

Interleukin 15 Promotes Antigen-independent *in Vitro* Expansion and Long-Term Survival of Antitumor Cytotoxic T Lymphocytes¹

Jun Lu, Robert L. Giuntoli, II, Ryusuke Omiya, Hiroya Kobayashi, Richard Kennedy, and Esteban Celis²

Departments of Immunology [J. L., R. O., H. K., E. C.] and Obstetrics and Gynecology [R. L. G.] and Mayo Graduate School [R. K., E. C.], Mayo Clinic, Rochester, Minnesota 55905

ABSTRACT

The survival and expansion of effector cytotoxic T lymphocytes (CTLs) during an immunological response are critical for the successful elimination of life-threatening attacks by microorganisms, parasites, or malignant cells. Among the numerous factors that regulate the immune response, interleukin (IL)-2, and its close relative, IL-15 are known to function as growth and survival factors for antigen-experienced T cells. However, major differences appear to exist between these lymphokines in their capacity to act on various T-cell types such as CD4+ *versus* CD8+ or effector *versus* memory T lymphocytes. Although several studies have been done in the mouse system, less information is available regarding the function of these lymphokines in the human system. Here, we report that IL-15 or high concentrations of IL-2 induced antigen-independent expansion of effector CD8+ CTLs. Neither IL-2 nor IL-15 induced the proliferation of CD4+ T cells. In the absence of antigen, at least one of these lymphokines was required for the long-term survival of the cells in tissue culture. Most significantly, the effector cytolytic activity of CTLs expanded and maintained in IL-15 for up to 60 days remained stable, indicating that these cells do not differentiate into a memory functional phenotype. The expression of IL-15R α , which was detected on CD8+ CTLs but not on CD4+ helper T cells, suggests that this receptor subunit somehow participates in the transduction of the mitogenic signals of IL-15. The present findings have practical implications for the propagation of antigen-specific T-cell lines *in vitro* and could be useful for expansion of therapeutic T cells for adoptive transfer.

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² To whom requests for reprints should be addressed, at Department of Immunology, GU421A, Mayo Clinic, Rochester, MN 55905. Phone: (507) 284-0124; Fax: (507) 266-5255; E-mail: celis.esteban@mayo.edu.

INTRODUCTION

The factors that influence the long-term survival and expansion of effector CTLs³ both *in vivo* and *in vitro* are not yet completely elucidated. It is well known that antigen-specific human CD8+ CTLs are difficult to maintain in tissue culture for long periods of time (>2–3 weeks) without the eventual loss of their viability or of their effector function (cytotoxicity). To avoid such problems, CTL cultures require periodic stimulation with either antigen or anti-TCR antibodies, which must be provided in combination with feeder cells that function as APCs. As a result of TCR stimulation, together with the appropriate lymphokines and costimulatory signals, CTLs can proliferate up to 1000-fold in a matter of 2 weeks and maintain specificity (1–3). After this growth spurge, the CTLs can be maintained in low concentrations of IL-2 (~50 IU/ml) for ~3–4 weeks without much additional increase in cell numbers but retaining their effector function.

Among the various factors that seem to control the expansion and viability of antigen-activated T lymphocytes, IL-2 and IL-15 are perhaps the most extensively studied. Although IL-2 and IL-15 share two of three receptor subunits on the T-lymphocyte surface, their biological function can be quite different depending on the activation state and the specific subset of T lymphocytes that is targeted by these lymphokines (4, 5). Although both of these lymphokines appear to function as T-cell growth and survival factors, in some circumstances IL-2 can also be involved in the down-regulation of T-cell responses mediated through mechanisms such as activation-induced cell death. On the other hand, IL-15 has been regarded by many as an antiapoptotic lymphokine that plays a critical role for the survival of memory CD8+ CTLs (6–8). Most significantly, in the presence of IL-15, tolerizing TCR stimuli result in a state of reversible T-cell anergy instead of apoptosis (9). In addition, IL-15 has also been reported to prevent *in vitro* apoptosis of T cells from patients after autologous progenitor cell transplantation (10). However, in HIV-infected individuals, IL-15 failed to function as an antiapoptotic factor in nave and memory CD8+ T cells, suggesting that the effects of this lymphokine may differ in some diseases affecting the immune system (11). In view of the above, we decided to evaluate the role of IL-15 in the maintenance of antigen-specific CTLs in culture and to determine whether these cells would retain their effector function or whether they would switch into a memory (nonlytic) phenotype,

³ The abbreviations used are: CTL, cytotoxic T lymphocyte; TCR, T-cell receptor; APC, antigen-presenting cell; DC, dendritic cell; IL, interleukin; IL-2R, IL-2 receptor; IL-15R, IL-15 receptor; PSMA, prostate-specific membrane antigen; CBMC, cord blood mononuclear cell; TIL, tumor-infiltrating lymphocyte.

as it has been reported to occur with mouse CTLs (12). The data presented herein demonstrate that human CD8⁺ CTLs expand in an antigen-independent fashion and retain their effector phenotype for up to 60 days, when IL-15 or high concentrations of IL-2 (1000 IU/ml) but not low concentrations of IL-2 (50 IU/ml) are included in the tissue culture medium. The effect of IL-15 on CD8⁺ T cells appears to be product of an increase in the rate of proliferation concomitant with a decrease in the numbers of cells undergoing apoptosis. In contrast, IL-15 and IL-2 (at either concentration) failed to stimulate the antigen-independent expansion of CD4⁺ T cells, indicating that signaling and survival pathways triggered by the IL-2R and IL-15R differ between these lymphocyte subsets.

