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Endogenous IL-2 Contributes to T Cell Expansion and IFN-γ Production During Lymphocytic Choriomeningitis Virus Infection[1]

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IL-2-deficient mice were used to examine the role of endogenous IL-2 for supporting T cell proliferative responses during infection with lymphocytic choriomeningitis virus (LCMV). The studies showed that, although virus-specific CTL activity was induced in the absence of IL-2, the overall magnitude of the response was profoundly inhibited. Examination of proportions and numbers of $CD8^+$ T cells demonstrated that the normal virus-induced expansion of these cells was virtually eliminated in spleens and dramatically decreased in lymph nodes from IL-2-negative mice. Absence of endogenous IL-2 also significantly inhibited virus-induced activated T cell production of IFN-γ, as well as increases in frequencies and numbers of IFN-γ-producing cells. Reductions in immune responses were accompanied by impaired viral clearance. Although T cell responses were dramatically reduced in IL-2-deficient, as compared with IL-2-containing mice, activation signals were being delivered in vivo because induced CTLs were sensitive to the cell cycle-specific toxin, hydroxyurea (HU), and $CD8^+$ T cells had induced expression of the IL-2R α- and β-chains. These studies demonstrated that, although low levels of T cell responses can be induced in the absence of IL-2, the factor plays a unique and critical role in supporting T cell proliferative responses in vivo and in optimizing induction of the biologic functions mediated by these cells. Furthermore, the results identify a role for IL-2 in promoting IFN-γ production in vivo. ***The Journal of Immunology,*** **1995, 155: 5690–5699.**

Interleukin-2 was originally identified and characterized based on its ability to support T cell proliferation in vitro (1, 2). This factor has been used extensively to expand T cell populations, including CTL. Much is known about the in vitro induction and function of IL-2. It is produced by activated T cells, and activated T cells are induced to express the IL-2R (2). This receptor is comprised of a number of molecules. Responses to IL-2 binding are dependent on the IL-2R β-chain, CD122. This molecule forms an intermediate affinity receptor with the IL-2R γ-chain. Cells expressing the IL-2R β- and γ-chains can respond to high concentrations of the factor (3, 4). An additional IL-2R α-chain, CD25, is required to form a high-affinity receptor for responses to low concentrations of the factor (5). IL-2 and signaling through the IL-2R have been shown to be essential for T cell proliferation in response to a variety of stimuli in culture.

Although the biologic functions of IL-2 have been extensively studied in vitro, the in vivo consequences of IL-2 expression remain controversial. Endogenous IL-2 can be demonstrated in a number of conditions associated with extensive T cell activation and proliferation in mouse and man (6, 7). Our laboratory has shown that IL-2 is induced in vivo during infections of mice with lymphocytic choriomeningitis virus (LCMV)[3] (8, 9). Peak expression of IL-2 mRNA and protein coincide with: 1) virus-specific, class I histocompatibility molecule-restricted CTL killing mediated by $CD8^+$ T cells; 2) total leukocyte expansion; 3) $CD8^+$ T cell proliferation; and 4) $CD4^+$ and $CD8^+$ T cell production of IFN-γ (10–13). As $CD8^+$ T cells elicited at times of endogenous IL-2 expression have the high-affinity form of the IL-2R and proliferate in response to low concentrations of IL-2 in vitro (14), these populations have been primed to utilize the factor. Thus, IL-2 is made during LCMV infections, and T cells activated under these conditions are equipped to respond to the factor. Given the complexity of endogenous immune responses to infection, however, it has been difficult to define the biologic functions depending on the induction of IL-2 and IL-2 responsiveness (15).

Mutated mice, made specifically deficient for IL-2 by homologous recombination, have been generated by Dr. Ivan Horak and his colleagues to investigate the functions requiring IL-2 in vivo (16, 17). Their studies have shown that IL-2 is not essential for induction of CTL activity during LCMV infections (17). Thus, factors in addition to IL-2 and/or instead of IL-2 can promote CTL activation. However, roles for the growth-promoting activity of IL-2 in supporting LCMV-induced leukocyte and $CD8^+$ T cell proliferation in vivo have not been examined. Furthermore, secondary effects on the magnitude of induced functions mediated by cells using IL-2 for expansion have not been defined.

The experiments reported here used mice with mutated, nonfunctional IL-2 genes to determine the effects of endogenous factor on LCMV-induced cell proliferation. In particular, total leukocyte and $CD8^+$ T cell proliferative responses were evaluated in IL-2-positive and -negative mice. The studies showed that the absence of endogenous IL-2 profoundly inhibited LCMV-induced T cell

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; HU, hydroxyurea; PE, phycoerythrin; PFU, plaque-forming unit; ELISPOT, enzyme-linked immunospot.

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expansion. Consequences of this inhibition included significant reductions in the magnitude of total CTL activity and the ability to clear viral infection. Studies of IFN-γ production also showed that IL-2 plays an important role in promoting expression of this factor. Although the absence of IL-2 dramatically inhibited a range of T cell responses, T cells were clearly receiving activation signals in the absence of the factor because they were induced to express IL-2Rs and sensitive to a cell cycle-specific toxin. These studies further define IL-2-dependent and -independent immune responses in vivo, and demonstrate a critical role for endogenous IL-2 in promoting proliferative and protective T cell responses to viral infections.

Materials and Methods

Mice

Mutated mice, deficient for IL-2, were originally derived by Dr. Ivan Horak at the Institute for Virology and Immunobiology at the University of Würzburg, Würzburg, Germany (16). With permission from Dr. Horak, heterozygous male (129 × C57BL/6) mice were obtained from Dr. Cox Terhorst of the Beth Israel Hospital, Boston, MA. The mice were bred to specific pathogen-free C57BL/6 females purchased from Taconic Laboratory Animals and Services, Germantown, NY. Brother × sister matings were set up, the male and female progeny of which were the mice used in these experiments. All breeding took place in strict isolation, with sterile food, water, and caging, in the animal facility at Brown University. Mice were typed by PCR amplification of wild-type or mutant alleles as +/+, +/−, and/or −/− for the normal gene. Mice used in the experiments were 4 to 8 wk of age. All of the reported results are from mice with no overt signs of illness, normal range hematocrits, and unremarkable bowel histology.

Treatment of mice

Animals were handled in accordance with institutional guidelines for animal care and use. Mice were uninfected or infected i.p. with 2×10^4 plaque-forming units (PFU) of LCMV, Armstrong strain, clone E350 on day 0 (18, 19). Uninfected mice and mice on day 7 or 9 after infection were killed by cervical dislocation. Spleens, kidneys, and/or inguinal lymph nodes were harvested. To evaluate responsiveness to added IL-2, mice were given human rIL-2 (Cetus Corp., Emeryville, CA). The factor had a sp. act. of 3×10^6 Cetus U/mg, and was diluted to 10^5 U/ml with 0.1% globulin-free mouse albumin (Sigma Chemical Co., St. Louis, MO) in PBS. Because of previous work from this laboratory demonstrating the kinetics of endogenous IL-2 expression during LCMV infection of normal mice (8), treatments were begun on day 4 after infection. A total of six injections of 10^4 units in 100 μl were given i.p. at 12-h intervals on days 4, 5, and 6 after LCMV infection. Samples were harvested from the treated mice on day 7 of infection, at 12 h after the last IL-2 injection. The replicative status of CTL was evaluated by examining sensitivity to the cell cycle-specific toxin, hydroxyurea (HU). Cells synthesizing DNA (i.e., in S phase) are killed by this drug (20, 21). Other cycling cells are blocked at the G1/S phase interface and enter into S phase in a semisynchronous fashion as the drug is cleared. Thus, double-dose treatments at appropriate intervals can eliminate the majority of cycling cells. Optimal conditions of treatment to eliminate cells induced to undergo DNA synthesis during LCMV infection have been previously described (20). Briefly, mice were given HU (Sigma Chemical Co.), dissolved at 30 mg/ml in PBS, i.p. at a dose of 1 mg/g body weight. Injections were done at 9 and 2 h before sample harvest on day 7 after LCMV infection. Control mice received injections of an equivalent volume of vehicle.

