm. The percentage of T cells expressing the DO.11 TCR was measured by staining with the clonotypic Ab KJ1-26 and flow cytometry and was routinely 85–95%. Equal numbers of viable KJ1-26⁺ CD4⁺ T cells were adoptively transferred into unmanipulated BALB/c or Rag-2⁻⁻ BALB/c mice by tail vein injection. The presence of surviving DO.11 T cells in recipients of activated DO.11 cells was assayed at the indicated time points after adoptive transfer. Peripheral lymph nodes (submandibular, axillary, brachial, inguinal, and popliteal) and spleen were harvested, and cell suspensions were blocked with anti-CD16/CD32 and stained with PE-conjugated KJ1-26 TCR clonotypic Ab and FITC- or CyC-conjugated anti-CD4 mAb (BD PharMingen). The percentage of KJ1-26⁺ CD4⁺ T cells was determined by flow cytometry. Absolute numbers of DO.11 T cells in lymph nodes and spleen were calculated from this percentage and duplicate counts of total cell numbers in a hemocytometer using trypan blue dye exclusion. The phenotype of KJ1-26⁺ CD4⁺ T cells was evaluated by staining with FITC-labeled Abs against CD25, CD44, and CD62L (BD PharMingen).

**Results**

**Role of IL-2 in proliferation, survival and differentiation of primed T cells**

In our initial experiments, we observed that IL-2⁻⁻⁻ T cells expressing the DO.11 TCR showed less accumulation at 1–2 wk after priming in vivo than did wild-type DO.11 cells (data not shown). It was, however, difficult to establish whether this effect of IL-2 was during the initial T cell response to Ag or during subsequent survival of primed cells. It has been suggested that autocrine IL-2 is not required for the initial Ag-driven expansion of CD4⁺ T cells in vivo (25). To assess the requirement of IL-2 for initial expansion vs long-term survival of primed T cells in vivo, we used a model where wild-type and IL-2⁻⁻⁻ DO.11 cells were primed in vitro and subsequently adoptively transferred to intact, Ag-free BALB/c mice. The first set of experiments was designed to characterize the
role of IL-2 during T cell priming in vitro. Therefore, IL-2−/− DO.11 T cells were activated with OVA peptide (1 μg/ml) and APCs, without and with added IL-2. Syngeneic splenocytes from IL-2−/− mice were used as a source of APCs to avoid any contamination with the cytokine. Assays of [3H]thymidine incorporation and viable cell recoveries showed that both were greatly increased by the presence of IL-2 during priming (Fig. 1). However, when DO.11 cells were labeled with CFSE, activated, and assayed for cell division by dye dilution within the live population, there was essentially no difference between cells activated without and with IL-2 (Fig. 1C). A likely explanation for these results is that IL-2 is not required for the progression of Ag-stimulated T cells through the cell cycle, but it may be important for recruiting cells into the cycle and for survival and, therefore, accumulation of the cells. In all of these assays, the responses of wild-type DO.11 cells, producing autocrine IL-2, were indistinguishable from those of IL-2−/− cells stimulated with exogenous IL-2.

Next, we analyzed the phenotypic and functional characteristics of T cells that were primed in the presence or absence of IL-2. Fig. 2A shows that IL-2 was required for maximal up-regulation of CD25 (26), but had a minor effect on the regulation of the activation markers CD44 and CD62L (Fig. 2A). Confirming earlier reports, IL-2 was required for increased expression of the IL-7Rα chain (CD127) and Bcl-2 (27, 28), two molecules that have been implicated in T cell survival. Finally, to assess the acquisition of effector function by T cells primed in the absence of IL-2, activated IL-2−/− and wild-type DO.11 cells were harvested from the cultures on day 4, equal numbers were restimulated with Ag plus APCs in the presence of IL-2 and cytokine production was measured. When primed in the absence of IL-2, Ag-stimulated T cells showed little differentiation to IFN-γ or IL-4-producing effector cells (Fig. 2B). Differences in cytokine production could not be attributed to poor survival of the IL-2−/− T cells in restimulation cultures as all populations proliferated comparably in response to exogenous IL-2 (Fig. 2C). This role of IL-2 in the development of effector cells has been described previously (29). Thus, in vitro priming of DO.11 T cells in the absence of IL-2 signals results in the generation of a viable population of T cells that have cycled and acquired an activated phenotype, but are deficient in effector cytokine production.

