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A Novel Role of IL-15 in Early Activation of Memory CD8\(^+\) CTL after Reinfecion

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A rapid induction of effector functions in memory T cells provides rapid and intensified protection against reinfection. To determine potential roles of IL-15 in early expansion and activation of memory CD8\(^+\) T cells in secondary immune response, we examined the cell division and cytotoxicity of memory CD8\(^+\) T cells expressing OVA\(_{257-264}/\text{K}^\alpha\)-specific TCR that were transferred into IL-15-transgenic (Tg) mice, IL-15 knockout (KO) mice, or control C57BL/6 mice followed by challenge with recombinant 

**Listeria monocytogenes** expressing OVA (rLM-OVA). In vivo CTL activities and expression of granzyme B of the transferred CD8\(^+\) T cells were significantly higher in the IL-15 Tg mice but lower in the IL-15 KO mice than those in control mice at the early stage after challenge with rLM-OVA. In contrast, there was no difference in the cell division in IL-15 Tg mice and IL-15 KO mice compared with those in control mice. In vivo administration of rIL-15 conferred robust protection against reinfection via induction of granzyme B in the memory CD8\(^+\) T cells. These results suggest that IL-15 plays an important role in early activation of memory CD8\(^+\) T cells.

IL-15 belongs to the four-helix bundle cytokine family and uses \(\beta\)- and \(\gamma\)-chains of IL-2R for signal transduction, and it thus has many properties that are the same as those of IL-2, despite the fact that it has no sequence homology with IL-2 (13–15). In contrast to IL-2, which is produced mainly by activated T cells, IL-15 is produced by a wide variety of tissues, including placenta, skeletal muscle, kidney, and macrophages upon stimulation with LPS (16, 17). IL-2 is known to be important in clonal expansion of Ag-specific CD8\(^+\) T cells during primary immune response, whereas IL-15 is not mandatory for the expansion of CD8\(^+\) T cells in the immune response. Recent studies have demonstrated that primary responses to lymphocytic choriomeningitis virus (LCMV)\(^3\) and OVA were readily generated in IL-15 knockout (KO) mice or IL-15\(^{/}\text{KO}\) KO mice to a level equal to that in control mice (18, 19). We have also reported that generation of listeriolysin O (LLO)\(_{91-99}\) specific CD8\(^+\) T cells in IL-15-transgenic (Tg) mice normally occurred after primary infection with 

**Listeria monocytogenes** (20). In contrast, IL-15 has potential roles in maintenance of memory phenotype CD8\(^+\) T cells, which are capable of slowly dividing without Ag stimulation (18, 19). We have demonstrated that the number of LLO\(_{91-99}\)-positive memory CD8\(^+\) T cells was significantly higher 6 wk after primary infection with 

**Listeria monocytogenes**, which resulted in increased levels of Bcl-2 expression and cell division without Ag stimulation (20). Thus, IL-15 plays an important role in long-term maintenance of Ag-specific memory CD8\(^+\) T cells in an Ag-independent manner. In addition, there are several lines of evidence that IL-15 is capable of stimulating NK cells and CD8\(^+\) CTL to exhibit increased cytotoxities (21–24). IL-15 has been reported to directly up-regulate expression of cytotoxic molecules such as granzyme B and perforin, mimicking TCR cross-linking in the induction of cytotoxic molecules and cytotoxicity of memory CD8\(^+\) T cells (25). These findings raise the possibility that IL-15 plays an important role in rapid elicitation of effector functions in

**Abbreviations used in this paper:** LCMV, lymphocytic choriomeningitis virus; KO, knockout; LLO, listeriolysin O; Tg, transgenic; rLM-OVA, recombinant 

**Listeria monocytogenes** expressing OVA; PEC, peritoneal exudate cell; LN, lymph node; TCM, central memory cell; TEM, effector memory cell.
in vivo administration of exogenous IL-15 conferred protection against secondary infection via induction of cytotoxic molecules in the Ag-specific memory CD8$^+$ T cells. These results suggest that IL-15 plays an important role in early activation of Ag-specific memory CD8$^+$ T cells following secondary infection with microbes.