Isolation of leukocytes

Leukocytes were isolated as previously described (8, 9, 18, 19). Splenic leukocytes were from macerated whole spleens that had erythrocytes osmotically lysed by treatment with ammonium chloride. Inguinal lymph node leukocytes were from organs that had been teased apart but not treated with ammonium chloride. Viable cell yields were determined by trypan blue exclusion.

Microcytotoxicity assay

Cytotoxicity was measured as release of isotope from target cells labeled with sodium chromate (^{51}Cr), as described (18, 19). Briefly, uninfected and LCMV-infected H-2^b histocompatible MC57G target cells were labeled with ^{51}Cr (Dupont-New England Nuclear Research Products, Boston, MA). Experiments were conducted with E:T cell ratios of 100, 33, 11, 3, and 1, in quadruplicate. Spontaneous release of ^{51}Cr was determined by incubating target cells with media, and maximum release was determined by adding 1% Nonidet P-40 to target cells. Assays were incubated for 5 to 8 h at 37°C, supernatant fluids were harvested, and cpm for each sample quantitated in an Isoflex Automatic Gamma Counter (ICN Micromedic Systems, Huntsville, AL). Spontaneous release was <15% of maximum release. Percent lysis was calculated as 100 × (cpm test sample − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release). Percent virus-specific lysis was calculated as the difference between lysis of infected and uninfected target cells. One LU was calculated as the number of leukocytes needed to obtain 15% virus-specific lysis of 10^4 target cells. Splenic cell yields were used to determine total LUs per spleen.

Flow cytometric analysis

Two-color flow cytometric analysis was done at the Roger Williams Cancer Center of Brown University using a FACScan (Becton Dickinson, San Jose, CA) with an argon laser output at 15 mW. The LYSIS I software package (Becton Dickinson) was used for analysis of data. $CD8^+$ T cells were identified using a phycoerythrin (PE)-conjugated rat monoclonal 53–6.7 (PharMingen, San Diego, CA), IgG2a, specific for mouse Lyt-2. CD8-positive cells were in a single peak of fluorescence at an intensity between 10^2 and 10^3 log. Cells expressing the IL-2R α-chain, CD25, on their surface were identified using the FITC-conjugated rat IgM monoclonal 7D4 (PharMingen) (22). CD25-positive cells had fluorescence peaks between 3×10^1 and 10^3 log intensity. Expression of the IL-2R β-chain, CD122, was identified using the FITC-conjugated rat IgG2a monoclonal TM-β1 (PharMingen) (23). The IL-2R-β-positive populations were characterized as dim and bright based on two different relative fluorescence intensities of 10^1 to 10^2 and 10^2 to 10^3 log, respectively. Background staining was assessed with control Abs (PharMingen) lacking specificity for murine determinants.

IFN-γ ELISA

Samples for quantitation of IFN-γ production were prepared by conditioning 10% FBS RPMI media, containing 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, with 10^7 splenic leukocytes per ml, for 24 h at 37°C (24).[4] Conditioned media were prepared without addition of exogenous Ags, mitogens, or growth factors. In experiments to evaluate cellular responsiveness to IL-2 for IFN-γ production, rIL-2 (100 U/ml) was added to the cultures during the 24-h incubation. A specific sandwich ELISA was used to measure IFN-γ. Immulon 4 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with the anti-IFN-γ mAb HB170 as the capture reagent and incubated overnight at 4°C. After a blocking step, standards and samples were added in duplicate and incubated at 37°C for 1 h. Plates were washed before adding a rabbit polyclonal anti-IFN-γ Ab, and then incubated at 37°C for 1 h. After extensive plate washings, peroxidase-conjugated donkey anti-rabbit Ig (Jackson ImmunoResearch, West Grove, PA) was added and incubated for 30 min at 37°C. Plates were washed thoroughly before adding the ABTS (2.2′-azino-di(3-ethyl-benzthiazoline sulfonate 6)) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Assays were developed at room temperature. Colorimetric changes were detected at multiple intervals using a Dynatech MR-4000 plate reader. Optical densities derived from IFN-γ standards (PharMingen) were plotted as a standard curve. Concentrations of sample IFN-γ were calculated by solving for the equation of the standard curve. Sensitivity of the ELISA was 19 pg/ml. Total values per spleen were calculated based on production per number of cells used × cell yield per spleen. Abs were the generous gift of Dr. Phillip Scott of the Department of Pathobiology, The School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA.

ELISPOT assay

Modifications of published ELISPOT procedures were used to quantitate numbers of IFN-γ producing cells (25, 26). Immulon 4 96-well plates (Dynatech) were coated, under sterile conditions, with capture anti-IFN-γ mAb, XMG1.2 (DNAX, Palo Alto, CA), or the isotype control mAb, GL113.1 (DNAX), at 10 μg/ml in coating buffer with 100 μl per well. Plates were covered and incubated overnight at 4°C and then washed thoroughly. Blocking was done with 150 μl per well of RPMI-5% FBS for a minimum of 1 h. Plates were washed. Cells were diluted to 10^6, 7.5×10^5,

[4] Orange, J. S., and C. A. Biron. An absolute and restricted requirement for IL-12 in NK cell IFN-γ production and antiviral defense: studies of NK and T cell responses in contrasting viral infections. *Submitted for publication.*