MATERIALS AND METHODS

Cytokines and Peptides. Human IL-2 was purchased from Chiron Corporation (Emeryville, CA). Recombinant human IL-4, IL-7 and IL-15 were purchased from Endogen (Woburn, MA). Human IL-10 was provided by Schering Corporation (Kenilworth, NJ). All of the peptides: gp100₂₀₉ (ITDQVPFSV), PSMA₄₆₉ (LMYSLVHNL), gp100₇₄ (GPTLIGANASFSIALN), and gp100₅₇₆ (SLAVVSTQLIMPGQE) were synthesized according to standard solid-phase synthesis methods using an Applied Biosystems apparatus and were purified by high-performance liquid chromatography. The purity (95%) and identity of peptides were determined by analytical high-performance liquid chromatography and mass spectrometry analysis, respectively. Peptides were dissolved at 10 mg/ml in DMSO containing 0.1% trifluoroacetic acid and were aliquoted in small volumes that were maintained frozen at -20°C until further use.

Cell Culture and Assessment of Cell Expansion. Antigen-specific human CD4⁺ and CD8⁺ T-cell lines or clones were induced from healthy donors using peptide-pulsed autologous DCs as described previously (3, 13–15). The DCs used to generate the antigen-specific T-cell lines and clones were prepared from adherent monocytes that were incubated in tissue culture for 7 days in the presence of granulocyte macrophage colony-stimulating factor (50 ng/ml) and IL-4 (1000 IU/ml), without additional maturation (3). CTL clone X283 specifically recognized HLA-A2-restricted peptide gp100₂₀₉ (ITDQVPFSV) derived from melanoma gp100 antigen (16), and CTL clone PSMA1126.14 was specific for HLA-A2-restricted peptide PSMA₄₆₉ (LMYSLVHNL) derived from PSMA.⁴ T-helper clones 10B1 and 3F12 recognize peptide gp100₇₄ (GPTLIGANASFSIALN) and peptide gp100₅₇₆ (SLAVVSTQLIMPGQE), respectively, both in the context of HLA-DR7 (15). All of the human T cells were maintained in tissue culture using cytokine-supplemented complete RPMI medium (RPMI 1640 plus 10% fetal bovine serum, L-glutamine, nonessential amino acids, sodium pyruvate, and lentamicin). Fresh medium supplemented with cytokines was added every 2 or 3 days. For reactivation, the T cells were stimulated with 30 ng/ml human anti-CD3 (Orthoclone OKT3; Ortho Biotech, Inc., Rar-

tan, NJ) in the presence of irradiated feeder cells and sufficient amount of IL-2 for 2 weeks before additional use (3). Effector T cells derived from CBMCs were generated by stimulation with 1 µg/ml OKT3 for a week and then purifying the CD8⁺ T cells using antibody-coated magnetic beads (Miltenyi Biotec, Auburn, CA). A CTL clone specific for peptide gp100₂₀₉, derived from TILs from a melanoma patient was kindly provided by Dr. Franco Marincola (Surgery Branch, NCI, NIH). These cells were maintained in complete medium containing 1000 IU IL-2/ml. The T2 cell line (HLA-A2⁺) was kept in complete RPMI medium and used as targets for cytotoxicity assays. To determine survival and expansion of T cells, the cultures were initiated at 5×10^5 cells/ml in complete RPMI medium with various cytokines. Every 3–5 days, the numbers of viable cells were estimated using trypan blue exclusion, and the cells were resuspended at the original cell concentration in fresh media containing the corresponding cytokine.

Cytotoxicity Assays and Target Cell Lines. Effector function of CD8⁺ T cells was evaluated at several time points by measuring the cytolytic activity using a standard 4–6 h chromium release assay as described previously (3). Approximately, 1×10^6 peptide-pulsed T2 cells were labeled with 100 µCi ⁵¹Cr for 1.5 h and, after extensive washing, were used as targets in the cytotoxicity assays at various E:T ratios. Target cell lysis was determined by measuring the amount of radioactivity released by the lysed target cells into the supernatant using a scintillation counter. The percentage of specific lysis was calculated by the formula $[(\text{cpm of the test sample} - \text{cpm of spontaneous release}) / (\text{cpm of the maximal release} - \text{cpm of spontaneous release}) \times 100]$. The transporter associated with antigen processing-deficient, HLA-A2⁺, T2 human cell line (17) was used as peptide-pulsed targets for the HLA-A2-restricted CTLs. The HLA-A2⁺, gp100⁺ human melanoma, and 624mel was provided by Dr. Steven A. Rosenberg (Surgery Branch, NCI, NIH). Both of these cell lines were maintained complete RPMI medium.

Cell Proliferation Assays. The function of CD4⁺ T cells was assessed by the capacity of these cells to respond to antigen by the incorporation of [³H]thymidine into DNA, which correlates with cell proliferation, as described previously (14, 15). Briefly, T cells (2×10^4 /well) were mixed with irradiated autologous peripheral blood mononuclear cell (1×10^5 /well) in the presence (and absence) of 3 µg/ml of the corresponding peptide in 96-well culture plates. The cultures were incubated at 37°C in a 5% CO₂ incubator for 72 h and during the last 16 h, each well was pulsed with 0.5 µCi/well of [³H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a liquid scintillation counter (TopCount; Packard Instruments) after harvesting the cell cultures onto glass fiber filters.

Antibodies and Flow Cytometry. Monoclonal antibody M161, specific for the human IL-15Rα chain, was originally developed by Immunex Corp. (Seattle, WA) and was kindly provided by Genmab A/S (Copenhagen, Denmark). The perforin antibody reagent set, phycoerythrin-conjugated anti-CD25 (IL-2Rα), CD122 (IL-2Rβ), CD132 (IL-2Rγ), and isotype-matched IgG controls were all purchased from BD PharMingen (San Diego, CA). Expression of IL-2R and IL-15R chains on T cells was determined by

⁴ E. Celis, unpublished results.