Materials and Methods

**Mice**

C57BL/6-background IL-15 Tg mice, which were constructed using originally described IL-15 cDNA, have been described previously (26). C57BL/6-background IL-15 KO mice were purchased from Taconic Farms. OT-I mice expressing the OVA$_{257-264}$/K$^b$-specific TCR and C57BL/6 Ly5.1-congenic mice were obtained from The Jackson Laboratory. To generate Ly5.1$^+$ OT-I mice, OT-I mice were crossed onto a B6-Ly5.1 background. All mice were used at 6–8 wk of age.

**Microorganism**

OVA expressing *L. monocytogenes* was kindly provided by Dr. Sabash Sald (Institute for Biological Sciences, Ontario, Canada) (27). Bacterial virulence was maintained by serial passages in C57BL/6 mice as described previously (20). Mice were infected i.p. with a sublethal dose of $5 \times 10^5$ CFU (0.1LD$_{50}$), or a lethal dose of $5 \times 10^6$ CFU (1LD$_{50}$), or 5 $\times 10^7$ CFU (10LD$_{50}$) of rLM-OVA in 0.2 ml of PBS on day 0. The spleen and liver were removed from each mouse and separately placed in homogenizers containing 3 ml of HBSS. These samples were spread on tryptophan-agar plates, and colonies were counted after incubation for 24 h at 37°C.

**Abs and reagents**

FITC-conjugated anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-CD25 (7D4), anti-CD6C (AL-21), and anti-IFN-γ (XMG1.2); PE-conjugated anti-Voα2(B20.1), anti-CD62L (MEL-14), CD122 (5H4), and CD132 (4G3); CyChrome-conjugated anti-CD8α (53-6.7) and streptavidin; allophycocyanin-conjugated streptavidin; and biotin-conjugated anti-Ly5.1 (A20) were purchased from BD Biosciences. PE-labeled anti-human granzyyme B mAb and isotype control Ab were obtained from Caltag Laboratories. CFSE was purchased from Molecular Probes. OVA$_{257-264}$ H-2K$^b$ tetramers were purchased from MBL.

**Analysis of intracellular granzyme B synthesis**

Spleen cells from infected mice were stained for surface markers for 30 min at 4°C and then subjected to intracellular cytokine staining using the Fast Immune Cytokine System according to the manufacturer’s instructions (BD Biosciences). For intracellular staining for granzyyme B expression, the cells were stained with PE-labeled anti-human granzyme B or an isotype control for 30 min at room temperature, and the fluorescence of the cells was analyzed using a flow cytometer.

**Generation of memory OT-I cells**

Memory OT-I cells were generated as follows. Purified CD8$^+$ T cells (2 × 10$^5$) of splenocytes from naive OT-I mice were injected i.v. into naive C57BL/6 hosts. Twenty-four hours later, these mice were infected with $5 \times 10^5$ CFU rLM-OVA. After 40 or more days, these mice were sacrificed, and the number of transgenic memory cells was determined by staining with anti-CD8 mAb, anti-Ly5.5 mAb, and OVA$_{257-264}$ H-2K$^b$ tetramer. In some experiments, mice harboring memory OT-I cells were injected i.p. with various doses of rIL-15 or PBS for control.

**Analysis of T cell proliferation following rLM-OVA infection in vivo**

CD8$^+$ T cells from naive OT-I mice or rLM-OVA-immune C57BL/6 mice harboring memory OT-I cells were purified by depleting nylon wool-enriched splenocytes with B220, CD4, and MHC II MicroBeads by MACS (Miltenyi Biotec) to >90% purity. For the analysis of naive or memory OT-I cell proliferation following rLM-OVA infection in vivo, purified CD8$^+$ T cells containing $2 \times 10^6$ naive OT-I cells or $5 \times 10^6$ memory OT-I cells were labeled with CFSE as described previously (20). Proliferation of transferred cells was visualized by FACs analysis of their CFSE profile. Transferred OT-I cells were identified by staining with a mAb to Ly5.1, CD8, and the Vα2 or OVA$_{257-264}$/K$^b$ tetramer.