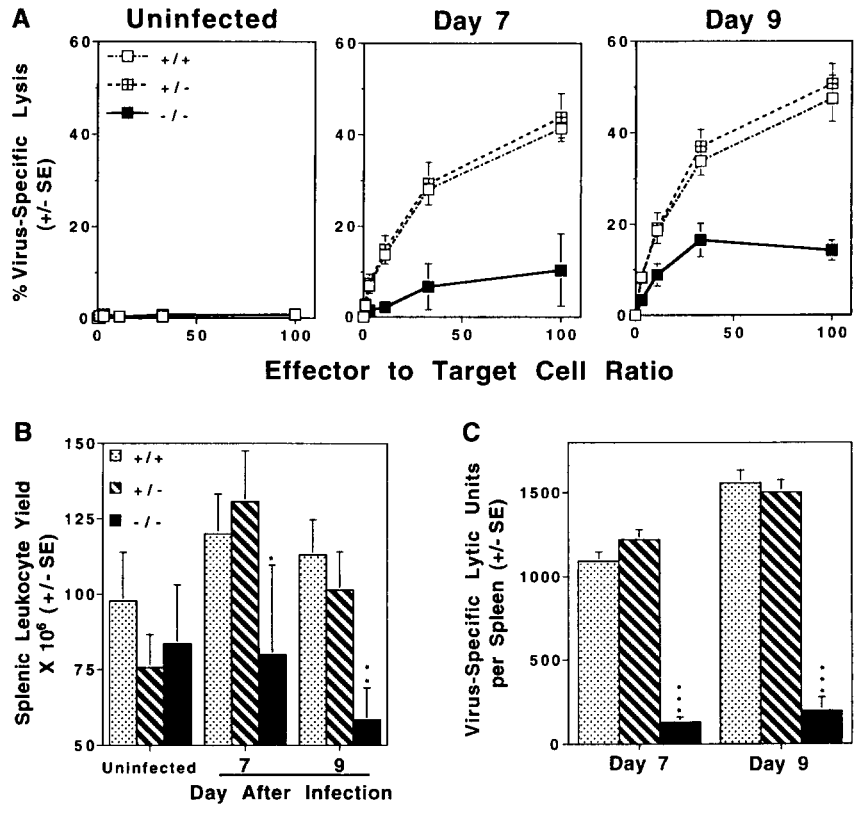

FIGURE 1. Effects of endogenous IL-2 on LCMV-induced total splenic CTL activity and leukocyte yields. Mice were bred and typed for inheritance of the normal (+) and mutated (−) IL-2 gene alleles, as described in *Materials and Methods*. Responses were examined in −/− mice lacking endogenous IL-2 and IL-2-positive +/+ and +/− littermates. Splenic leukocytes were harvested from uninfected mice or mice that had been infected with LCMV for 7 or 9 days. Numbers of mice in each group were as follows: *A*, uninfected 6 +/+, 6 +/−, and 5 −/−; *B* and *C*, uninfected 4 +/+, 3 +/−, and 2 −/−; *A*, *B*, and *C*, day-7 infected 4 +/+, 3 +/−, and 3 −/−; and *A*, *B*, and *C*, day-9 infected 3 +/+, 3 +/−, and 3 −/− mice. Results shown are means ± standard errors. *A*, Virus-specific CTL activities were evaluated on a per cell basis with splenic leukocytes, from +/+ (-·-□-·-), +/− (–⊞–), and −/− (—■—) mice, in a 5-h ^{51}Cr release assay against LCMV-infected and uninfected, histocompatible MC57G target cells. Data presented are specific lysis of virus-infected cells. *B*, Total viable leukocyte yields per spleen, in +/+ (▨), +/− (▧), and −/− (■) mice, were determined on the basis of trypan blue exclusion. *C*, Total virus-specific LUs per spleen, in +/+ (▨), +/− (▧), and −/− (■) mice, were calculated on the basis of number of cells required to mediate 1 LU of activity and total number of splenic leukocytes. Differences of results with samples from −/− as compared with +/+ or +/− mice were statistically significant: $*p < 0.1$, $**p < 0.05$, and $***p < 0.01$.

5×10^5, 2.5×10^5, 10^5, 5×10^4, 10^4, 5×10^3 in 100 μl of medium, tested in duplicate at 100 μl per well, and incubated at 37°C for 24 h. After washing, 100 μl per well of the secondary mAb (polyclonal rabbit anti-mouse IFN-γ, a gift from Dr. Phillip Scott) in PBS-Tween-5% FBS was added. Plates were incubated 1 h and washed. Alkaline phosphatase-conjugated donkey anti-rabbit Ab (Jackson ImmunoResearch) was diluted to the manufacturer's recommendations in PBS-Tween-5% FCS, and 100 μl was added per well. Plates were incubated 30 min at 37°C before washing. BCIP (5′-bromo-4-chloro-3-indolyl phosphate; 1 mg/ml; Sigma Chemical Co.) substrate was dissolved in 0.1 M AMP buffer (diluted from 1.5 M buffer from Sigma Chemical Co.) containing 0.6% SeaPlaque agarose (FMC Bioproducts, Rockland, ME) at 45°C. Substrate was added at 200 μl per well. Assays were developed overnight at room temperature. Positive cells were observed as spots of substrate precipitate and counted under low-power magnification (10–30X).

Viral plaque assay

LCMV burdens in infected mice were quantitated as titers of infectious viruses forming plaques on confluent monolayers of Vero cells (18). Renal or splenic tissue homogenates were prepared using cold Teflon pestle homogenizers (Wheaton, Millville, NJ). Supernatants were isolated from the homogenate by centrifugation at 4°C for 20 min at 1000 × *g*. Dilutions of supernatant fluids were prepared under sterile conditions, and 100 μl incubated with 1 ml of media on each Vero monolayer, in polystyrene 60-mm culture dishes, for 1 h at 37°C. Infected monolayers were overlaid with 1X media 199 (Life Technologies, Grand Island, NY) containing 5% FBS and 0.5% ME agarose (FMC Bioproducts). The plates were incubated for 4 days at 37°C. Plaques were visualized after staining of viable cells by the addition of a second overlay of 2 ml of 1X media 199 containing 5% FBS, 0.5% ME agarose, and 0.034% neutral red (Life Technologies). Experimental samples, negative controls, and LCMV standards were run in parallel.

Statistical analysis

The Student's *t*-test was performed where indicated.

Results

Total leukocyte expansion and CTL activity

LCMV infection of normal mice induces dramatic increases in lymphocyte proliferation and results in increases in total leukocyte yields (8, 9, 18). To evaluate the effects of absence of IL-2 on overall expansion and function, splenic leukocyte cell yields and CTL responses as virus-specific LUs per spleen were measured in IL-2-deficient −/− mice and their IL-2-containing +/+ and +/− littermates. Splenic leukocytes were isolated from mice that were uninfected or i.p. infected with LCMV for 7 or 9 days. Virus-specific CTL activity was evaluated against histocompatible target cells in ^{51}Cr release assays (Fig. 1*A*). Splenic leukocytes isolated from +/+ or +/− mice were induced to mediate, at E:T ratios of 100:1, approximately 40 and 50% virus-specific killing on days 7 and 9 after infection, respectively. The induced CTL activity was significantly decreased by >70% in IL-2-deficient mice; virus-specific lysis reached only 10 to 15% at an E:T ratio of 100:1. Thus, the relative induction of killing on a per cell basis in IL-2-deficient, as compared with IL-2-containing, mice was approximately one-third, and was comparable to levels reported by others (17).

When the yields of viable splenic leukocytes were examined, however, it was found that the absence of IL-2 dramatically inhibited LCMV-induced cell expansions (Fig. 1*B*). Splenic leukocyte yields from uninfected +/+, +/−, and −/− mice were not significantly different. On days 7 and 9 after infection, IL-2-containing +/+ mice had respective average increases of 22 and 15 million cells, and +/− mice had increases of 55 and 25 million cells. In contrast, infected IL-2-deficient −/− mice had modest

Table I. *Effect of IL-2 deficiency on LCMV-induced cell expansion in inguinal lymph nodes*

	Total Leukocyte Yields × 10^4 (±SE)[a]			CD8$^+$ T Cell Yields × 10^4 (±SE)[a,b]		
		LCMV-infected			LCMV-infected	
Genotype[c]	Uninfected	Day 7	Day 9	Uninfected	Day 7	Day 9
+/+[d]	152 ± 32	423 ± 38	345 ± 49	35 ± 5	78 ± 1	67 ± 2
+/−[d]	132 ± 49	461 ± 76	665 ± 160	35 ± 13	70 ± 2	132 ± 22
−/−[e]	153 ± 53	120	265 ± 55	46 ± 14	11	60 ± 12

[a] Yields shown are per lymph node.
[b] CD8$^+$ T cell yields were quantitated based on proportions of positive cells measured by flow cytometric analysis, as described in *Materials and Methods*.
[c] Inheritance of normal IL-2 gene was determined by PCR analysis.
[d] For each time point, there were 3 +/+ and 3 +/− mice per group.
[e] There were two, one, and two mice for the day 0, 7, and 9 groups, respectively.

