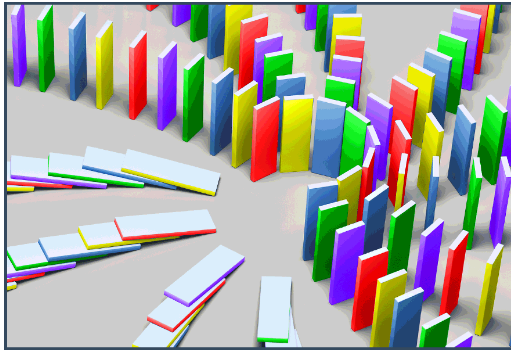
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Memory-Type CD8$^+$ T Cells Protect IL-2 Receptor α-Deficient Mice from Systemic Infection with Herpes Simplex Virus Type 2[1]

Hironaka Tsunobuchi,* Hitoshi Nishimura,* Fumi Goshima,† Tohru Daikoku,† Yukihiro Nishiyama,† and Yasunobu Yoshikai[2]*

IL-2Rα-deficient (IL-2R$\alpha^{-/-}$) mice exhibit an impaired activation-induced cell death for T cells and develop abnormal T cell activation with age. In our study, we found that IL-2R$\alpha^{-/-}$ mice at the age of 5 wk contained an increased number of CD44$^+$CD69$^-$CD8$^+$ T cells in lymph nodes, which expressed a high intensity of IL-2Rβ and vigorously proliferated in response to a high dose of IL-15 or IL-2. The T cells produced a large amount of IFN-γ in response to IL-15 plus IL-12 in a TCR-independent bystander manner. When IL-2R$\alpha^{-/-}$ mice were inoculated i.p. with HSV type 2 (HSV-2) 186 strain, they showed resistance to the infection accompanied by an increased level of serum IL-15. The depletion of CD8$^+$ T cells by in vivo administration of anti-CD8 mAb rendered IL-2R$\alpha^{-/-}$ mice susceptible to HSV-2-induced lethality. These results suggest that memory-type CD8$^+$ T cells play a novel role in the protection against HSV-2 infection in IL-2R$\alpha^{-/-}$ mice. *The Journal of Immunology,* **2000, 165: 4552–4560.**

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Interleukin 2 and its receptor (IL-2R) have been known to play a major role in the activation and proliferation of NK (1), T (2), and B cells (3). The high affinity IL-2R is composed of three subunits, α-, β-, and γ-chains (4). IL-2Rα is expressed only on activated lymphocytes, whereas IL-2Rβ and IL-2Rγ chains are constitutively expressed on resting lymphocytes and form the low affinity IL-2R. The IL-2Rα chain interacts exclusively with IL-2, whereas the β-chain has been shown to bind to IL-15 as well as IL-2 (5–8). The IL-2Rγ chain, called the common γ-chain, is shared among receptors for IL-4, IL-7, IL-9, and IL-15 (6, 9–11). IL-2Rα-deficient (IL-2R$\alpha^{-/-}$)[3] mice have no apparent defect in T or B cell development (12), suggesting that IL-2Rα is not required for the generation of mature T and B cells. In contrast, these mice exhibit an impaired activation-induced cell death for T cells and consequently develop abnormal T and B cell activation and autoimmune disorders, including hemolytic anemia and inflammatory bowel disease, with age (12). These results suggest that the predominant role of IL-2Rα in vivo is to deliver negative regulatory signals within the peripheral lymphoid compartment.

HSV is an important human pathogen that causes a variety of diseases, ranging from mild skin disorders to life-threatening encephalitis (13, 14). Animal models of infection with HSV have been established to study mechanisms by which the virus causes disease, and studies have demonstrated that the cellular immune system contributes to the recovery from infection (14, 15). Protective mechanisms against primary infection with HSV are mediated by two major waves of host responses: innate immunity and adaptive immunity. Innate immunity depends mainly on phagocytes, including neutrophils (16, 17) and macrophages (18, 19), which preexist and destroy the virus within hours in an Ag-nonspecific way. Adaptive immunity depends on immune responses mediated by class I-restricted CD8$^+$ CTL (20) and class II-restricted CD4$^+$ Th1 cells secreting IFN-γ (21), which contribute to the final clearance of the virus. Recently, a novel host defense mechanism linking innate and acquired immunity has been proposed (22). NK cells (23–25) and TCR-$\gamma\delta$ T cells (26) play important roles in protection against murine HSV type 1 infection. Because IL-2 plays important roles in enhancing cellular proliferation and promoting functional differentiation of NK and T cells, IL-2 is thought to be a key mediator in protection against HSV infection. In fact, in vivo administration of rIL-2 augmented the protective immunity against HSV type 1 (27). In contrast, immune responses are relatively normal in mice lacking the IL-2 or IL-2Rα chain. It has been shown that mice lacking IL-2 show normal CTL responses against lymphocytic choriomeningitis virus or vaccinia virus and exhibit only marginally reduced Th cell responses against vesicular stomatitis virus (28). These findings suggest that other cytokines compensate for the lack of IL-2 function in mice lacking this cytokine after viral infection.

IL-15 has recently been shown to bind to the β- and γ-chains of the IL-2 receptor (5–8). In IL-2/IL-15Rβ-deficient mice, NK cells, NK1.1$^+$TCR-$\alpha\beta^+$ T cells, CD8$\alpha\alpha^+$TCR-$\alpha\beta^+$, or TCR-$\gamma\delta^+$ intestinal intraepithelial lymphocytes are disrupted in development, although early development of the thymus is normal (29, 30). Lodelce et al. (31) showed that IL-15Rα knockout mice are deficient in NK cells, NK1.1$^+$ T cells, and TCR-$\gamma\delta$ intraepithelial lymphocytes. In addition to these cells, memory phenotype CD8$^+$ T cells are severely reduced in IL-15Rα knockout mice. Memory-type CD8$^+$ T cells are reported to expand in response to exogenous IL-15 or various infectious agents that can elicit IL-15 production (32). It therefore appears that IL-15 has potential roles in the development and maintenance of significant fractions of lymphocytes, including NK, NK T, $\gamma\delta$, and memory-type CD8$^+$ T cells.

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[3] Abbreviations used in this paper: IL-2R$\alpha^{-/-}$ mice, IL-2Rα-deficient mice; PEC, peritoneal exudate cells; LN, lymph node; MACS, magnetic cell separator.

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In this study, we characterized memory-type $CD8^+$ T cells increasing in naive IL-2R$\alpha^{-/-}$ mice and examined the roles of the T cells in the host defense mechanisms against systemic infection with HSV type 2 (HSV-2) in IL-2R$\alpha^{-/-}$ mice. Our results revealed that IL-2R$\alpha^{-/-}$ mice showed resistance against HSV-2 infection accompanied by marked increases in memory $CD8^+$ T cells and IL-15 production. The susceptibility was significantly increased in IL-2R$\alpha^{-/-}$ mice depleted of $CD8^+$ T cells by in vivo treatment with anti-CD8 mAb. The implications of these findings for potential roles of IL-15-dependent memory-type $CD8^+$ T cells in protection against HSV-2 infection are discussed.