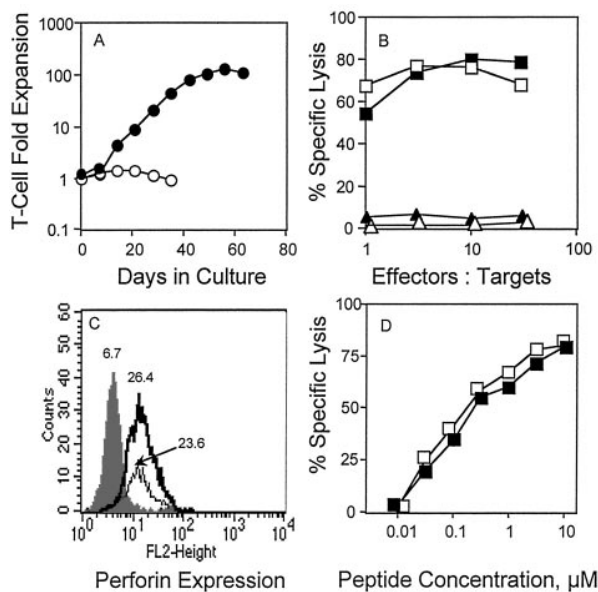


Fig. 1 IL-15 but not IL-2 induces the proliferation of human CTLs in the absence of antigen. **A**, an HLA-A2-restricted, PSMA-specific CD8+ CTL clone was cultured in either 50 IU/ml IL-2 (○) or 10 ng/ml IL-15 (●), and numbers of viable cells were determined at various time points. **B**, antigen-mediated cytotoxicity of CTLs maintained in IL-15 against PSMA₄₆₉ peptide-pulsed T2 cells was determined on day 0 (□) and on day 60 (■). No significant cytotoxicity was observed against T2 target cells pulsed with a control irrelevant peptide (day 0: △; day 60: ▲). **C**, intracellular perforin content of CTLs grown in IL-15 was measured by flow cytometry with a perforin-specific antibody on day 0 (thick line) and day 60 (thin line). The shaded area represents staining using an antibody isotype control, and the numbers above each curve correspond to the mean fluorescence intensity. **D**, peptide titration curve of CTLs that were incubated for 0 days (□) or 30 days (■) in 10 ng/ml IL-15. Targets were ⁵¹Cr-labeled T2 cells that were allowed to incubate with various concentrations of peptide PSMA₄₆₉ for 1 h before adding CTLs at a 1:1 E:T ratio.

fluorescent antibody staining and flow cytometry using a FACScan machine and CellQuest software (Becton Dickinson, Biosciences, San Jose, CA). Cell cycle analysis was performed by staining permeabilized T cells with propidium iodide (PharMingen) and subsequently running the samples in the FACScan machine and analyzing the results with the ModFit LT v2.0 software (Verity Software House, Topsham, ME).

RESULTS

For our initial studies, we first selected an HLA-A2-restricted clone reactive with a peptide derived from PSMA, which was activated in tissue culture using anti-CD3 antibodies, irradiated peripheral blood mononuclear cell, and an optimal concentration of IL-2 (6 ng/ml or 50 IU/ml). Two weeks later, after a ~700-fold expansion, the cells were harvested, washed, resuspended in fresh media containing either IL-2 or IL-15, and the cell numbers were monitored 3–4 times/week. As routinely observed in our laboratory, cells resuspended in IL-2 did not expand any further but were able to maintain their viability for ~2 weeks (Fig. 1A). Interestingly, the CTLs resuspended in IL-15 (10 ng/ml) continued to expand at an accelerated rate for

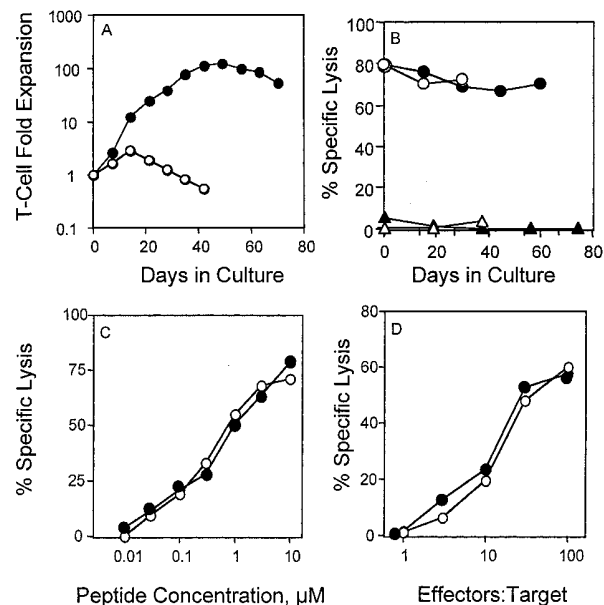


Fig. 2 Assessment of the antigen-independent proliferative effects of IL-15 on a gp100-specific CD8+ CTL clone. **A**, CTLs were grown in medium with IL-2 (○) or IL-15 (●), and cell numbers were monitored at various time points. **B**, cytotoxicity activity of CTLs maintained in IL-2 (○) or IL-15 (●) against peptide gp100₂₀₉-pulsed T2 target cells was measured at the indicated time points at E:T ratio of 10:1. No significant cytotoxicity was observed toward targets pulsed with a control peptide (IL-2, △; IL-15, ▲). **C**, peptide titration curve of CTLs that were incubated for 0 days (○) or 30 days (●) with 10 ng/ml IL-15. Targets were ⁵¹Cr-labeled T2 cells that were allowed to incubate with various concentrations of peptide gp100₂₀₉ for 1 h before adding CTLs at a 1:1 E:T ratio. **D**, same CTLs as in **C** tested for cytotoxicity against the HLA-A2+, gp100 + and 624mel (same symbols as in **C**). These CTLs failed to kill an HLA-A2+, gp100– melanoma (data not shown).