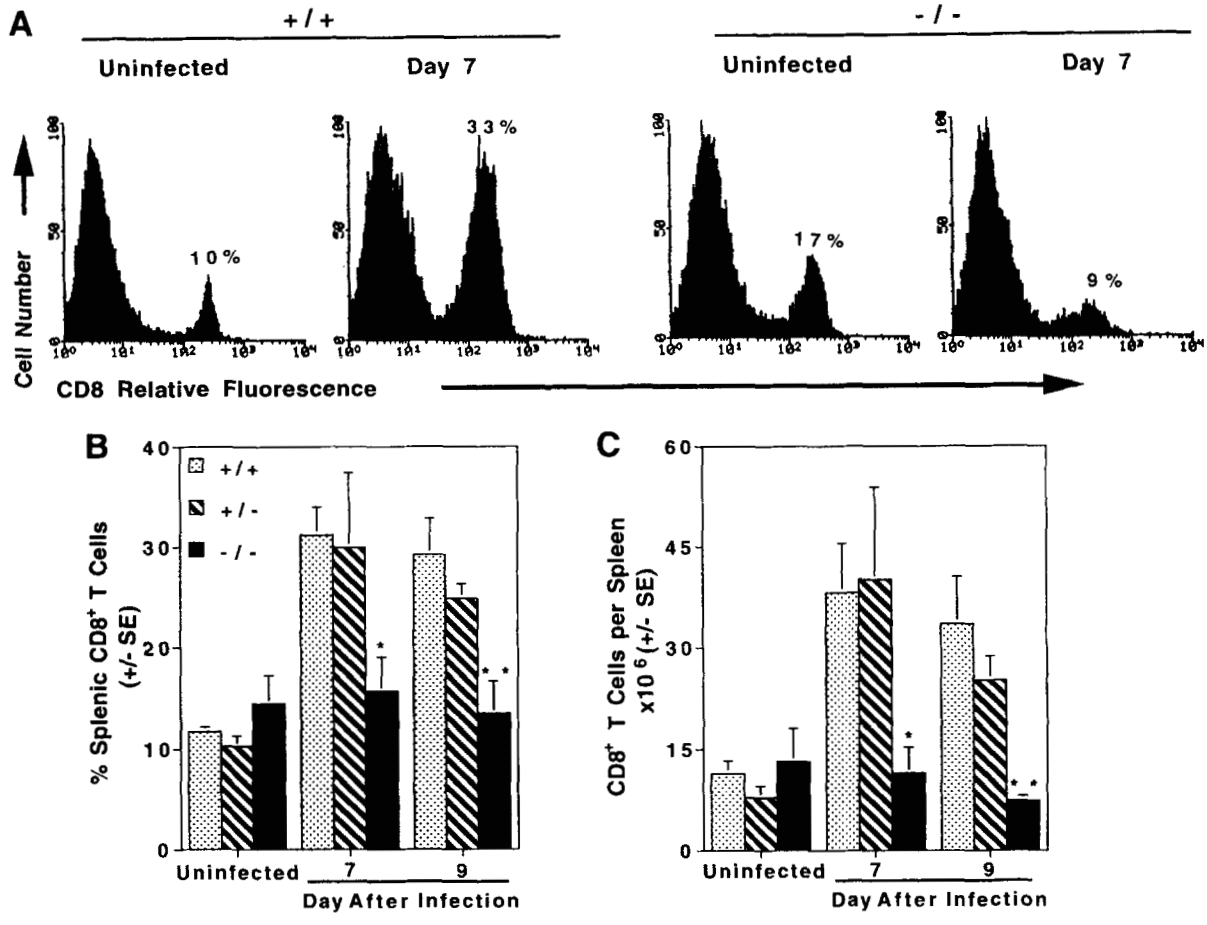

FIGURE 2. Effects of endogenous IL-2 on LCMV-induced splenic CD8$^+$ T cell expansion. Mice were infected with LCMV as in Figure 1. Proportions of splenic CD8$^+$ T cells were evaluated by flow cytometric analyses with PE-conjugated anti-CD8 Ab, as described in *Materials and Methods*. Experiments were carried out with −/− mice lacking endogenous IL-2 and their IL-2-positive +/+ and +/− littermates. *A*, Representative histograms of splenic CD8$^+$ T cell proportions in IL-2-positive (+/+) and IL-2-negative (−/−) mice that were uninfected or LCMV-infected for 7 days are shown. *B*, Means of percentages of CD8$^+$ T cells ± standard error in +/+ (▨), +/− (▧), and −/− (■) mice are presented. *C*, The numbers of CD8$^+$ T cells per spleen were calculated by multiplying total spleen leukocyte yields by fraction of CD8$^+$ T cells. Means ± standard error are shown. For the data in *B* and *C*, numbers of mice in each group were as follows: uninfected, four +/+, three +/−, and two −/−; day 7 LCMV-infected, four +/+, three +/−, and three −/−; and day 9 LCMV-infected, three +/+, three +/−, and three −/− mice. Differences of results with samples from −/− as compared with +/+ or +/− mice were statistically significant: $*p < 0.05$, and $**p < 0.01$.

decreases in splenic leukocyte yields on day 7 and average decreases of 25 million cells on day 9 after infection. The reduction in virus-induced leukocyte expansion was not limited to this compartment. Significant decreases in virus-induced cell expansion were also observed in peripheral lymph nodes (Table I). Thus, the absence of IL-2 resulted in a complete inhibition in expansion of splenic leukocyte yields and a profound decrease in lymph node cell expansion during LCMV infection.

To evaluate the overall CTL lytic capacity per spleen, CTL activity was calculated as LU, i.e., number of cells required to mediated 15% virus-specific lysis, and the total LUs per spleen were calculated on the basis of leukocyte yields per spleen (Fig. 1*C*). In

Table II. *Administration of exogenous IL-2 to support CD8⁺ T cell expansion during LCMV infection of IL-2-deficient mice*[a]

Genotype for Normal IL-2 Gene[b]	$CD8^+$ T Cell Yields $\times 10^{6}$[c]
+/+	30 ± 2
+/−	17 ± 1
−/−	26 ± 8[d]

[a] Mice were treated with human rIL-2 on days 4, 5, and 6 after infection with LCMV, as described in *Materials and Methods*. On day 7 after infection, spleens were harvested. Experiments were carried out with three animals per group. Means ± standard error are shown.

[b] Genotype was determined by PCR.

[c] Percentages of $CD8^+$ T cells were quantitated based on flow cytometric analysis, then multiplied by total spleen yield.

[d] There were no statistically significant differences between −/− and +/+ or +/− results.

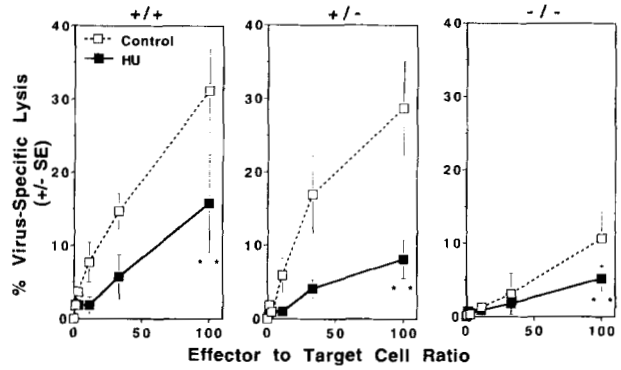

FIGURE 3. HU sensitivity of CTL activity in LCMV-infected IL-2-positive and -negative mice. Mice were infected with LCMV for 7 days. Animals were control-treated (--□--) or HU-treated (—■—) at 9 and 2 h before being killed, as described in *Materials and Methods*. Splenic leukocytes were harvested and used as effector cells in a ^{51}Cr release assay against uninfected and LCMV-infected targets. The results shown are the means of virus-specific lysis ± standard error. Numbers of mice in each group were: three +/+, four +/−, and three −/− control-treated, and three +/+, four +/−, and five −/− HU-treated mice. Differences of results with HU-treated as compared with control-treated samples, at E:T ratios of 100:1, were statistically significant for all of the groups: $^{**}p < 0.05$.

both the day 7 and 9 IL-2-deficient mice, LUs per spleen were decreased by up to 9.4-fold in comparison to those in IL-2-containing littermates. These results demonstrate that, although CTL activity can be induced in the absence of IL-2 during viral infection, the presence of the factor is required for leukocyte expansion and contributes the development of total CTL activity.