Materials and Methods

Mice

Mice genetically deficient in IL-2Rα gene expression (12) bred to the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). A homogeneous population was established by backcrossing heterozygotes to C57BL/6 mice of more than five generations. The resultant heterozygotes were bred to obtain homozygotes. In each experiment, age-matched female mice were used. In some experiments, siblings from the same mother were used. Mice were maintained under specific pathogen-free conditions and given food and water ad libitum. All mice were used at 5 to 6 wk of age.

Abs and reagents

PE-conjugated anti-TCR-$\gamma\delta$, anti-NK1.1, anti-CD44, and anti-CD8 mAb; FITC-conjugated anti-CD3, anti-CD69, anti-Ly-6c, and anti-IL-2Rβ mAb; biotin-conjugated anti-TCR-$\alpha\beta$ and anti-CD8 mAb; Cy-Chrome-conjugated anti-CD4 mAb; and Cy-Chrome-conjugated streptavidin were purchased from PharMingen (San Diego, CA). Anti-TCR-$\alpha\beta$ mAb (H57-597) was a gift from Dr. R. Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Purified anti-murine IL-15 mAb (rat IgG1) and biotin-conjugated anti-murine IL-15 mAb (rat IgG1) were purchased from PharMingen. Human rIL-15 and rIL-12 were purchased from Peprotech (London, U.K.). Human rIL-2 was purchased from Takeda Chem (Osaka, Japan).

Virus and infections

The 186 strain of wild-type HSV-2 was originally obtained from Fred Rapp, Pennsylvania State University College of Medicine, Hershey, PA (33). The viral stock was grown in monolayer cultures of Vero cells overlaid with MEM supplemented with 5% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The stock was stored frozen at 2–5 $\times 10^6$ PFU/ml. The virus was diluted into PBS just before infection. Mice were infected at 5–6 wk of age. Mice were injected i.p. with 5×10^3 PFU strain 186 in 200 μl PBS. In some experiments, 300 μg of anti-CD8 (clone 2.43, rat IgG2b) mAb or isotype control rat IgG were administered to mice 2 days before an i.p. challenge with HSV.

Cell preparation

Peritoneal exudate cells (PEC) were prepared by centrifuging peritoneal exudates at $110 \times g$ for 10 min and suspending them at a concentration of 10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. Cells were plated in the wells of 100-mm tissue culture dishes (Nunc, Roskilde, Denmark) and allowed to adhere for 1 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. In some experiments, nonadherent cells were passed over a nylon wool column and subsequently purified by using a magnetic cell separator (MACS) column according to the manufacturer's instructions. $CD4^+$ or $CD8^+$sorting was performed with directly coupled CD4 or CD8 magnetic beads, respectively. The purified $CD4^+$ or $CD8^+$ T cells were >98% as assessed by FACS analysis.

Flow cytometry

Lymph node (LN) cells and nonadherent PEC were stained with FITC-, PE-, and biotin-conjugated mAbs. To block FcR-mediated binding of the mAb, 2.4 G2 (anti-FcgR mAb) was added. All incubation steps were performed at 4°C for 30 min. To detect biotin-conjugated mAb, cells were stained with Cy-Chrome-conjugated streptavidin after incubation with a primary mAb. The stained cells were analyzed by a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). Small lymphocytes were gated by forward and side scatter.

Cell culture

Lymphocytes from LN were cultured in 200 μl complete culture medium in a 96-well flat-bottom plate (Falcon, Beckon Dickinson, Oxford, U.K.) at a density of 5×10^5–1×10^6 cells/well with rIL-2, rIL-15, and/or rIL-12 or anti-TCR-$\alpha\beta$ mAb (100 μg/ml) that had been immobilized on the plates by prior incubation for 1 h [^{3}H]TdR incorporation was measured by liquid scintillation counting following a 1 μCi [^{3}H]TdR pulse for the last 6 h. To estimate cytokine production, the supernatant were collected at the indicated times.

Intracellular cytokine staining

The collected cells were washed with PBS/0.1% NaN_3/1% FCS (FACS buffer) and stained with cell surface markers. After washing a further two times, the cells were then resuspended in a 1% paraformaldehyde in PBS, incubated for 30 min at 4°C, and then thoroughly washed with FACS buffer. For intracellular staining, the fixed cells were resuspended in PBS/0.1% saponin/1% FCS and stained with FITC anti-mouse IFN-γ or FITC rat IgG1 as an isotype control (PharMingen) for 30 min at 4°C. The cells were then spun at $300 \times g$ for 5 min and washed twice in 1 ml FACS buffer. The stained cells were analyzed by a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

T cell responses to HSV Ag

The enriched $CD4^+$ or $CD8^+$ T cells from PEC were cultured in 200 μl complete culture medium in 96-well flat-bottom plates (Falcon) at a density of 5×10^5 cells/well, with the same number of mitomycin-treated spleen cells from B6 mice with or without 2.5×10^4 PFU heat-inactivated HSV-2 (56°C for 1 h) or with anti-TCR-$\alpha\beta$ mAb (100 μg/ml) that had been immobilized on the plates by prior incubation for 1 h. After culturing for 48 h, the supernatant was collected to estimate cytokine production.

Cytokine ELISA

The cell-free culture supernatants were collected at the indicated times. The cytokine activity in the culture supernatant was assayed by an ELISA using an IL-2 or IFN-γ DuoSet ELISA Development System (Genzyme Diagnostics, Cambridge, MA). ELISA for mouse IL-15 was performed in triplicate using purified anti-mouse IL-15 mAb (capture mAb, G277-3588, PharMingen), biotin-conjugated anti-mouse IL-15 mAb (second mAb, G277-3960, PharMingen), and peroxidase-conjugated streptavidin (detection reagent, Genzyme).

Statistical analysis

The statistical significance of the data was determined by Student's t test except for lethality data, which were analyzed by the generalized Wilcoxon test. $p < 0.05$ was taken as significant.

Results

Characteristics of $CD8^+$ T cells in IL-2R$\alpha^{-/-}$ mice

IL-2R$\alpha^{-/-}$ mice have been reported to develop massive enlargement of LN due to expansion of lymphocytes consisting of otherwise normal proportions of $CD4^+$ and $CD8^+$ T cells (12). Consistent with this, the absolute number of $CD3^+$ T cells was markedly increased in LN of IL-2R$\alpha^{-/-}$ mice at the age of 5 wk compared with those in IL-2R$\alpha^{+/-}$ mice of the same age (data not shown). However, the proportion of $CD8^+$ T cells in IL-2R$\alpha^{-/-}$ mice was 2-fold higher than that in IL-2R$\alpha^{+/-}$ mice (Fig. 1*A*). As shown in Fig. 1*B*, most of the $CD8^+$ T cells in IL-2R$\alpha^{-/-}$ mice expressed a memory phenotype ($CD44^+$, $CD69^-$, and Ly-$6c^+$) and IL-2/IL-15Rβ.