60 days, increasing ~120-fold in cell number (Fig. 1A). In separate experiments, we observed that CTL cultures in IL-4, IL-7, or IL-10 (10 ng/ml each) or in the absence of lymphokines gradually decreased in cell numbers and failed to maintain their viability (data not presented). Most importantly, the cytolytic activity of the CTLs did not decrease after 60 days in culture with IL-15 (Fig. 1B), and the levels of intracellular perforin remained constant (Fig. 1C), indicating that the effector phenotype of these cells was stable. Moreover, the quality of the cytolytic activity of these CTLs, as assessed by peptide titration curves, remained constant after 60 days in IL-15 (Fig. 1D). The functional characterization of the CTLs maintained in IL-2 could not be assessed at day 60 because by that time these cells had all died, but these cells retain their effector function during the first 2 weeks (data not shown).

Very similar results were obtained using another CTL clone, this one specific for an HLA-A2-restricted epitope from the melanoma gp100 antigen (Fig. 2). Again, the CTLs maintained in IL-15 expanded ~100-fold, whereas the cells in IL-2 had a limited proliferative potential (Fig. 2A). The cytolytic activity of the cells grown in either IL-2 or IL-15 was preserved while the cells retained viability (Fig. 2B) and the expression of intracellular perforin remained high after 60 days in culture in

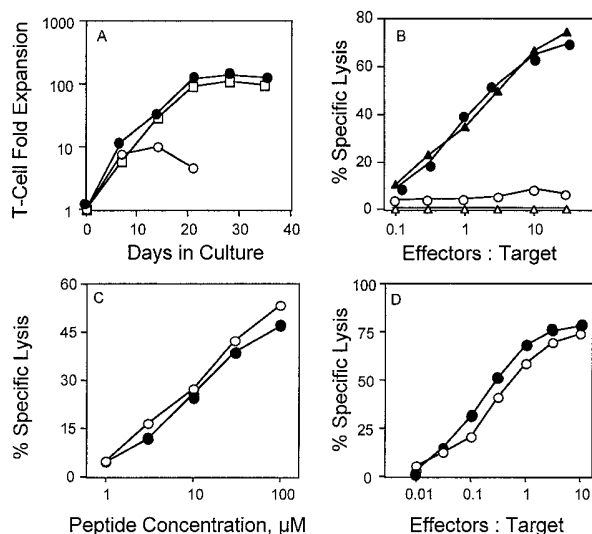


Fig. 3 A gp100-specific, HLA-A2-restricted CTL clone isolated from a melanoma patient's TIL proliferates to IL-15 or high concentrations of IL-2 in an antigen-independent way. **A**, patient CTLs were resuspended in medium containing 50 IU/ml IL-2 (○), 1000 IU/ml IL-2 (□), or 10 ng/ml IL-15 (●), and cell numbers were estimated as described in the legend of Fig. 1. **B**, cytotoxicity of patient's CTLs maintained in 1000 IU/ml IL-2 (circles) or IL-15 (triangles) was measured on day 15 against T2 cells pulsed with peptide gp100₂₀₉ (filled symbols) or control peptide (open symbols). **C**, peptide titration curve of CTLs that were incubated for 0 days (○) or 30 days (●) with 10 ng/ml IL-15. Targets were ⁵¹Cr-labeled T2 cells that were allowed to incubate with various concentrations of peptide gp100₂₀₉ for 1 h before adding CTLs at a 1:1 E:T ratio. **D**, same CTLs as in **C** tested for cytotoxicity against the HLA-A2+, gp100 + 624mel (same symbols as in **C**). These CTLs failed to kill an HLA-A2+, gp100- melanoma (data not shown).

IL-15 (data not shown). Most significantly, the quality of the cytolytic activity of these CTLs did not decrease after 30 days in culture in IL-15, as determined by the capacity of the CTLs to kill melanoma tumor cells (Fig. 2D) and in peptide titration curves (Fig. 2C).

In both examples presented above, it became evident that CTL cultures in IL-15 did not have the ability to expand endlessly because in all cases the cells ceased to proliferate ~40 days after the initiation of the experiments (Figs. 1A and 2A). From that time on, the cultures maintained constant cell numbers for ~20 additional days and then the cell numbers slowly decreased until the viability was lost (~day 80). However, when the CTLs that had reached stationary growth in IL-15 (~60 days) were stimulated with anti-CD3 antibodies, APC and IL-2 (50 units/ml), they expanded ~300–500-fold during a period of 2 weeks, and if placed in IL-15 after this time period, they expanded an additional 100-fold in a matter of 30 days (data not presented). Throughout this time, the CTLs continued to maintain their cytolytic function (data not shown).