Virus-induced $CD8^+$ T cell expansion

In normal mice, $CD8^+$ T cells undergo extensive proliferation during LCMV-infection (8, 9, 18). To examine $CD8^+$ T cell expansion in the absence of IL-2, splenic $CD8^+$ T cell percentages were enumerated in IL-2-positive and -negative mice by flow cytometric analysis (Fig. 2*A*, representative histograms; Fig. 2*B*, summary of multiple experiments). Uninfected IL-2-positive +/+ and +/− mice had 10 and 12% $CD8^+$ T cells, respectively, in their splenic leukocyte populations. The baseline proportions in the IL-2 −/− mice were a little higher, averaging about 14%, but this difference was not statistically significant. Consistent with reported results in normal mice, proportions of $CD8^+$ T cells were elevated threefold in IL-2-containing +/+ and +/− littermate mice on days 7 and 9 after LCMV infection, such that about 30% of the splenic leukocytes were $CD8^+$ T cells. In contrast, percentages of $CD8^+$ T cells did not increase in IL-2-deficient −/− mice during LCMV infection (Fig. 2, *A* and *B*). The difference in $CD8^+$ T cell percentages between virus-infected, IL-2-negative and -positive mice were highly significant, i.e., $p < 0.05$. Calculation of total $CD8^+$ T cell yields per spleen demonstrated that virtually all of the cellular expansion in spleens of infected IL-2-containing mice was a consequence of $CD8^+$ T cell expansion, and that absence of IL-2 completely inhibited the apparent virus-induced $CD8^+$ T cell expansion (Fig. 2*C*). Differences in $CD8^+$ T cell numbers per spleen in IL-2-deficient- as compared with IL-2-containing mice were highly significant, i.e., $p < 0.05$. Decreases in virus-induced $CD8^+$ T cell expansion were also observed in peripheral lymph nodes (Table I).

To prove that $CD8^+$ T cells in infected IL-2-deficient mice were able to expand in response to IL-2, exogenous IL-2 was administered during LCMV infection. Treatment protocols were designed on the basis of earlier work from this laboratory demonstrating the kinetics of endogenous IL-2 expression and T cell proliferation in normal LCMV-infected mice (8). Infected mice were given 10^4 U of rIL-2 at 12-h intervals on days 4, 5, and 6, and yields of $CD8^+$ T cells per spleen were evaluated on day 7 after LCMV infection (Table II). When exogenous IL-2 was provided, the yields of $CD8^+$ T cells per spleen in IL-2-deficient −/− mice were not significantly different from those in +/+ or +/− littermates (Table II). Thus, the $CD8^+$ T cells in IL-2-deficient mice were able to expand during LCMV infection if IL-2 was provided. Taken together, these data show that endogenous IL-2 plays a significant role in promoting the in vivo proliferation of $CD8^+$ T cells during infection.

Status of CTL cell cycling

Earlier studies of responses in normal mice have demonstrated that LCMV-induced CTL activity is mediated by proliferating cells with increased DNA turnover rates and sensitivity to cell cycle-specific toxins (20). The results reported above showed that, although expansion of $CD8^+$ T cells was limited in IL-2-deficient mice, these cells were receiving signals to promote CTL induction. To determine whether CTLs elicited in the absence of IL-2 were proliferating, their sensitivity to the cell cycle specific-toxin HU was examined. Based on previously determined optimal conditions for elimination of cells undergoing DNA synthesis in vivo (20), HU was administered i.p. at 9 and 2 h before killing of mice on day 7 after infection. The HU treatments caused statistically significant reductions in CTL activity in both IL-2-containing and IL-2-deficient mice. The levels of HU sensitivity were proportionally equivalent in all three groups examined; the virus-specific lysis was reduced by 49%, 73%, and 51% in IL-2 gene +/+, +/−, and −/− mice, respectively (Fig. 3). These data demonstrate that CTLs induced in the absence of IL-2 are undergoing DNA synthesis, and that the turnover rates for these cells are similar to those for CTL induced in the presence of IL-2.

$CD8^+$ T cell expression of IL-2Rs

The aforementioned data, demonstrating the cycling of CTL, suggested that these cells were receiving activation signals during LCMV infection. To examine priming for proliferation in the IL-2-positive and -negative mice, induction of IL-2R expression was evaluated. $CD8^+$ T cell expression of the IL-2R α-chain (CD25) required to form the high-affinity receptor, and the IL-2R β-chain (CD122) forming an intermediate affinity receptor and required for signal transduction, were studied using two-color flow cytometric analysis. In the IL-2-containing mice, the percent of $CD8^+$ T cells