Stimulation of LN cells from IL-2R$\alpha^{-/-}$ mice by IL-2/IL-15

We next examined the effect of IL-2 or IL-15 on the expansion of $CD44^+CD8^+$ T cells constitutively expressing an IL-2/IL-15Rβ chain in IL-2R$\alpha^{-/-}$ mice. We have previously reported that memory-type $CD8^+$ T cells can proliferate rapidly in response to immobilized anti-TCR-$\alpha\beta$ mAb for 24 h (34). Therefore, the LN cells from IL-2R$\alpha^{-/-}$ or IL-2R$\alpha^{+/-}$ mice were cultured with immobilized anti-TCR-$\alpha\beta$ mAb or a high dose of human rIL-2 (1 μg/ml) or human rIL-15 (1 μg/ml) for 24 h. LN cells from IL-2R$\alpha^{+/-}$ mice proliferated on TCR triggering but only marginally

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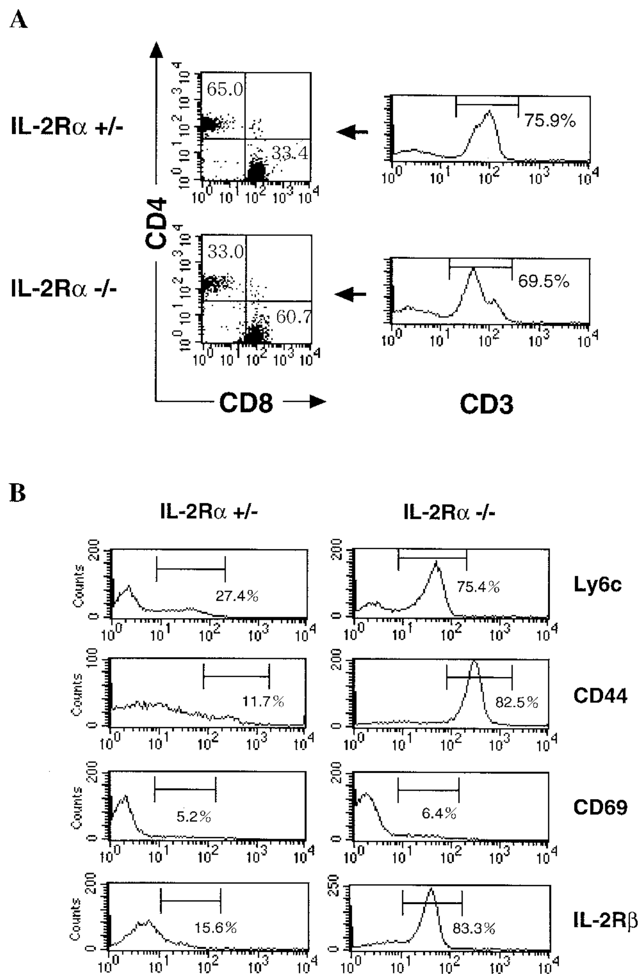

FIGURE 1. Flow cytometric analysis of LN cells from IL-2Rα-deficient mice. LN cells from IL-2R$\alpha^{-/-}$ mice and their littermates (+/−) were collected for flow cytometric analysis. *A*, Cells were stained with FITC-anti-CD3, PE-anti-CD8α, and Cy-Chrome-anti-CD4 mAbs. Dot plot analysis was gated on CD3-positive cells. *B*, CD8α-positive cells were gated, and histograms represent staining with the indicated Abs (Ly-6c, CD44, CD69, and IL-2Rβ). Lymphocytes were gated by forward (FSC) and side (SSC) scatter. Representative data from three independent experiments are shown.

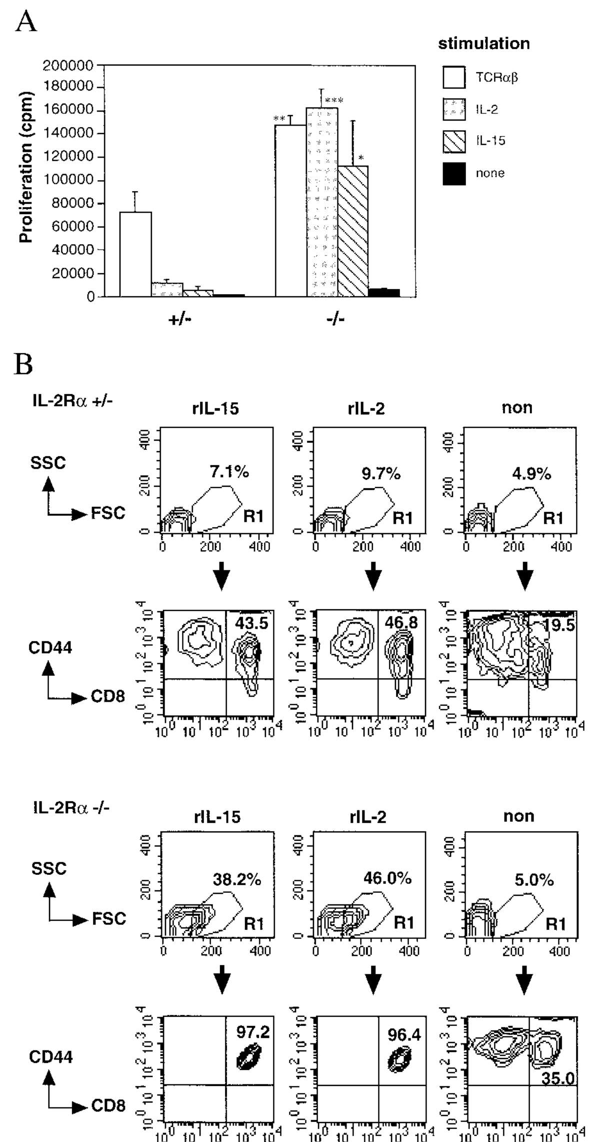

FIGURE 2. Expansion of CD44$^+$CD8$^+$ T cells in IL-2Rα-deficient mice by exogenous IL-2 and IL-15. *A*, Proliferation of LN cells from wild-type and IL-2Rα-deficient mice in response to immobilized anti-TCR-αβ mAb, exogenous IL-2, or IL-15. LN cells were cultured with or without immobilized TCR-αβ mAb, human rIL-2 (1 μg/ml), or human rIL-15 (1 μg/ml) for 24 h at 37°C. [^{3}H]TdR incorporation was measured by liquid scintillation counting following a 1-μCi [^{3}H]TdR pulse for the last 6 h. There was a statistically significant difference between the two groups (*, $p < 0.005$; **, $p < 0.001$; and ***, $p < 0.0001$ by Student's *t* test). *B*, Flow cytometric analysis of cultured LN cells from wild-type and IL-2Rα-deficient mice. LN cells were cultured with or without rIL-2 (1 μg/ml) or rIL-15 (1 μg/ml) for 24 h at 37°C. The cells were then harvested and stained with anti-CD8α and anti-CD44 mAb. Blastoid cells were gated on the basis of forward and side scatter as R1. CD44 expression on the responding cells, as evidenced by their blastoid appearance to rIL-2 or rIL-15, is presented as typical two-dimensional profiles. Representative data from three independent experiments are shown.

responded to rIL-2 or rIL-15. In contrast, those from IL-2R$\alpha^{-/-}$ mice proliferated vigorously in response to a high dose of IL-2 or IL-15 (Fig. 2*A*). To identify the responding cells in LN from IL-2R$\alpha^{+/-}$ mice, the blastoid cells were analyzed by forward scatter (cell size) and side scatter (granularity) on a flow cytometer. Consistent with the results of the proliferation assay with [^{3}H]thymidine, rIL-15 or rIL-2 significantly increased the proportion of large blastoid cells after 48 h culture of LN cells from IL-2R$\alpha^{-/-}$ mice (Fig. 2*B*). Flow cytometric analysis with anti-CD44 and anti-CD8 mAbs revealed that the blastoid cells after culture with rIL-15 or rIL-2 represented CD44$^+$CD8$^+$ T cells. These results indicate that CD44$^+$CD8$^+$ T cells in IL-2R$\alpha^{-/-}$mice proliferated in response to either rIL-15 or rIL-2.