The results presented above were obtained using antigen-specific CTLs that were generated *in vitro* from normal individuals using peptide-pulsed DCs (3). To determine whether these observations would also be applicable to CTL derived from cancer patients, we compared the capacity of IL-2 and IL-15 to expand a CTL clone derived from melanoma TILs. In

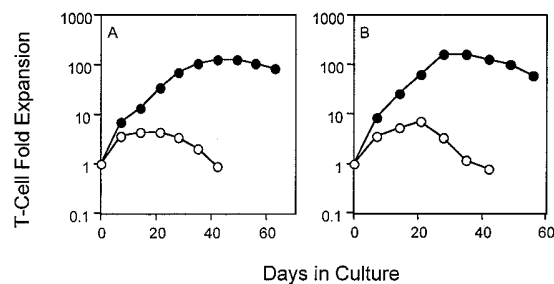


Fig. 4 Expansion and survival of CD8+ T cells obtained from CBMCs in the presence of IL-2 or IL-15. CBMCs from two different cord blood donors (**A** and **B**) were activated with 1 µg/ml anti-CD3 for 1 week, and subsequently, the CD8+ T cells were purified using anti-CD8 antibody-coated magnetic beads. The cells were then transferred into fresh media containing either 50 IU/ml IL-2 (○) or 10 ng/ml IL-15 (●) and monitored for cell growth.

these experiments, we also included a high-concentration IL-2 condition because TILs have been reported to require high concentrations of this lymphokine to grow *in vitro* (18, 19). The results observed with the melanoma patient-derived CTLs were very similar to those obtained with the peptide-pulsed DC-generated CTLs. The data presented in Fig. 3A indicate that IL-15 or the high concentration of IL-2 (120 ng/ml or 1000 units/ml) was equally effective in stimulating the melanoma patient's CTLs to proliferate *in vitro* up to 100-fold in the absence of antigen, and the cell viability was maintained constant for at least 35 days. On the other hand, a low-dose IL-2 (50 units/ml) induced a much lower degree of proliferation (~10-fold), and the cell viability declined from day 15 onwards. Most significantly, the cytotoxic activity of the TIL-derived CTLs that were maintained in either IL-15 or high-dose IL-2 for 15 days remained high (Fig. 3B). Moreover, after 30 days in culture in IL-15, the TIL continued to recognize peptide and tumor cells in a high efficient manner (Fig. 3, C and D).

Of note, in all of the above experiments, the CD8+ CTLs were all able to expand ~120–100-fold in a period of 40–50 days and then proliferation appeared to stop. Because the CTLs used in these experiments had all been continuously propagated in cell culture for 3–6 months, the possibility existed that the cells ceased to expand because they had reached a senescence state. However, as mentioned above, TCR stimulation was able to reinitiate the proliferation of CTLs that had reached stationary growth in IL-15. To determine whether CD8+ T cells with a short proliferation history would be able to expand in IL-15 for longer periods of time than long-term cultured CTLs, we evaluated the capacity of CD8+ T cells obtained from CBMCs to proliferate and survive in IL-15 and IL-2. The growth curves presented in Fig. 4, using purified short-term TCR-activated CD8+ T cells that were isolated from CBMC of two separate donors, show very similar patterns to those obtained using long-term cultured CTLs. Once more, the cells maintained in IL-15 expanded close to 120-fold in a matter of 40 days, whereas the cells in IL-2 had a more limited expansion potential. The small, but significant (~10-fold) expansion of these cells in IL-2, could be because these cells remained in a highly activated state because they had been stimulated with anti-CD3 antibody

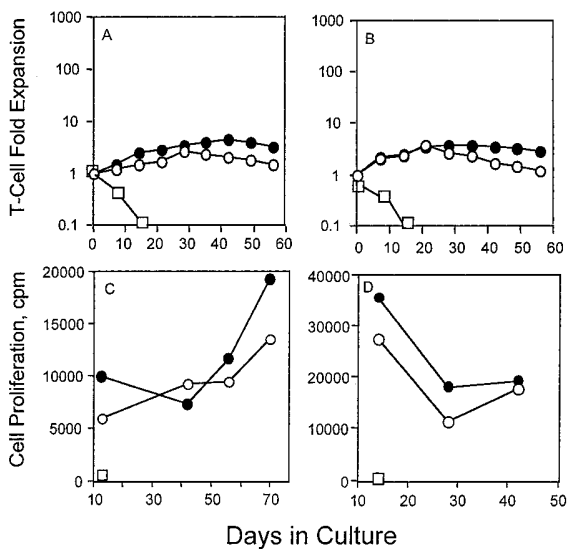


Fig. 5 Human CD4+ T lymphocytes do not proliferate but are able to survive in the presence of IL-15 or IL-2. Two human CD4+ T-helper clones specific for peptides gp100₇₄ (A and C) or gp100₅₇₆ (B and D) were monitored for their expansion and viability (A and B) or their proliferative response to 1 μ g/ml peptide presented by autologous APC (C and D) while being cultured in media with 50 IU/ml IL-2 (○), 10 ng/ml IL-15 (●), or no lymphokine (□). Proliferation in the absence of peptide (but in the presence of APC) was in all cases < 500 cpm (data not shown). Proliferation assays of cells grown without lymphokines could not be done past day 15 because cells did not survive.

only 7 days before the initiation of the experiment. These results suggest that the cell expansion potential after TCR stimulation of CTLs incubated in IL-15 appears to be independent of the proliferative past of the cell cultures.