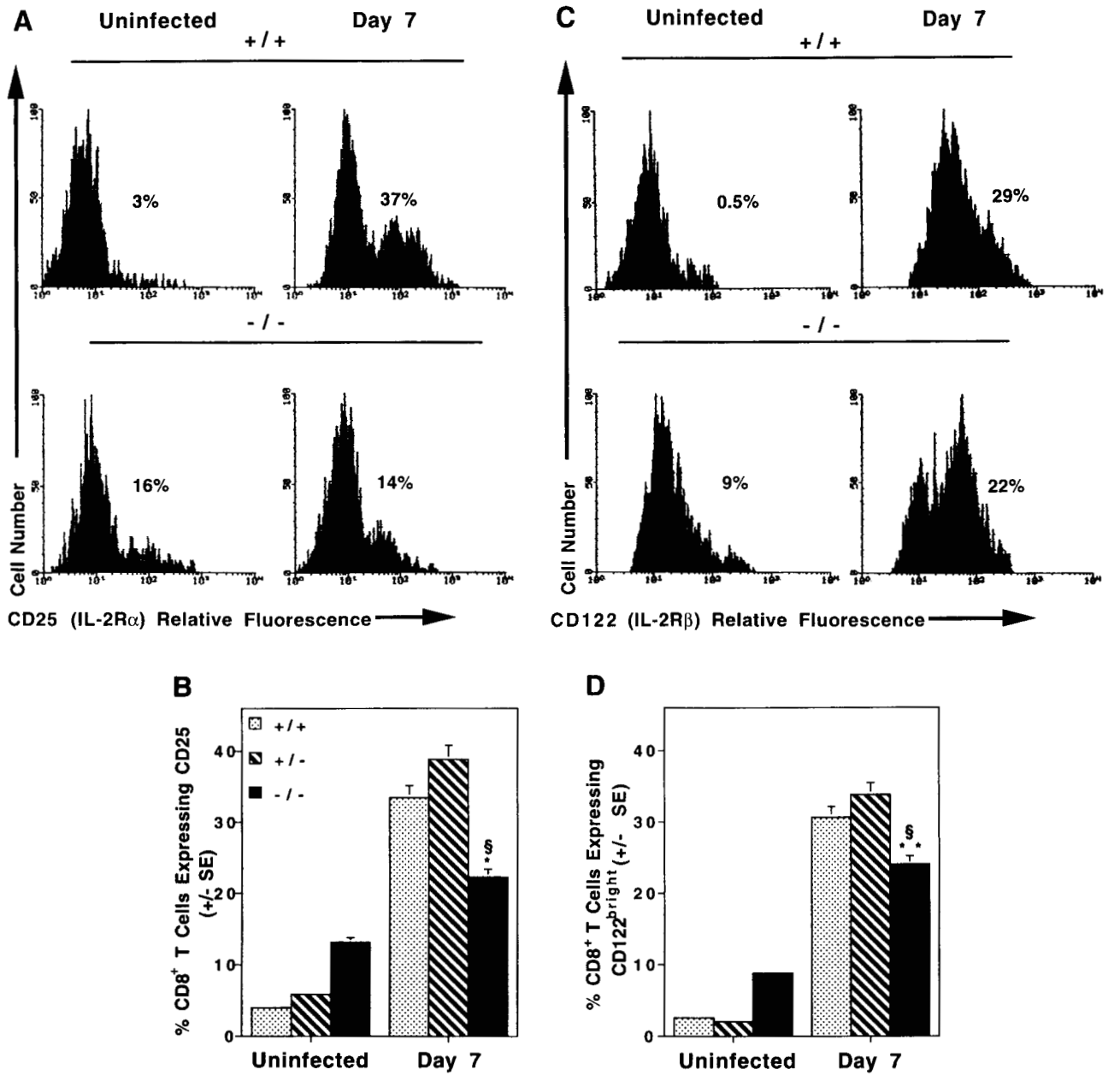

FIGURE 4. Effects of endogenous IL-2 on virus-induced $CD8^+$ T cell expression of IL-2R α- and β-chains. Splenic leukocytes were harvested from uninfected or day 7 LCMV-infected mice. Two-color flow cytometric analyses were done with PE-conjugated anti-CD8 Ab in combination with either FITC-conjugated anti-CD25 Ab or FITC-conjugated anti-CD122 Ab, as described in *Materials and Methods*. Analyses were done by gating on the $CD8^+$ T cell population and examining relative fluorescence of IL-2R molecules. Experiments were carried out with −/− (■) mice lacking endogenous IL-2 and their IL-2-positive +/+ (▨) and +/− (▧) littermates. Histograms were generated by gating on the $CD8^+$ cells and examining the CD25 or CD122 relative fluorescence within this population. *A*, Representative histograms of CD25, the IL-2R α-chain, expression on splenic $CD8^+$ T cells in IL-2-positive (+/+) and IL-2-negative (−/−) mice that were uninfected or LCMV-infected for 7 days are shown. Percent of $CD8^+$ T cells expressing CD25 is given. *B*, Mean percentages of $CD8^+$ T cells expressing CD25, ± standard error, are presented. *C*, Representative histograms of CD122, the IL-2R β-chain, expression on splenic $CD8^+$ T cells in IL-2-positive (+/+) and IL-2-negative (−/−) mice that were uninfected or LCMV-infected for 7 days are shown. Percent of $CD8^+$ T cells expressing $CD122^{bright}$ is given. *D*, Mean percentages of $CD8^+$ T cells expressing $CD122^{bright}$, ± standard error, are presented. For the data shown in *B* and *D*, numbers of mice used in each group were as follows: uninfected, 9 +/+, 9 +/−, and 6 −/−; and day 7 LCMV-infected, 11 +/+, 10 +/−, and 8 −/−. Differences of results with samples from infected −/− mice as compared with infected +/+ or +/− mice were statistically significant: $^{\S}p < 0.05$. Differences of results with samples from infected −/− mice as compared with uninfected −/− mice were also significant: $^{*}p < 0.05$ and $^{**}p < 0.01$.

expressing CD25 increased by approximately 10-fold from average percentages in uninfected mice of 4 to 6% to 33 to 39% on day 7 after infection (Fig. 4*A*, representative histograms; Fig. 4*B*, summary of multiple experiments). In uninfected IL-2-deficient mice, the proportions of $CD8^+$ T cells expressing CD25 were greater, averaging 13%. Upon infection, the percentage increased <twofold to an average of 22%. Although this level was induced in comparison with that in uninfected IL-2-negative mice, the value was significantly smaller than that obtained with cells from infected IL-2-positive mice (Fig. 4*B*).

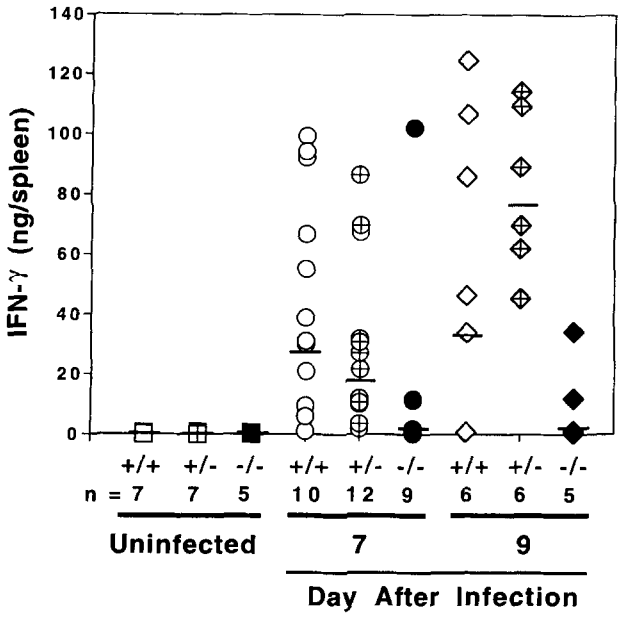

FIGURE 5. Effects of endogenous IL-2 on IFN-γ production during LCMV infection. Conditioned media were prepared for 24 h, as described in *Materials and Methods*, with splenic leukocytes harvested from uninfected mice and mice on days 7 or 9 after infection with LCMV. IFN-γ production was quantitated by ELISA. Samples are plotted individually and the geometric means for each group represented by a solid bar. Solid symbols are data from IL-2-deficient −/− mice. Open symbols are data from IL-2-containing +/+ and +/− littermates. The numbers of mice in each group are shown. Differences of results with samples from infected IL-2-negative mice as compared with combined infected IL-2-positive mice were statistically significant as follows: day 7, $p < 0.05$ and day 9, $p < 0.005$.

Relative fluorescence of expression of CD122, the IL-2R β-chain, distinguished negative, dim, and bright populations, respectively, as having $<10^1$, 10^1–10^2, and $>10^2$ log intensities (Fig. 4*C*). $CD8^+$ T cells from uninfected IL-2-containing mice were primarily CD122 negative. On day 7 after LCMV infection, most of the $CD8^+$ T cells moved into positive populations, and an average of 31 and 34% of these were $CD122^{bright}$ (Fig. 4*C* representative histograms; Fig. 4*D* summary of multiple experiments for $CD122^{bright}$ expression). Uninfected mice lacking IL-2 had higher proportions of $CD8^+$ cells with dim fluorescence, and an average of 9% of these cells were $CD122^{bright}$ (Fig. 4, *C* and *D*). On day 7 after infection of the IL-2-negative mice, a large proportion of the $CD8^+$ T cells increased in relative intensity of IL-2R β-chain expression (Fig. 4*C*), and an average of 24% of these were $CD122^{bright}$ (Fig. 4, *C* and *D*). Differences between $CD8^+CD122^{bright}$ T cells in IL-2-containing and IL-2-deficient mice were statistically significant (Fig. 4*D*). Taken together, these results demonstrated that molecules of the IL-2R are induced during viral infection in the absence of the IL-2, with the IL-2R β-chain being somewhat generally expressed at low levels, and the proportions of cells expressing high levels of IL-2R β and α-chains being similar. The absence of IL-2, however, does reduce the proportions of $CD8^+$ T cells expressing high levels of these molecules.

Induction of IFN-γ production

Another result of T cell activation during LCMV infection of normal mice is the induction of IFN-γ production by these cells (10–13). To evaluate the contribution of endogenous IL-2 to this response, 24-h conditioned media were prepared with splenic leukocytes isolated from IL-2-positive and -negative mice that were uninfected or infected with LCMV for 7 and 9 days. Sample IFN-γ levels were measured by ELISA, and values per spleen calculated on the basis of splenic cell yields. Because there was some variability in the magnitude of responses between samples, all individual mouse values were plotted with the geometric mean for each group represented by a solid bar (Fig. 5). Cells from uninfected IL-2 +/+ or +/− mice had no detectable IFN-γ production, and those from −/− mice averaged <1 ng/spleen. On day 7 after infection, the geometric means of IFN-γ production by splenic leukocytes from +/+ and +/− mice were 27 and 19 ng/spleen, respectively. In contrast, cells from −/− mice had significant 90% reductions in IFN-γ production on day 7 after infection; the geometric mean was 2.3 ng/spleen with samples from these animals. Similarly on day 9, the geometric mean of IFN-γ production by splenic leukocytes from +/+ and +/− mice was 33 and 78 ng/spleen, respectively, whereas mean production by cells from −/− mice was only 2.4 ng/spleen.