**IL-2 during priming is required for long-term T cell survival in vivo**

In recent years, several activation parameters have been proposed to influence the longevity of primed T cells after removal of the antigenic stimulus. The strength of TCR signaling (24), the level of effector cell differentiation (30), and the number of cycles transited during priming (31) all appear to be determinants of subsequent survival in vivo. We asked whether IL-2 signals during priming were required for long-term T cell survival in vivo. Therefore, equal numbers of in vitro-activated DO.11 T cells, primed with or without IL-2, were adoptively transferred into normal syngeneic (BALB/c) recipients and the numbers of DO.11 T cells in lymphoid tissues were determined 6 wk later by staining with the clonotypic Ab KJ1-26. Fig. 3A shows that viable DO.11 cells were readily detected in lymphoid tissue if they had been primed with

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**FIGURE 2.** Phenotype and recall responses of primed IL-2−/− T cells. Wild-type (WT) and IL-2−/− DO.11 T cells were cultured with OVA peptide (1 μg/ml) and APCs, as in Fig. 1, and harvested on day 4. A, Primed cells (bold line) and freshly isolated naïve cells (thin line) were stained with KJ1-26, anti-CD4, and Abs against CD25, CD44, CD62L, and CD127 (IL-7Rα). Intracellular staining for bcl-2 (bold line) was performed on primed cells only, using an isotype control Ab (thin line). The histograms show expression of the respective markers, gating on CD4+ KJ1-26+ cells. Data are representative of three to five experiments. B, Wild-type DO.11 cells, or IL-2−/− DO.11 cells primed in the absence or presence of IL-2 were restimulated with peptide plus APCs and IL-2 (10 ng/ml). Culture supernatants harvested on day 2 were assayed for IFN-γ and IL-4 levels by ELISA. C, 2.5 × 10^6 viable recovered cells were restimulated with OVA peptide, APCs, and 10 ng/ml IL-2, and [3H]thymidine incorporation was assayed on day 2. Data represent two independent experiments.
IL-2, but not if they were primed without IL-2. The fact that IL-2−/− DO.11 cells primed with IL-2 for 4 days survived as well as wild-type cells demonstrated also that long-term maintenance of previously activated T cells in vivo was not due to continued autocrine IL-2 production. When the recovered cells were restimulated with Ag, with or without IL-2, the long-lived IL-2−/− T cells remained IL-2 dependent (Fig. 3B). As we have previously shown (32), “memory” CD4+ T cells show increased [3H]thymidine incorporation in response to IL-2 alone, even without Ag. Thus, temporary exposure to IL-2 during priming promotes prolonged survival but does not convert the cells to an IL-2-independent state. This ex vivo restimulation assay cannot be done with IL-2−/− T cells primed without the growth factor, because of poor survival in the transfer recipients.

The cells that survived for 6 wk in vivo showed low expression of CD25, high CD44, and biphasic CD62L (Fig. 3C). Thus, these cells have the phenotypic characteristics of memory T cells (33–35).

Kinetic analysis of T cell survival in vivo following adoptive transfer showed that the initial homing and survival were similar for wild-type and IL-2−/− T cells 2 days posttransfer. Wild-type T cells further accumulated in the first week but then slowly decreased and reached a steady state at 3 wk (Fig. 4A). The numbers of IL-2−/− T cells decreased sharply in the first week, and the cells were virtually undetectable by 2–3 wk. At 7 days posttransfer, even the few IL-2−/− T cells that had survived showed a phenotype consistent with that of memory T cells (Fig. 4B). Thus, T cells acquire the phenotypic characteristics of memory cells even when they are activated without IL-2, but the presence of this cytokine during priming is essential for the subsequent long-term survival of the cells.