Next, we compared the capacity of IL-15 and IL-2 to mediate the antigen-independent *in vitro* expansion and survival of CD4+ T lymphocytes. For these experiments we selected two different antigen-specific MHC class II restricted helper T cell clones which were previously stimulated for 2 weeks using anti-CD3 antibodies APC and IL-2. The cells were then resuspended in fresh media containing, IL-15, IL-2 or none. As shown in Fig. 5, both T cell clones exhibited a limited expansion potential (~3-fold max) in the presence of IL-15 or IL-2. Nonetheless, both IL-2 and IL-15 were able to preserve the viability of the cell cultures up to day 58 when this experiment was terminated (Fig. 5, A and B). Conversely, the CD4+ T-helper cell cultures that were maintained in the absence of exogenous lymphokines rapidly decreased in cell numbers, and most of the cells were dead by day 15. The function of the CD4+ T cells cultured in either IL-2 or IL-15 was preserved as assessed by antigen-induced proliferation assays (Fig. 5, C and D). These responses were slightly higher on the CD4+ T cells maintained in IL-15 as compared with the cells grown in the presence of IL-2. The variability in the proliferative responses observed between different time points, which in some cases appeared to increase (Fig. 5C) and in other cases decrease (Fig. 5D), could be because of variances in the proliferation assays because these were performed in different days. Never-

theless, at each individual time point, the responses of the T cells were very similar regardless of whether they were maintained in IL-2 or IL-15. The overall results indicate that IL-15 displays different effects in the expansion of CD8+ and CD4+ T cells as previously suggested (6). Nonetheless, it is evident that IL-2 and IL-15 can serve as viability preserving factors for both CD4+ and CD8+ T cells.

The high degree of expansion of CD8+ T-cell cultures maintained in IL-15 as compared with the subtle expansion obtained in IL-2 could be because of differences in the capacity of these lymphokines to induce antigen-independent proliferation of the T cells. Another possible explanation for these results could be if both lymphokines stimulated the proliferation of the CD8+ T cells, but IL-15 was more effective than IL-2 at inhibiting apoptosis. To explore these possibilities, we performed cell cycle analyses by propidium iodide staining cultures of CD8+ T cells grown in IL-2 or IL-15 for different time periods (7, 14, and 21 days). The results of these experiments indicate a higher proportion of cells undergoing cell division (S + G₂-M) was present in those cultures maintained in IL-15 as compared with those kept in IL-2 (Fig. 6). Furthermore, the proportion of cells having DNA debris (which is an indication of apoptotic cell death) that was evident in the cultures maintained in IL-15 was significantly lower than in the IL-2 cultures. Similar results were obtained with other T-cell lines and clones (data not presented). These results indicate that the expansion of CD8+ T cells in IL-15 is the product of an increase rate of proliferation and decrease in the degree of cell apoptosis.

The disparity in proliferative responses to IL-15 observed between CD8+ and CD4+ T lymphocytes could be explained by differences in the expression of the cell surface cytokine receptors between these cell types. Although both the receptors for IL-2 and IL-15 share two protein chains (β and γ), each cytokine receptor has a unique α chain (20–22). We measured the expression of the various components of the IL-2 and IL-15 receptors on the surface of recently (2-week) TCR-stimulated T-cell clones by flow cytometric analysis. The results indicate that the IL-15R α chain was detected in only CD8+ T cells (Fig. 7A) and not on the CD4+ T cells (Fig. 7B). Although both CD4+ and CD8+ T-cell subsets expressed the IL-2R α chain, the levels of expression on CD4+ cells were substantially lower than those observed on the CD8+ T cells (Fig. 7, C and D). Lastly, no apparent differences were detected in the levels of expression of the shared β and γ chains (Fig. 7, E–H). Interestingly, when CD8+ T cells were resuspended in medium containing IL-15, the expression of the IL-15R α was lost after a few days, but the expression of the β and γ chains remained unaltered (data not shown), suggesting that the IL-15R α chain may be down-regulated by IL-15.

The above results indicate that the capacity of CD8+ T cells to proliferate to IL-15 may be because of the expression of IL-15R α , which is evident on these cells but not on CD4+ T lymphocytes. These results suggest that IL-15 may directly transmit a proliferative signal to the T cell by binding to IL-15R α . Alternatively, IL-15R α , which has a very high affinity for IL-15 (10^{11}) may stabilize the binding of lymphokine to the $\beta\gamma$ complex, which then transmits the proliferative signal. The latter would explain why high concentrations of IL-2 could mimic the effects of IL-15 on TIL-derived CTLs (Fig. 3A). This scenario would predict that