Table III. *Effects of IL-2 deficiency on frequency of IFN-γ producing cells on day 7 after LCMV infection*

IL-2 Genotype[a]	n[b]	IFN-γ$^+$ Cells per 1 × 10^6	IFN-γ$^+$ Cells per Spleen
+/+	3	131 ± 21	13528 ± 1766
+/−	4	110 ± 25	10964 ± 1603
−/−	3	61 ± 11[c]	5300 ± 1131[d]

[a] IFN-γ production by splenic leukocytes isolated on day 7 after LCMV infection was determined by ELISPOT assays, as described in *Materials and Methods*.
[b] The number of mice in each group is shown.
[c] Significance is $p < 0.05$ for −/− compared with +/− or +/+.
[d] Significance is $p < 0.01$ for −/− compared with +/− or +/+.

Experiments were conducted adding exogenous IL-2 to prove that the cells from IL-2-negative mice could be induced to synthesize IFN-γ if the factor was replaced. Although it was not possible to support normal IFN-γ responses by administering IL-2 using the protocols described above, addition of IL-2 during the 24-h culture for IFN-γ production did reconstitute factor production. Supplementation of cultures of cells, from day-7 LCMV-infected mice, with 100 U/ml rIL-2 resulted in the production of 45.7 ng/spleen IFN-γ with cells from IL-2-containing +/+ mice and 36.0 ng/spleen with cells from IL-2-deficient −/− mice. Thus, cells from IL-2-negative mice could be induced to produce a high level of IFN-γ if exogenous IL-2 was added. Taken together, these results demonstrate that virus-induced IFN-γ production is IL-2 dependent.

The decreases in IFN-γ production in the absence of IL-2 could have resulted from either reductions in levels of IFN-γ production by individual cells and/or an inhibition in expansion of IFN-γ-producing cells. To distinguish between these possibilities, ELISPOT assays were conducted to quantitate the frequency and number of IFN-γ-producing cells per spleen (Table III). Frequencies of cells producing IFN-γ were decreased by 45 to 53% in IL-2-deficient −/− mice as compared with IL-2-containing +/+ or +/− littermates. On a per spleen basis, the numbers of IFN-γ-producing cells were decreased by two- to threefold. Thus, although the decrease in IFN-γ-producing cells was substantial, it did not account for the 10-fold reduction of IFN-γ production in conditioned media. Because the spots produced by IFN-γ-producing cells from the IL-2-negative −/− mice were of lower intensity than many of those from the IL-2-positive +/+ and +/− mice (not shown), the results of the ELISPOT assay were consistent with reductions in both cell frequencies and levels of IFN-γ production per cell. The

Table IV. *Effects of IL-2 deficiency on regulation of LCMV infection*[a]

Genotype	Log PFU (±SE)			
	Spleen		Kidney	
	Day 5	Day 7	Day 5	Day 7
+/+	5.35 ± 0.27 (5)[b]	1.69 ± 0.98 (2)	2.07 ± 0.26 (4)	<1.33 ± 0.14 (9)
+/−	5.16 ± 0.26 (5)	1.65 ± 0.07 (2)	2.60 ± 0.23 (5)	<1.35 ± 0.19 (9)
−/−	5.55 ± 0.58 (4)[c]	2.55 ± 0.15 (2)[d]	2.70 ± 0.48 (2)[c]	2.80 ± 0.23 (7)[e]

[a] Spleen or kidney homogenates were prepared and viral titers were quantitated as infectious plaques on confluent monolayers of Vero cells, as described in *Materials and Methods*. PFU shown are per spleen or pair of kidneys.
[b] The numbers of mice per group are indicated in parentheses.
[c] Significance is $p > 0.05$ for −/− as compared with +/+ or +/−.
[d] Significance is $p < 0.01$ as compared with +/−.
[e] Significance is $p < 0.01$ as compared with +/+ or +/−.

studies demonstrate that absence of IL-2 decreases frequencies and numbers of IFN-γ-producing cells, and suggest that cells induced to produce IFN-γ in the absence of IL-2 may release lower levels of factor per cell.

LCMV replication in IL-2-deficient mice

To assess the biologic consequences of the immunologic defects observed in IL-2 −/− mice, viral burdens were quantitated. Infectious LCMV PFUs in the spleens and kidneys of IL-2-deficient −/− mice were compared with those in IL-2-containing +/+ and +/− mice on days 5 and 7 after infection. As viral titers were similar in IL-2-positive and -negative mice on day 5 after infection, LCMV infections appeared to proceed at equivalent rates throughout this time. However, by day 7 the viral titers in mice lacking IL-2 were significantly higher than those in IL-2-containing mice; titers in +/+ and +/− littermates had decreased by almost 4 log in the spleen and to below detectable levels in the kidneys, but −/− mice had reductions of only 3 log in the spleen and no detectable change in the kidneys (Table IV). Viral burdens were still detectable in the kidneys of IL-2-negative mice on day 9 after infection (data not shown). Thus, viral infections can be regulated in the absence of IL-2, but the factor contributes to the ability to clear the virus efficiently.

Discussion

The studies presented in this report used IL-2-deficient mice to demonstrate that endogenous IL-2 is required for the profound total leukocyte increases, $CD8^+$ T cell expansion, and peak activation of CTL function and IFN-γ production induced during LCMV infections. In the absence of IL-2, virus-induced expansion of $CD8^+$ T cells was virtually eliminated, total CTL activation reduced by 90%, and IFN-γ production resulting from T cell activation was dramatically inhibited. As infected IL-2-deficient mice had prolonged viral replication, the observed reductions in T cell expansion and T cell-mediated immune responses were biologically significant. Treatments with the cell cycle-specific toxin HU demonstrated that, although T cell expansion was inhibited, the cells mediating virus-specific CTL activity in IL-2-deficient mice were induced to enter into cell cycle. $CD8^+$ T cells elicited in the absence of IL-2 were also shown to have induced expression of the IL-2R α- and β-chain components. These results show that, although T cells can be activated in vivo during viral infections through IL-2-independent mechanisms, the factor is required for extensive T cell proliferation and optimal induction of functions mediated by T cells.

The studies extend previous work from this laboratory demonstrating that, during LCMV infection, IL-2 transcription and production are induced at times corresponding with proliferation of $CD8^+$ T cells (8, 9). The profound virus-induced $CD8^+$ T cell proliferation was measured in those studies as increases in cell yields and ex vivo DNA synthesis (8, 9). The experiments showed that extended ex vivo proliferation of in vivo-activated cells was dependent on culture-produced IL-2 (8). Additional experiments with cyclosporin A, an inhibitor of IL-2 transcription, suggested that IL-2 also contributed to the extensive in vivo $CD8^+$ T cell proliferation (9). Because this drug can inhibit expression of other cytokines and intracellular signals (27, 28), however, it was not possible to define precisely IL-2 effects on the basis of sensitivity to cyclosporin A. The studies presented here demonstrate that specific deletion of IL-2 virtually eliminates the profound virus-induced $CD8^+$ T cell expansion.