Competitive survival advantage of activated T cells

Experiments in lymphopenic mice, where competition between lymphocytes for survival factors is limited, suggested that IL-2 and other common γ-chain cytokines are not required for memory CD4+ T cell homeostasis (36). To test the idea that IL-2 confers a competitive survival advantage on T cells, we transferred DO.11 cells primed with Ag in the absence or presence of IL-2 into normal or Rag−/− BALB/c mice and followed the kinetics of cell survival. As shown before, for the cells to survive in normal BALB/c recipients, they had to be primed in the presence of IL-2. In striking contrast, IL-2−/− cells primed without IL-2 survived and even increased in number in Rag−/− recipients to the same extent as wild-type cells (Fig. 5A). DO.11 cells recovered from the Rag−/− recipients all showed the phenotype of memory cells, regardless of whether or not IL-2 was present during priming (Fig. 5B). Thus, IL-2 is not required for primed T cells to survive and expand in the absence of competing lymphocytes. It is unclear whether the function of IL-2 in intact recipients is to restore or reinforce the same signal that is required for homeostasis in lymphopenic mice, or whether homeostasis in intact and lymphopenic mice is regulated by entirely different, i.e., IL-2-dependent vs IL-2-independent mechanisms. Taken together, our data show that IL-2−/− DO.11 cells primed without IL-2 are not doomed to die due to an intrinsic survival defect, but suggest that these cells are unable to receive mandatory survival signals in an intact immune system. Therefore, one of the major functions of IL-2 during priming might be to imprint a long-lasting competitive survival advantage in T lymphocytes, thereby facilitating the development of a memory population.

**FIGURE 3.** In vivo recovery of DO.11 T cells from Ag-primed wild-type (WT) and IL-2−/− DO.11 cells. A, Wild-type and IL-2−/− DO.11 cells were primed with Ag in vitro for 4 days as in Fig. 1B. 1.5 × 10⁵ (Expt. 1, △), or 2.5 × 10⁵ (Expt. 2, ■) viable cells from each culture were transferred into BALB/c mice. Lymph nodes and spleens were harvested 6 wk later, and cell suspensions were stained with KJ1-26 and anti-CD4 and analyzed by flow cytometry. Numbers of recovered DO.11 cells were calculated. Results are from individual mice from two representative experiments of four. B, Splenocytes (5 × 10⁷), containing 8 × 10⁶ DO.11 cells from recipients that received primed wild-type (WT) cells or IL-2−/− DO.11 cells primed in the absence of IL-2 were restimulated with OVA plus APCs in the absence or presence of rIL-2 (10 ng/ml). Incorporation of [3H]thymidine was measured on day 3 and results are shown as means of triplicate cultures ± SD. C, Spleen cells from recipients of primed wild-type DO.11 cells or IL-2−/− DO.11 cells primed in the presence of IL-2 were stained with KJ1-26, anti-CD4, and Abs against CD25, CD44, and CD62L and analyzed by flow cytometry. Dot plots show expression of the indicated markers gated on the CD4+ population. A representative example of four experiments is shown.
Discussion

Although it has been shown previously that IL-2 is required for the proliferation and differentiation of primed T cells in vitro (27, 29, 37), it is not known whether IL-2 signals contribute to the long-term survival of activated T cells in vivo. To answer this question, we primed wild-type and IL-2−/− DO.11 T cells in vitro and adoptively transferred the activated T cells into unmanipulated recipients. This experimental system can be used to clearly distinguish between the function of IL-2 during priming and the subsequent, Ag-free survival phase. Our in vitro experiments showed that IL-2 was not required for the cycling of individual cells in response to TCR stimulation (Fig. 1C). Conventional population assays that are commonly used to measure cell “proliferation,” such as the incorporation of [3H]thymidine or the recovery of viable cells, gave the expected result that IL-2 was essential for both. The discrepancy with the cell cycling results, as assayed by CFSE dye dilution, suggest that the population assays measure not only mitogenic activity in cells but also the number of viable cells at the end of the assay. Collectively, these results are consistent with the conclusion that IL-2 is essential for the survival of Ag-stimulated T cells but not for cell cycle transit and division. Whether IL-2 is an essential survival factor for CD4+ T cells in vivo is less clear. Studies using IL-2−/− and IL-2Rα−/− DO.11 T cells showed conflicting results on the role of IL-2 in initial T cell expansion in response to TCR stimulation (25, 38). Furthermore, IL-2−/− or IL-2Rα−/− mice accumulate activated, autoreactive T cells (39, 40), but show reduced viral responses (41). A recent study suggests that the timing of IL-2 exposure critically determines its effect on T cells: administration of exogenous IL-2 during the expansion vs contraction phase of an antiviral response limited or enhanced virus-specific CD4+ and CD8+ T cell numbers (42). Thus, it is likely that IL-2 plays both positive and negative roles in CD4+ T cell responses in vivo. Careful analysis of IL-2 effects on CD4+ T cells using in vivo priming systems is therefore complicated and further obscured by the interference of IL-2 function on other T cell populations, e.g., regulatory T cells, and the difficulty to control for effects of bystander, for instance DC-derived IL-2 (43).