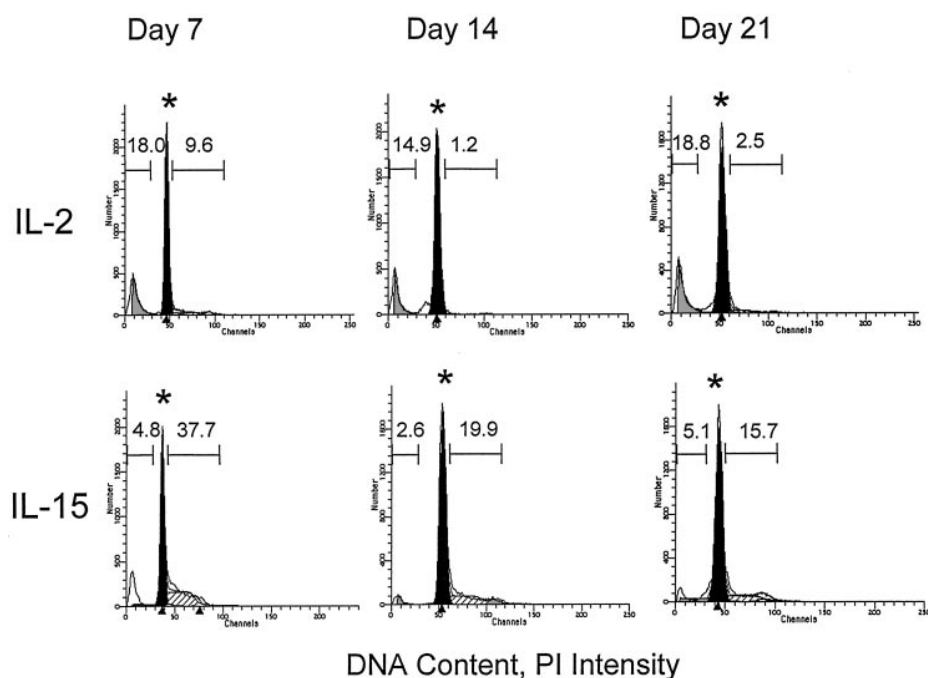


Fig. 6 IL-15 increases the proliferation and inhibits apoptosis of CD8+ T lymphocytes. The gp100-specific CTL clone X283 was maintained in either 10 ng/ml IL-15 (top panels) or 50 IU/ml IL-2 (bottom panels), and at the times noted, cell cycle analyses were performed as described in "Materials and Methods." The main black peak in each histogram corresponds to cells in G₀-G₁ (haploid) phase (marked with an *). A gate was set to the right of the G₀-G₁ peak to quantify the proportion of dividing cells in S + G₂-M phase, and another gate to the left of G₀-G₁ to assess the proportion of cells undergoing apoptosis (DNA debris). Numbers represent the percentages of cells (events) in these gates.

CD4+ T cells should proliferate to high concentrations of IL-2 because the levels of expression of their IL-2/15 β and IL-2/15 γ chains were similar to those found in CD8+ T cells (Fig. 7, E-H). On the other hand, if CD4+ T cells fail to proliferate to high concentrations of IL-2, this would suggest that IL-15R α does play a role in the transduction of the proliferative signal in CD8+ T cells. To examine these possibilities, we measured the capacity of CD4+ T cells to proliferate in an antigen-independent way to high concentrations of IL-2 (1000 IU/ml). The results presented in Fig. 8, showing that CD8+ but not CD4+ T cells proliferated to the high-dose IL-2 (and to IL-15), indicate that IL-15R α actively participates in the proliferative response of CD8+ T cells in the absence of antigen.

DISCUSSION

The multiple complex roles that IL-2 and IL-15 play in mediating the expansion and survival of T lymphocytes continue to draw much attention (4, 23). Not only do these lymphokines share some of their receptor subunits, but they also overlap in several of their functions such as serving as growth/survival factors for CD4+ and CD8+ T lymphocytes. However, some major functional differences exist between these sister lymphokines. For instance, it has been reported in the mouse system that IL-15, but not IL-2, induces *in vitro* proliferative responses of memory CD8+ T cells and that these effects do not translate to memory CD4+ T lymphocytes (6, 8). Furthermore, through the action of IL-15, mouse memory CD8+ T cells are able to divide slowly *in vivo* and to survive for long periods of time without the need of antigen stimulation (7, 24). IL-2, on the other hand, has been reported to inhibit the *in vivo* long-term survival of mouse memory CD8+ T cells (24). Also in the mouse system, Manjunath *et al.* (12) reported that antigen-

stimulated CD8+ T cells differentiate *in vitro* into memory cells in the presence of IL-15 or proceed to become cytolytic effector cells in the presence of IL-2. Interestingly, these studies also showed that effector CTLs revert to a noncytolytic memory phenotype after only 7 days in culture with IL-15 (12). Although our *in vitro* studies using human T cells share some similarities with the observations in the mouse system, major differences between these systems were observed. In our hands, IL-15 enabled CD8+ CTLs to proliferate and survive *in vitro* in the absence of antigen, allowing these cells to expand and survive for relatively long periods of time (>60 days) without the loss of their effector function and without reverting into memory (noncytolytic) cells (Figs. 1-3 and 6). As reported in the mouse system, human CD4+ T cells did not respond to IL-15 in the same manner as CD8+ T cells did (Figs. 5 and 8). Our data also shows that although IL-15 was unable to induce the expansion of CD4+ T cells, it was capable of functioning as a survival factor in a similar way as IL-2 (Fig. 5). In contrast to mouse CD8+ memory cells where antigen-independent proliferation presumably continues throughout the animal's life, the human CD8+ effector cells ceased to divide in culture after 30-40 days and their viability declined unless they received TCR activation signals, which may be required for the re-expression of IL-15R α (6). Thus, it is possible that effector and memory CD8+ T cells may respond differently to IL-15. Alternatively, it is possible that memory CD8+ T cells continue to express IL-15R α without the need of TCR stimulation for much longer periods of time than effector CTLs. Another possible explanation for the differences observed between the *in vivo* mouse and the *in vitro* human systems is that *in vivo* T cells are likely to receive additional signals from other cells or the extracellular matrix, which help to maintain their responsiveness to IL-15.