To assess cytokine production by CD44$^+$CD8$^+$ T cells in IL-2R$\alpha^{-/-}$mice, we examined cytokine activity in the culture supernatant of LN cells stimulated with immobilized anti-TCR-αβ mAb or rIL-15. Memory-type T cells are characterized by early cytokine production after TCR engagement (35, 36). Consistent with these reports, IFN-γ was rapidly produced by LN cells in IL-2R$\alpha^{-/-}$mice within 24 h after stimulation with immobilized anti-TCR-αβ mAb, whereas LN cells from IL-2R$\alpha^{+/-}$ mice produced only a marginal level of IFN-γ 24 h after stimulation (Fig. 3*A*). LN cells from either IL-2R$\alpha^{-/-}$ or IL-2R$\alpha^{+/-}$ mice produced only small amounts of IFN-γ in response to rIL-15 alone or rIL-12 alone, whereas those from IL-2R$\alpha^{-/-}$mice produced a large amount of IFN-γ in response to rIL-15 plus rIL-12 (Fig. 3*B*). Other

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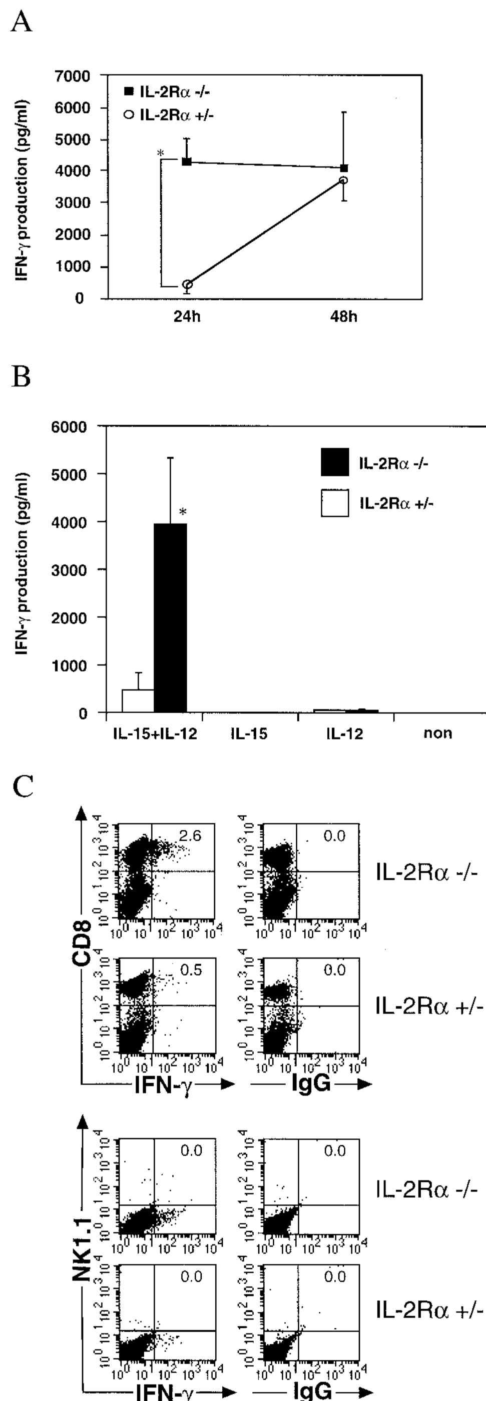

FIGURE 3. IFN-γ production of LN cells in response to immobilized anti-TCR-$\alpha\beta$ mAb or rIL-15 plus rIL-12 in IL-2Rα-deficient mice. LN cells (1×10^6 cells/well) from IL-2R$\alpha^{-/-}$ and IL-2R$\alpha^{+/-}$ mice were cultured with immobilized TCR-$\alpha\beta$ mAb for 24 or 48 h (*A*) or with rIL-15 (100 ng/ml) or rIL-12 (100 ng/ml) or rIL-15 plus rIL-12 (100 ng/ml) for 48 h at 37°C (*B*). At the indicated times, culture supernatants were collected for IFN-γ measurement (*, $p < 0.0001$ by Student's *t* test). Representative data from three independent experiments are shown. *C*, LN cells cultured with rIL-15 plus rIL-12 (100 ng/ml) for 48 h were collected. For intracellular cytokine staining, 10 μg/ml brefeldin A was added for the last 4 h. Then the cells were harvested and stained with PE-anti-NK1.1 and biotin-anti-CD8α mAbs. After surface staining, cells were stained for the expression of intracellular IFN-γ described in *Materials and Methods*.

cytokines, including IL-4, IL-10, TNF-α, and IL-13, were not detected in the culture supernatants. To identify the IFN-γ-producing cells in response to IL-15 plus IL-12, we performed intracellular cytokine staining. The results shown in Fig. 3*C* indicated that cells producing IFN-γ in response to IL-15 plus IL-12 were mainly CD8$^+$ T cells but not NK1.1$^+$ cells in IL-2R$\alpha^{-/-}$ mice. These results suggest that memory-phenotype CD44$^+$CD8$^+$ T cells in IL-2R$\alpha^{-/-}$ mice can expand and produce IFN-γ in response to exogenous IL-15 plus IL-12.

Susceptibility to HSV-2 infection in IL-2R$\alpha^{-/-}$ mice

We next define the roles of the memory-type CD8$^+$ T cells in protection against systemic infection with HSV-2. As shown in Fig. 4, all IL-2R$\alpha^{+/-}$ and IL-2R$\alpha^{-/-}$ mice survived for >30 days after an i.p. inoculation with 500 PFU of strain 186, and 50% of the IL-2Rα+/− mice and 40% of the IL-2R$\alpha^{-/-}$ mice survived after an i.p. inoculation with 5000 PFU of strain 186. A significant difference was not observed between the survival rates of IL-2R$\alpha^{+/-}$ mice and IL-2R$\alpha^{-/-}$ mice (NS by the generalized Wilcoxon test). Thus, IL-2R$\alpha^{-/-}$ mice showed resistance against systemic infection with HSV-2.