The formal test for the ability of activated T cells to survive long-term is to follow them after transfer into unmanipulated, Ag-free recipients. These experiments showed that in order for activated T cells to survive in vivo, they have to be primed in the presence of IL-2. Importantly, even IL-2−/− T cells that are activated with IL-2 and survive in vivo remain dependent on IL-2 for in vitro recall responses. Therefore, the presence of IL-2 during priming does not permanently alter the properties of the T cells. Rather, the presence of the cytokine during priming “imprints” a survival program in the cells. Interestingly, this enhanced survival ability is most evident in the presence of normal lymphocytes, because activated T cells survive and expand in lymphocyte-deficient recipients regardless of the presence of IL-2 during priming (Fig. 5A). This implies that in a lymphopenic environment the requirements for survival of primed T cells are less stringent than in an intact lymphoid compartment. A likely explanation for this is that competition for specified niches or obligatory survival signals is limited in the absence of other lymphocytes. Several authors have described the phenomenon of “homeostatic proliferation” in lymphopenic animals (21–23). Naive and memory T cells transferred to animals that are depleted of lymphocytes genetically or by irradiation, spontaneously start to proliferate, presumably to restore the lymphoid compartment. Curiously, transferred naive T cells acquire a memory phenotype during homeostatic proliferation (44, 45). The accumulation of much higher numbers (2.5–3 × 10^6 in Rag−/− vs 7 × 10^5 in BALB/c, Fig. 5A) of memory phenotype cells we observed from both wild-type and IL-2−/− cells in lymphopenic animals after 6 wk indicates that these cells underwent homeostatic proliferation. Our results raise concerns regarding the validity of using lymphopenic recipients to study the survival of memory cells. Indeed, the apparently lowered threshold for survival in these animals might obscure the real requirements for memory cell survival signals in normal animals. It is striking in this respect that Lantz et al. (36) state that signals from IL-2 or other common γ-chain cytokines during priming are not required for memory cell survival. However, these studies were conducted in alymphoid hosts, thus supporting our data in Rag−/− recipients, but not excluding the need for IL-2 signals for survival in BALB/c mice.

One can envisage two main strategies by which primed T cells survive after removal of the Ag: the acquisition of a cell-autonomous resistance to passive apoptosis and the capacity to respond to external survival factors such as cytokines. Cell-intrinsic resistance
The observation that IL-2 to acquire cytokine responsiveness and effector function in vitro. In support of this, it has been shown that naive T cells require IL-2 into IFN-α-producing T cells after immunization (25) and the delayed development of an IgG response to vesicular stomatitis virus (VLV) mice (50) also indicate that IL-2 is required for the efficient generation of effector cells in vivo. Effector cell differentiation and memory cell generation appear to be linked phenomena (30). The previously described role of IL-2 in the differentiation of effector cells on the one hand and its herein proposed role in the induction of competitiveness for survival on the other hand suggests that IL-2 facilitates the development of a long-lived memory population. It is hereby also noteworthy that since the extent of cycling during priming was comparable for wild-type and IL-2−/− T cells (Fig. 1C), it appears likely that cell cycling alone cannot be the sole determinant of the magnitude of memory cell generation, contrary to what other authors have suggested (31, 45, 51).

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References