Protection against LCMV infection is dependent on $CD8^+$ T cell responses and activation of virus-specific, class I histocompatibility-restricted CTL activity mediated by these cells (29–32). Our characterization of the CTL response in the absence of IL-2 is consistent with, but extends, the earlier report from Dr. Kündig and his colleagues (17). In both their studies and ours, CTL activity induced in IL-2-deficient mice on a per cell basis was about one-third of that observed in the presence of IL-2 (17; Fig. 1*A*). Our experiments, however, demonstrated that, if cell yields and LUs per organ are compared, the total CTL activity was less than one-tenth of that achieved in IL-2-containing mice (Fig. 1*C*). Furthermore, the evaluation of viral load presented here indicates that differences in immune responses developed in the absence, as compared with the presence, of IL-2 are biologically significant, because such responses are less efficient at clearing the infection (Table IV). Thus, our studies show that IL-2 plays an important role in T cell proliferation and amplification of responses depending upon these cells.

Our results are an interesting contrast to those that evaluate peptide Ag induction of CTL activation and T cell expansion (33). Using IL-2-deficient mice transgenic for a TCR specifically recognizing a presented peptide, those studies showed that CTL induction by peptide immunization is IL-2 dependent. Given the variety of cytokines known to be induced during acute viral infections (15), a likely explanation for the observed CTL induction reported here is that the conditions of infection induce alternative factors to activate CTL. The studies also differ, however, in the IL-2 requirement for T cell proliferation. Although the factor is required for the extensive virus-induced proliferation of $CD8^+$ T cells observed here (Fig. 2), the more limited T cell expansion induced by peptide stimulation of TCR-transgenic mice can proceed in the absence of IL-2 (33). The differences in the two systems may result from peptide activation of other growth factors,

perhaps as a consequence of Ag presentation by different accessory cell populations. Alternatively and more likely, they may be a result of the magnitude of T cell clonal expansion required. Frequencies of Ag-specific precursor cells are high in the transgenic mice, but low in normal mice (34). Thus, virus-induced T cell proliferation during infections of normal mice requires more cell expansion.

Results presented here contribute to the understanding of cytokine regulation and function during viral infections and reveal a previously unappreciated role for IL-2 in the development of T cell IFN-γ responses. Although T cell production of IFN-γ during viral infections has been appreciated for some time (10–13, 15),[4] the mechanisms responsible for inducing this response are poorly understood. On the basis of an observed IL-12 requirement for induction of Th1 $CD4^+$ T cells producing IFN-γ in response to in vitro stimuli (35, 36) and in vivo infections with nonviral agents (37), it has been suggested that IL-12 is the pivotal cytokine for inducing T cell production of IFN-γ (38). Studies from our laboratory have recently shown, however, that there are IL-12-independent pathways for eliciting T cell production of IFN-γ.[4] The studies shown here demonstrate that the absence of IL-2 has a profound negative effect on induction of IFN-γ production by T cells activated during LCMV infection (Fig. 5 and Table III). The data suggest that IL-2 mediates effects on IFN-γ production at two different levels. The first is at the level of IFN-γ-producing cell frequency (Table III). This could be a result of reduced IL-2-supported expansion of IFN-γ-producing cells. The effect appears to account for about 60% of the reduction in response. However, it does not explain the >90% inhibition of IFN-γ production by these cells (Fig. 5), nor the apparent responses of cells to produce high levels of factor upon addition of IL-2 (*Results*). Thus, IFN-γ-producing cells induced in the absence of IL-2 also appear to produce less factor per cell than those produced in the presence of IL-2.

A number of cell types, including $CD4^+$ T, $CD8^+$ T, and NK cells, can make IFN-γ. At days 7 to 9 after LCMV infection of normal mice, however, T cells are responsible for the detectable IFN-γ production (10, 11, 15). Both $CD4^+$ and $CD8^+$ T cells have been reported to contribute to this response (10). Our studies did not characterize the phenotype of the less frequent IFN-γ-producing cells induced in the IL-2-deficient mice. Nevertheless, they definitively established that the normal T cell response was reduced.

Although the present studies were not conducted with inbred mice, the results and conclusions were not influenced by variability in genetic backgrounds between IL-2-containing and IL-2-deficient mice. The mice used were bred to be predominately C57BL/6, but still had some 129 background (see *Materials and Methods*). Because the C57BL and 129 mice are both $H\text{-}2^b$, their CTLs recognize the same infected target cells. The other reported differences were not influenced by genetic variability because: 1) studies in inbred C57BL/6 and 129 mice demonstrated that LCMV infection of either genetic background induced significant $CD8^+$ T cell expansion (L. P. Cousens, H. C. Su, and C. A. Biron, unpublished observations); 2) addition of exogenous IL-2 reconstituted the T cell-proliferative response (Table II) in, and enhanced IFN-γ production by, cells (*Results*) from IL-2-deficient mice; and 3) the data demonstrating reduced T cell responses in IL-2-negative as compared with IL-2-positive mice were obtained with replicate animals and multiple experiments with different litters (Figs. 1, 2, 4, and 5, and Tables I and IV). Complications that may have resulted from endogenous diseases reported to develop in the IL-2-deficient mice (16, 39) were limited by conducting all of the studies with young mice that had no overt signs of illness, normal hematocrits, and unremarkable bowel histology at the time of harvest (see *Materials and Methods*).

In the experiments examining lymph node cell yield (Table I) and IFN-γ production (Fig. 5), there appeared to be effects of IL-2 gene dosage on the immune responses. In IL-2 +/+ mice, these responses peaked on day 7 and were either subsiding or at a plateau on day 9 after infection. However, in IL-2 +/− mice, the responses were continuing to rise on day 9 as compared with day 7. Experiments to characterize the mechanisms responsible for differences between +/+ and +/− mice are beyond the scope of this study. However, if IL-2 availability regulates the kinetics of peak responses, a mechanism for the observed differences might be reduced IL-2 production resulting from a half-gene dose.

The studies of IL-2R expression on $CD8^+$ T cells suggest a number of interesting hypotheses about regulation of these molecules and the cells that express these molecules (Fig. 4). First, because virtually all of the $CD8^+$ T cells in normal mice, and most of those in IL-2-deficient mice, expressed intermediate levels of IL-2R β during LCMV infection, it appears that this chain can be induced at low levels even if T cells have not been specifically activated through the TCR. Second, because the proportions of cells induced to express high levels of IL-2R β are roughly equivalent to those induced to express IL-2R α, the populations expressing both chains may be the cells specifically stimulated by Ag. Third, the lower proportions of $CD8^+$ T cells expressing IL-2R α- and β-chains in IL-2-deficient as compared with IL-2-containing infected mice suggest that a consequence of IL-2 expression may be the preferential expansion of Ag-specific cells expressing high-affinity receptors for the factor. These possibilities are being further investigated in other studies.

As T cells are induced to mediate lower levels of CTL activity, enter into cell cycle, and express IL-2R molecules in the absence of IL-2, there must be other cytokines that can induce these responses. Both IL-4 and IL-7 have been shown to utilize the γ-chain (40–42), and a recently identified cytokine, IL-15, has been shown to use the β- and γ-chains of the IL-2R for signal transduction (43–45). It has been clearly shown that these factors can induce and/or support the expansion of CTL (45–48). As studies in this laboratory have documented that the induction of IL-4 at times overlaps with IL-2 expression, during LCMV infections of normal mice (H. C. Su and C. A. Biron, manuscript in preparation), there is a possibility that this factor may contribute to the activation of at least some of the observed responses.

In summary, we demonstrate here that during viral infection IL-2 is required for in vivo $CD8^+$ T cell proliferation and promotes the magnitude of the CTL and IFN-γ production responses. These changes in endogenous immune responses have biologic consequences and result in a reduced ability to clear the viral infection. The studies also show that, although the absence of IL-2 has profound negative effects on endogenous immune responses, T cells do receive alternative in vivo activation signals because they are induced to express IL-2Rs and enter into the cell cycle. The results are consistent with a model by which other factors are induced during viral infection to activate the immune response. The data clearly show, however, that IL-2 is unique in its ability to sustain extensive $CD8^+$ T cell proliferation in vivo.

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