Flow cytometric analysis of PEC from IL-2R$\alpha^{-/-}$ mice after HSV-2 infection

To compare the cellular responses to systemic infection with HSV-2 in IL-2R$\alpha^{-/-}$ and IL-2R$\alpha^{+/-}$ mice, the expressions for NK1.1, CD3, TCR-$\alpha\beta$, TCR-$\gamma\delta$, CD4, and CD8 were examined on the PEC by means of flow cytometer. Typical results of flow cytometric analysis before and on days 3 and 6 after infection with HSV-2 wild-type strain 186 (5000 PFU) are shown in Fig. 5, and mean ± SE values based on the absolute cell number of lymphocytes in each group are shown in Table I. The emergence of NK1.1$^+$CD3$^-$ cells, which are representative of NK cells, and TCR-$\gamma\delta^+$CD3$^+$ cells was detected in the peritoneal cavity of IL-2R$\alpha^{+/-}$ mice on day 3 after HSV-2 infection, whereas such increases, if any, were only marginal in IL-2R$\alpha^{-/-}$ mice ($p < 0.01$). The percentage and absolute number of CD3$^+$ cells in the peritoneal cavity of IL-2R$\alpha^{-/-}$ mice were much higher than those in the littermates (IL-2R$\alpha^{+/-}$ mice) before infection ($p < 0.05$). Similar to the case of LN cells, a markedly increased percentage of CD8$^+$ T cells was found in the peritoneal cavity of IL-2R$\alpha^{-/-}$ mice before infection (Fig. 5, Table I; $p < 0.05$). Thus, the appearance of NK cells or TCR-$\gamma\delta^+$ T cells was impaired during the course of

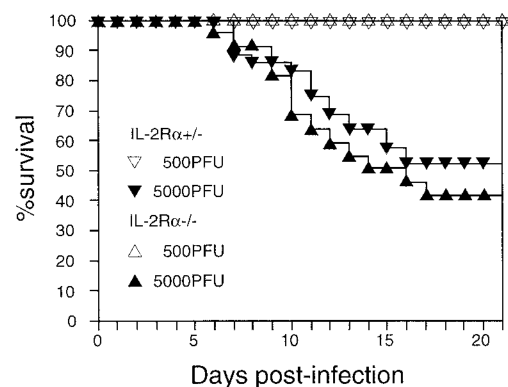

FIGURE 4. Survival rates of IL-2Rα-deficient mice after infection with HSV-2 strain 186. IL-2R$\alpha^{-/-}$ mice and their littermates (+/−) (35 mice in each group) were inoculated i.p. with 5×10^2 or 5×10^3 PFU HSV-2 strain 186 and monitored for survival. There were no statistically significant differences between the survival rates of IL-2R$\alpha^{-/-}$mice and their control littermates (NS by the generalized Wilcoxon test).

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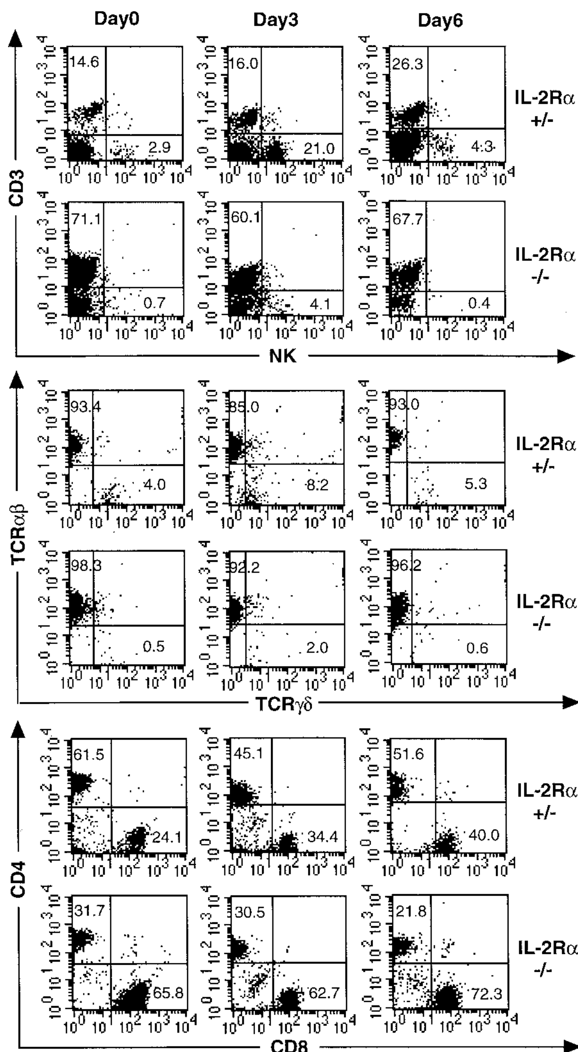

FIGURE 5. Flow cytometric analysis of PEC from IL-2Rα-deficient mice on days 3 and 6 after infection with HSV-2 strain 186. IL-2Rα$^{-/-}$ mice and their littermates (+/−) were inoculated i.p. with 5×10^3 PFU of HSV-2 strain 186. At 3 days and 6 days later, non-plastic-adherent PEC were recovered and stained with anti-CD3ε and anti-NK1.1 mAbs, or with anti-CD3ε, anti-TCR-γδ and anti-TCR-αβ mAbs, or with anti-CD3ε, anti-CD4, anti-CD8α mAbs. Cells gated on CD3$^+$ cells were analyzed for their expression of TCR-αβ and TCR-γδ or of CD4 and CD8α. Numbers represent the percentage of total cells found in each quadrant. Representative data from three independent experiments are shown.

HSV-2 infection, whereas a markedly increased number of CD8$^+$ T cells were present in the peritoneal cavity of IL-2Rα$^{-/-}$ mice before and after systemic HSV-2 infection.

IL-15 production in IL-2Rα$^{-/-}$ mice after an i.p. inoculation with HSV-2

We monitored the IL-15 and IL-2 production in the serum of IL-2Rα$^{-/-}$ mice after an i. p. inoculation with HSV-2. IL-15 production was detected after infection in both IL-2Rα$^{-/-}$ and IL-2Rα$^{+/-}$ mice, but a significantly higher level of IL-15 was detected in IL-2Rα$^{-/-}$ mice on days 3 and 6 during HSV-2 infection (Fig. 6). IL-2Rα$^{-/-}$ mice spontaneously produced IL-2 before infection, and a high level of IL-2 was sustained after HSV-2 infection. We also monitored serum IL-12p70 and IFN-γ levels after systemic HSV-2 infection. However, no cytokine activity was detected in serum by our ELISA method.

Impaired generation of protective Th1 cells producing IFN-γ in IL-2Rα$^{-/-}$ mice after HSV-2 infection

Ag-specific Th1 response producing IFN-γ is important for protection against HSV infection. To determine whether HSV-2-specific Th1 cell responses occurred in IL-2Rα$^{-/-}$ mice after systemic infection with HSV-2, we compared IL-2 and IFN-γ productions by the peritoneal T cells in response to HSV-2 in IL-2Rα$^{-/-}$ mice and IL-2Rα$^{+/-}$ mice on day 6 after systemic HSV-2 infection. We separated CD4$^+$ T cells and CD8$^+$ T cells from the peritoneal exudate T cells of the infected mice by MACS. The purity of each population was confirmed to be >98% by FACS analysis. As shown in Fig. 7, IFN-γ production by CD4$^+$ T cells from IL-2Rα$^{-/-}$ mice in response to HSV-2 was significantly lower than that in the IL-2Rα$^{+/-}$ mice, whereas a higher level IL-2 production in response to HSV-2 was detected in IL-2Rα$^{-/-}$ mice. IL-4 production was undetectable in the culture supernatants of CD4$^+$ T cells derived from either IL-2Rα$^{-/-}$ mice or IL-2Rα$^{+/-}$ mice. Thus, HSV-2-specific CD4$^+$ T cells producing IL-2 were generated, but the generation of Th1 cells capable of producing IFN-γ was impaired in IL-2Rα$^{-/-}$ mice infected with HSV-2. In contrast, CD8$^+$ T cells from HSV-2-infected IL-2Rα$^{+/-}$ mice or IL-2Rα$^{-/-}$ mice did not respond to exogenous HSV-2 Ag (data not shown).