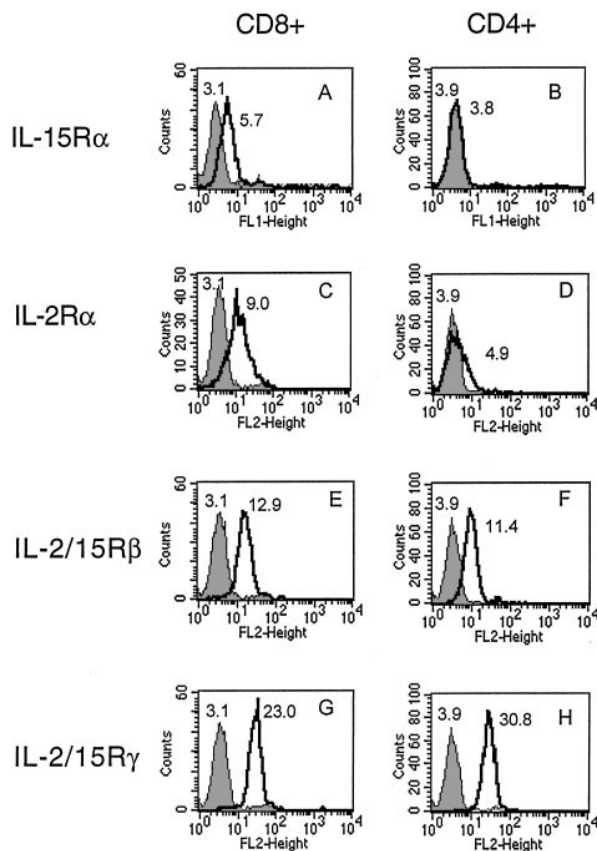


Fig. 7 Evaluation of the cell surface expression of the IL-2 and IL-15 receptor components on CD8+ and CD4+ human T lymphocytes by flow cytometric analysis. A CD8+ CTL clone (same one studied in Fig. 1) and a CD4+ T helper clone (from Fig. 5A) were stimulated with anti-CD3 antibody and APC. After a 2-week culture in IL-2, the cells were stained with fluorescent-labeled monoclonal antibodies specific to IL-15R α , IL-2R α (CD25), IL-2R β (CD122), and IL-2R γ (CD132). The levels of expression of each antigen were assessed as described in "Materials and Methods," and the numbers above each curve correspond to the mean fluorescence intensity. Similar results were obtained with other CTLs and T-helper lines (data not shown).

The expansion of CD8+ T cells observed in IL-15 and not with IL-2 appears to be derived from both an increase in the rate of cell proliferation and a decrease in cell death (Fig. 6). The mechanism by which IL-15 triggers antigen-independent cell proliferation in CD8+ T cells, but not in CD4+ T cells, may be complex. Because the surface expression of IL-15R α in mouse memory CD8+ T cells is undetectable, and the levels of IL-2/15R β are much higher (10–30-fold) than those observed on memory CD4+ T cells, it has been proposed that IL-15 triggers cell division in these cells through the IL-2/15R $\beta\gamma$ receptor complex (6). On the other hand, the presence of the IL-15R α chain on human CD8+ CTLs (Fig. 7A), which alone can bind IL-15 with a K_a 100-fold higher than the IL-2/15R $\beta\gamma$ complex (25), suggests that the proliferative signals of IL-15 on human CTLs could be mediated via the IL-15R α chain. This possibility is reinforced by the observations that effector CD4+ human T cells, which did not express the IL-15R α chain (Fig. 7), failed to

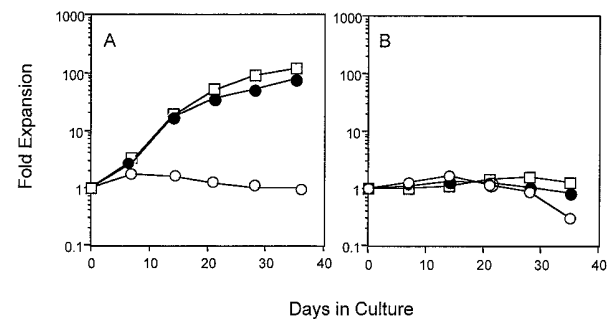


Fig. 8 High concentrations of IL-2 stimulate the antigen-independent expansion of CD8+ but not CD4+ T cells. A, the expansion and survival of a CD8+ CTL clone (from Fig. 1) and (B) a T-helper clone (from Fig. 5A) were evaluated in the presence of 50 IU/ml IL-2 ($\circ$), 1000 IU/ml IL-2 ($\bullet$), or 10 ng/ml IL-15 ($\square$).

proliferate in the presence of this lymphokine (Fig. 5, A and B). Although another possible explanation for these results is that IL-15R α simply facilitates the binding of the lymphokine to the IL-2/15R $\beta\gamma$ complex, which is then responsible for signal transduction for the proliferative response. However, if this were the case, high concentrations of IL-2 should be able to trigger the proliferation of the T cells because this lymphokine binds to the $\beta\gamma$ complex with a K_a of $\sim 10^9$ M. Indeed, IL-2 at 1000 units/ml (6.8×10^{-9} M) mimicked the effect of IL-15 in CD8+ T cells, triggering their antigen-independent proliferative response. Nevertheless, the same high concentration of IL-2 did not have an effect on CD4+ T cells, although these cells expressed similar levels of IL-2/15R β and IL-2/15R γ . Thus, under this scenario, one would have to propose that differences existing between CD4+ and CD8+ T cells are in the capacity of their IL-2/15R $\beta\gamma$ complexes to transduce a proliferative signal. In other studies (26), it was reported that IL-15 was able to activate memory CD4+ T cells in addition to naïve and memory CD8+ T cells, as determined by increase in the expression of the CD69 marker and the synthesis of DNA measured by [^{3} H]thymidine incorporation. However, these authors used much higher concentrations of IL-15 (100 ng/ml) than those used in the present studies and did not assess the long-term effects of IL-15 or the actual expansion of the cell cultures. We have observed that T-cell activation and even [^{3} H]thymidine incorporation into DNA do not necessarily imply that the cells will be able to successfully proliferate and expand in cell numbers, because in some instances the cells undergo apoptosis.⁴

The present findings suggest that in addition to maintaining CD8+ T cell memory *in vivo* (6–8, 12, 24), IL-15 may also help to enhance and prolong the effector function of CTLs. It is possible that once a pathogen has been apparently eliminated by the immune system, some of the effector cells may remain functional and even continue to expand in the peripheral tissues for some period of time as a safeguard against pathogen resurgence. In addition to the possible physiological role that IL-15 plays on effector CD8+ T-cell responses, our results bear some practical implications for the maintenance and propagation of antigen-specific T-cell lines *in vitro*. The use of IL-15 should enable researchers to grow CTLs for longer periods of time

without loss of effector function while avoiding the cumbersome process of periodic antigen restimulation. This technique could also be useful for the expansion of therapeutic CTLs to be used for adoptive transfers.

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