Effect of CD8$^+$ T cell depletion on protection against HSV-2 infection

To test whether the increased number of CD8$^+$ T cells contribute to the resistance against HSV-2 infection in IL-2Rα$^{-/-}$ mice, CD8$^+$ T cell-depleted mice were prepared by in vivo administration of anti-CD8 mAb. IL-2Rα$^{-/-}$ and IL-2Rα$^{+/-}$ mice were injected with 300 μg purified anti-CD8 mAb or isotype control rat IgG 2 days before infection. We confirmed by FACS analysis that CD8$^+$ T cells were completely depleted in the peritoneal cavity of IL-2Rα$^{-/-}$ and IL-2Rα$^{+/-}$ mice 6 days after i.p. challenge with HSV-2. As shown in Fig. 8, there was no statistically significant difference between the survival rates of IL-2Rα$^{+/-}$ mice treated with anti-CD8 mAb and those treated with control IgG. In contrast, all IL-2Rα$^{-/-}$ mice depleted of CD8$^+$ T cells died within 10 days after HSV-2 infection, whereas 40% of IL-2Rα$^{-/-}$ mice treated with control IgG survived ($p < 0.05$ by the generalized Wilcoxon test). These results suggested that CD8$^+$ T cells appearing in IL-2Rα$^{-/-}$ mice play an important role in the protection against systemic infection with HSV-2.

Discussion

In this study, we found that IL-2Rα$^{-/-}$ mice had remarkably increased numbers of memory phenotype CD8$^+$ T cells, which produced IFN-γ in response to IL-15 and IL-12. IL-2Rα$^{-/-}$ mice showed resistance to systemic infection with HSV-2 accompanied by increases in memory CD8$^+$ T cells and IL-15 production. The results of a study with in vivo deletion of CD8$^+$ T cells suggest that the memory phenotype CD8$^+$ T cells protect against systemic infection with HSV in IL-2Rα$^{-/-}$ mice. The absence of an IL-2Rα chain suggests that memory-type CD8$^+$ T cells have a unique role in protective mechanisms against HSV infection.

IL-2 or the high-affinity IL-2R is thought to play a key role in immunoregulation by influencing the balance between clonal expansion and cell death after T cell activation (37). Willerford et al. (12) reported that mice deficient in IL-2Rα develop massive enlargement of peripheral lymphoid organs associated with polyclonal T and B cell expansion. The 5-wk-old IL-2Rα$^{-/-}$ mice in the present study had twice the number of CD8$^+$ T cells as CD4$^+$ T cells in LN, and most of the CD8$^+$ T cells were of the

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Table I. *Absolute cell number of each subset in the nonadherent PEC of IL-2Rα$^{-/-}$ and IL-2Rα$^{+/-}$ mice before and after infection with HSV-2 strain 186*[a]

	CD3$^+$		CD3$^+$		
	CD4$^+$	CD8$^+$	TCRαβ$^+$	TCRγδ$^+$	NK$^+$CD3$^-$
IL-2Rα$^{-/-}$					
Day 0	(3.1 ± 0.2) × 10^5*	(7.7 ± 2.2) × 10^5*	(11.0 ± 2.3) × 10^5*	(0.8 ± 0.2) × 10^4	(0.6 ± 0.1) × 10^4*
Day 3	(5.3 ± 1.2) × 10^5	(11.0 ± 1.7) × 10^5**	(16.0 ± 2.8) × 10^5*	(1.9 ± 0.3) × 10^4**	(6.5 ± 3.1) × 10^4**
Day 6	(7.3 ± 2.2) × 10^5	(15.8 ± 2.1) × 10^5*	(20.2 ± 3.2) × 10^5	(2.2 ± 0.4) × 10^4**	(3.2 ± 1.1) × 10^4**
IL-2Rα$^{+/-}$					
Day 0	(1.5 ± 0.0) × 10^5	(0.5 ± 0.1) × 10^5	(2.0 ± 0.1) × 10^5	(1.3 ± 0.0) × 10^4	(3.8 ± 0.8) × 10^4
Day 3	(2.7 ± 0.5) × 10^5	(2.2 ± 0.4) × 10^5	(5.6 ± 1.1) × 10^5	(4.5 ± 0.3) × 10^4	(43.7 ± 4.0) × 10^4
Day 6	(11.7 ± 0.5) × 10^5	(8.4 ± 0.7) × 10^5	(20.7 ± 0.9) × 10^5	(13.6 ± 1.4) × 10^4	(48.9 ± 7.8) × 10^4

[a] Data were obtained from three to nine mice and are expressed as mean ± SE. Numbers represent absolute numbers of each subset in the nonadherent PEC per mouse. Asterisks, Significantly different from the values of control mice (*, $p < 0.05$; **, $p < 0.01$).

CD44$^+$Ly-6c$^+$CD69$^-$ memory phenotype. IL-15 has recently been shown to be important in expansion and maintenance of memory CD8$^+$ T cells (31, 32) as well as NK, NK T cells and a significant fraction of γδ T cells. We recently constructed transgenic mice expressing IL-15 cDNA encoding a secretable isoform, and we found that the IL-15-transgenic mice contained a larger number of memory-type CD8$^+$ T cells expressing CD44^{high}CD62L$^-$Ly-6c$^+$ in the peripheral lymphoid tissues (34). It therefore appears that IL-15 may play an important role in the expansion of memory-type CD8$^+$ T cells in naive IL-2Rα$^{-/-}$ mice. However, the results of the present study indicated that serum IL-2, but not IL-15, was spontaneously detected in naive IL-2Rα$^{-/-}$ mice and that in vitro stimulation with a high dose of IL-2 as well as IL-15 can induce massive proliferation of memory CD8$^+$ T cells, suggesting that spontaneous IL-2 production may be responsible for expansion of the memory-type CD8$^+$ T cells in naive IL-2Rα$^{-/-}$ mice. Recently, a small amount of IL-15

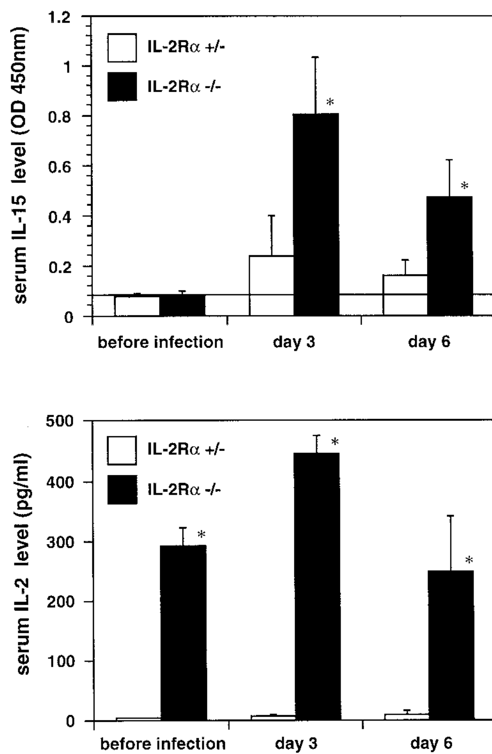

FIGURE 6. IL-15 and IL-2 production in IL-2Rα-deficient mice after an i.p. challenge with HSV-2 strain 186. Sera were collected from IL-2Rα$^{-/-}$ and IL-2Rα$^{+/-}$ mice on days 3 and 6 after an i.p. challenge with HSV-2. The IL-15 and IL-2 protein levels were determined by mouse ELISA. The level of IL-15 was expressed as net $OD_{450\ nm}$ (mean ± SD of 10 mice). The IL-15 level at the line in the figure is shown as the amount of the detection limit. There was a statistically significant difference between the two groups (*, $p < 0.05$ by Student's *t* test).

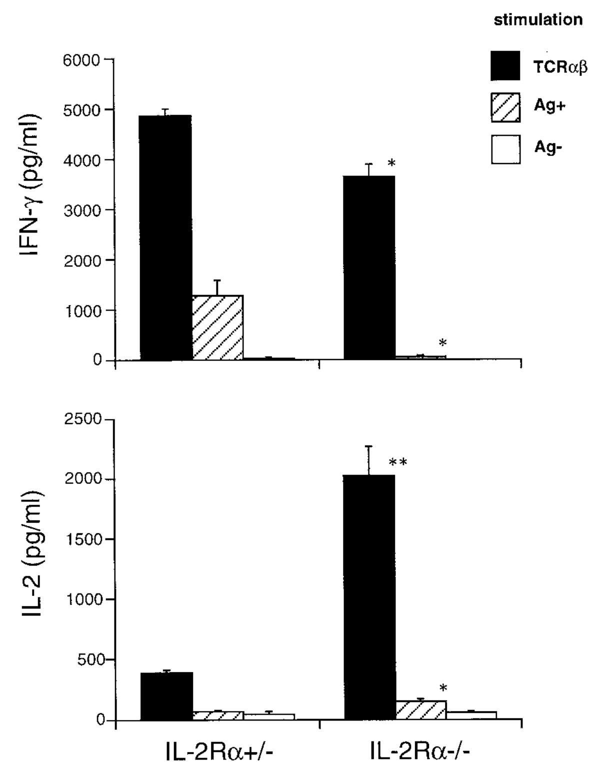

FIGURE 7. Cytokine production by peritoneal exudate CD4$^+$ T cells in response to HSV. PEC were collected from IL-2Rα$^{-/-}$ and IL-2Rα$^{+/-}$ mice on day 6 after an i.p. challenge with HSV-2. Purification of CD4$^+$ T cells were performed by using a nylon wool column and MACS column, and the purified CD4$^+$ T cells were >98% CD4$^+$ as assessed by FACS analysis. The CD4$^+$ T cells were cultured in the presence of mitomycin-treated spleen cells with or without heat-inactivated HSV. As a positive control, T cells were cultured in the presence of immobilized anti-TCR-αβ mAb (100 μg/ml). Cytokine levels in the supernatants were determined by ELISA. Data are means ± SD of 15 mice in each group. There was a statistically significant difference between the two groups (*, $p < 0.005$ and **, $p < 0.0001$ by Student's *t* test). Representative data from two independent experiments are shown.

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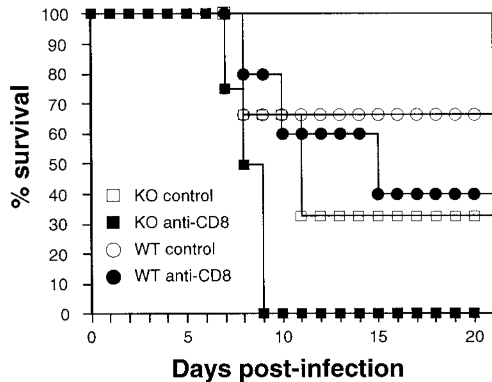

FIGURE 8. Effect of CD8$^+$ T cell depletion on the survival rate of IL-2Rα-deficient mice after infection with HSV-2 strain 186. IL-2R$\alpha^{-/-}$ and IL-2R$\alpha^{+/-}$ mice (10 mice in each group) were monitored for survival after i.p. inoculation with 5000 PFU HSV-2. Two days before infection, both groups of mice were injected with 300 μg purified anti-CD8 mAb or isotype control rat IgG. A statistically significant difference was found between CD8-depleted IL-2R$\alpha^{-/-}$ mice and nondepleted IL-2R$\alpha^{-/-}$ mice ($p < 0.05$ by the generalized Wilcoxon test). KO, Knockout; WT, wild-type.

was found in the Golgi apparatus and in early endosomes, suggesting that IL-15 is quickly absorbed by juxtacrine loops, although there is no activity of IL-15 in the supernatants (38). Therefore, it is alternatively possible that IL-15 may be secreted in a very low amount and thus act in a narrow intracellular range in naive IL-2R$\alpha^{-/-}$ mice. Willerford et al. (12) showed that the populations of CD4$^+$ and CD8$^+$ T cells were present in normal proportions in LN of IL-2R$\alpha^{-/-}$ mice between 4 and 6 wk of age, whereas in the present study, CD8$^+$ T cells were predominant over CD4$^+$ T cells in the IL-2R$\alpha^{-/-}$ mice at 5 wk of age. This difference may be partly due to the differences in genetic background and in stimulation with environmental Ag. The proliferation to optimal doses of anti-CD3 mAb for 48 h was also reported to be severely reduced in T cells from IL-2R$\alpha^{-/-}$ mice (12), whereas the proliferation in response to anti-TCR-$\alpha\beta$ mAb for 24 h was rather augmented in T cells from IL-2R$\alpha^{-/-}$ mice in the present study. An increase in the proportion of memory type CD8$^+$ T cells may be responsible for the early increased response of T cells to TCR engagement.

Most viral infections are rapidly terminated by the induced antiviral immune response, and MHC class I-restricted CD8$^+$ CTL are generally thought to play a predominant role in this process (39). In fact, CD4$^+$ T cells do not seem pivotal to the induction of an antiviral CTL response and clearance of most acute viral infections (39–43). However, in the case of infection with HSV, CD4$^+$ Th1 cells are indispensable for the host defense mechanism against HSV infection. CD4$^{-/-}$ mice have been reported to show susceptibility to HSV type 1 infection compared with CD8$^{-/-}$ mice (44). HSV expresses several proteins that can independently block the MHC class I presentation pathway by which antigenic peptides are presented to CD8$^+$ T cells. An HSV-infected cell protein, ICP47, is known to block the transporter associated with Ag presentation, so that antigenic peptides cannot be transported into the endoplasmic reticulum, the site of assembly of MHC class I molecules (45–48). Thus, generation of HSV-specific CD8$^+$ T cells is severely impaired during the course of HSV infection. In contrast, HSV-specific CD4$^+$ Th1 cells are generated and produce large amounts of IFN-γ, which have effects such as blocking of viral replication, activation of macrophages and NK cells (24), and enhancement of cytotoxicity by CD8 killer T cells (49, 50). Mice deficient in IFN-γ are susceptible to the development of cutaneous zosteriform lesions and to encephalitis (51). In addition to these, IFN-γ overcomes HSV-mediated MHC class I down-regulation and permits lysis of HSV-infected cells by CD8$^+$ CTL (52). Overexpression of IFN-γ in photoreceptor cells by transgenes protects mice from intraocular HSV infection (53). These results indicate that IFN-γ is an essential parameter for the clearance of HSV. Van Parijs et al. (54) reported that the T cells from IL-2Rα-deficient mice cannot differentiate into Th1 cells against protein Ags such as OVA. Consistent with this finding, we found that the T cells from IL-2R$\alpha^{-/-}$ mice infected with HSV-2 produced an appreciable level of IL-2 but no IFN-γ in response to HSV-2. These finding suggest that HSV-2-specific pre-Th cells can be generated in IL-2R$\alpha^{-/-}$ mice after infection with HSV-2 but that differentiation of the pre-Th cells to Th1 cells may be severely impaired in the absence of high-affinity IL-2R. Nevertheless, IL-2R$\alpha^{-/-}$ mice showed relatively strong resistance to HSV-2 infection. IL-2R$\alpha^{-/-}$ mice depleted of CD8$^+$ T cells became susceptible to HSV-2-induced lethality, suggesting that the memory-type CD8$^+$ T cells protect against systemic HSV-2 infection in IL-2R$\alpha^{-/-}$ mice.

There are several possible mechanisms by which the memory-type CD8$^+$ T cells protect against systemic HSV-2 infection. The first is a bystander activation of the CD8$^+$ T cells by endogenous IL-15 or IL-2 and presumably IL-12 during the course of HSV-2 infection. There are several lines of evidence for strong bystander stimulation of CD8$^+$ T cells in vivo elicited by various infectious agents (39, 55–61). Lymphocytic choriomeningitis virus infection induced activation and expansion of CD8$^+$ T cells. Limiting dilution analysis to quantities virus-specific CTL has shown that only a small fraction (at most 1–5%) of the activated CD8$^+$ T cells are Ag specific at the peak of primary response (39, 55, 56). Thus, memory phenotype CD8$^+$ T cells are subject to non-Ag-specific bystander stimulation through contact with various cytokines released during infection with pathogens. We found that the memory-type CD8$^+$ T cells in IL-2R$\alpha^{-/-}$ mice produced IFN-γ in response to exogenous IL-15 plus IL- 12 in vitro. Marked IL-15 production was evident after HSV-2 infection in IL-2R$\alpha^{-/-}$ mice, and IL-2 production was sustained in these mice after HSV-2 infection. The generation of HSV-2-specific Th1 or CTL producing type 1 cytokines was severely impaired in IL-2R$\alpha^{-/-}$ mice after HSV-2 infection. Taken together, it is likely that most of the CD8$^+$ T cells expand polyclonally in a TCR-independent manner but also in a bystander manner by endogenous IL-15 and/or IL-2 and produce IFN-γ in the presence of IL-12 during the course of HSV-2 infection. The second possibility is a reactivation of the memory CD8$^+$ T cells cross-reacting with Ag during the course of HSV-2 infection. The memory CD8$^+$ T cells in IL-2R$\alpha^{-/-}$ mice rapidly produced IFN-γ under TCR triggering, suggesting that Ag-induced early activation of the memory CD8$^+$ T cells may be responsible for the protection against HSV-2 infection. Welch et al. (62) proposed that reactivation of memory CD8$^+$ T cells by heterologous viral infection provides "natural immunity" to viral infections. A significant fraction of CD8$^+$ T cells from viable *Listeria monocytogenes*-immune mice have been reported to recognize not only *Listeria*-specific Ag in the context of MHC class I but also *N*-formylated peptides and phospholipid-associated Ags in the context of H-2 M3 (63). Human $\gamma\delta$ T cells have been reported to recognize conserved Ag such as alkylamine derived from various bacteria and cells (64). NK T cells can recognize glycolipid such as glycosylphosphatidylinositol common to prokaryotes and eukaryotes in the context of CD1d (65). Therefore, it is also possible that the memory phenotype CD8$^+$ T cells recognize the conserved Ags that are common to previously encountered Ag such as environmental Ag, including intestinal microbial flora or self Ags such

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as stress-induced proteins, and that they are in turn activated rapidly as a secondary immune response after primary infection with HSV-2. A recent report has shown that a subset of $CD8^+$ T cells expresses NKG2D, which was identified as an activating receptor on the cell surface of $\gamma\delta$ T cells and NK cells specific for stress-inducible MHC class I-related molecule MICA expressed on stress cells or tumor cells in humans (66). IL-15 can induce the expression of the NK receptor family, including NKG2A, on human NK cells (67). Hence, the third possibility for the protective mechanism against HSV infection by memory $CD8^+$ T cells is that memory phenotype $CD8^+$ T cells expressing NKG2D interact with a stress-induced ligand on HSV-2-infected cells and protect against HSV-2 infection. Lastly, although we could not detect HSV-2-specific $CD8^+$ T cells producing IFN-γ in IL-2R$\alpha^{-/-}$ mice in the present study, it is not completely precluded that memory phenotype $CD8^+$ T cells specific for HSV-2 may serve to protect against HSV-2 infection. However, these are only speculations and additional experiments are required to clarify the mechanisms controlling HSV-2 by memory phenotype $CD8^+$ T cells.

IL-15 production was more markedly increased after HSV-2 infection in IL-2R$\alpha^{-/-}$ mice than those in IL-2R$\alpha^{+/-}$ mice. However, at present, the mechanisms for increased IL-15 production in IL-2R$\alpha^{-/-}$ mice after HSV-2 infection remains unknown. It has been reported that NK (23–25) and $\gamma\delta$ T cells (26) play protective roles in HSV infection, and our results revealed that IL-2R$\alpha^{-/-}$ mice showed a severely impaired response of these cells after HSV-2 infection. In normal mice after HSV-2 infection, IL-15 may be rapidly absorbed by NK, NK T, $\gamma\delta$ T and memory $CD8^+$ T cells expressing IL-15Rα, which appeared at the early stage after infection. In contrast, because NK and $\gamma\delta$ T cell responses were impaired but a higher level of IL-2 was produced in IL-2R$\alpha^{-/-}$ mice, it is possible that IL-15 is not consumed rapidly by these cells after infection with HSV-2. We also found the higher level of serum IL-2 in IL-2R$\alpha^{-/-}$ mice before and after infection. The sustained level of IL-2 in IL-2R$\alpha^{-/-}$ mice may be explained by IL-2 accumulation due to lack of IL-2Rα^+ cells.

In conclusion, our data suggest that there are novel roles of memory phenotype $CD8^+$ T cells in host defense against HSV-2 infection. Memory phenotype $CD8^+$ T cells produce a large amount of IFN-γ in response to IL-15 and IL-12 and protect IL-2R$\alpha^{-/-}$ mice against HSV-2-induced lethality. IL-15 seems to provide protective immunity against HSV-2 infection via activation of memory phenotype $CD8^+$ T cells.

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