**In vivo cytotoxicity assay**

Analysis of in vivo cytolytic activity was carried out by a protocol similar to those previously reported (28, 29). B6-Ly5.1$^+$Ly5.2$^+$ splenocytes were divided into two populations and labeled with a high concentration (5 µM) and a low concentration (0.5 µM) of CFSE. Next, CFSE$^{low}$ cells were pulsed with 5 µg/ml OVA$_{257-264}$ peptide for 1 h at 37°C and CFSE$^{high}$ cells remained unpulsed. After washing, these groups were mixed in equal proportions and then injected i.v. into mice infected with rLM-OVA 2 days previously. Spleens from recipients were taken 5 h later for flow cytometric analysis to measure in vivo killing activities. Percent specific lysis was calculated according to the formula $1 - (\text{ratio primed/ratio unprimed}) \times 100$, where the ratio unprimed = percent CFSE$^{low}$/percent CFSE$^{high}$ cells remaining in noninfected recipients, and ratio primed = percent CFSE$^{low}$/ percent CFSE$^{high}$ cells remaining in infected recipients.

**Results**

**Expansion and activation of naive CD8$^+$ T cells in naive IL-15 KO or IL-15 Tg hosts in primary response to L. monocytogenes**

We first examined the role of IL-15 in expansion and activation of naive CD8$^+$ T cells during primary immune responses using a system of adoptive transfer of OT-I Tg CD8$^+$ T cells (Ly5.1$^+$) that express a TCR specific for H-2K$^b$-restricted OVA$_{257-264}$ epitopes into naive Ly5.2$^+$ IL-15 Tg, IL-15 KO, or control C57BL/6 hosts that were subsequently infected with rLM-OVA. The absolute numbers of OT-I cells in the spleens of IL-15 KO and IL-15 Tg mice, as assessed by staining with an H-2K$^b$ tetramer coupled with an OVA-derived SIINFEKL peptide or with anti-Vα2 mAb, were almost the same as those in control mice on day 5 or 7 after primary infection with rLM-OVA (Fig. 1A). The absolute numbers of OT-I cells in the peritoneal exudate cells (PEC) and lymph node (LN) from IL-15 Tg and IL-15 KO mice were also the same as those in control mice after primary infection (data not shown). We next examined that expansion of OT-I cells in IL-15 Tg and IL-15 KO mice during the primary response. CFSE-labeled naive OT-I cells were adoptively transferred i.v. into naive IL-15 KO mice, IL-15 Tg mice or C57BL/6 mice that were subsequently infected with rLM-OVA. As shown in Fig. 1B, OT-I cells began to proliferate equally in all groups on day 4, and a massive expansion of OT-I cells occurred on day 5 after rLM-OVA infection. Although all of the cells were CFSE negative at this stage, indicating that most of the cells had divided five to eight times. Thus, we confirmed by using an adoptive transfer system that IL-15 is not essential for expansion of Ag-specific effector CD8$^+$ T cells after primary infection.

To determine the role of IL-15 in activation of effector CD8$^+$ T cells during primary immune response, we examined the expression levels of activation markers and granzyyme B of OT-I cells after primary infection with rLM-OVA. The OT-I cells, of CD4$^+$, CD122 (IL-2 R$^+$), and CD132 (c-Myb) phenotypes, did not contain intracellular granzyyme B in IL-15 KO and IL-15 Tg mice on day 3 and were equivalently increased on OT-I cells on day 5 after rLM-OVA infection (Fig. 1C and data not shown).

Only the CFSE-negative OT-I cells from each mouse contained
high levels of granzyme B, indicating that naive OT-I cells required clonal expansion to differentiate into fully functional effector CTL that secrete cytotoxic molecules. We further analyzed the ability of transferred naive OT-I cells to produce IFN-γ upon restimulation with peptide on day 5 after rLM-OVA infection. The proportions of intracellular IFN-γ production by OT-I cells in the spleens from IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). These results indicated that Ag-specific naive CD8+ T cells might expand and acquire effector function in an IL-15-independent manner during primary Listeria infection.

Expansion and activation of memory CD8+ T cells transferred into naive IL-15 KO or IL-15 Tg hosts in secondary response to L. monocytogenes

We next examined the role of IL-15 in expansion and activation of memory CD8+ T cells during secondary immune responses using a system of adoptive transfer of memory OT-I cells into naive IL-15 Tg, IL-15 KO, and C57BL6 hosts. Because the magnitude of the secondary expansion of memory CD8+ T cells is related to the precursor frequency of Ag-specific memory CD8+ T cells and because numbers of endogenous OVA325-334-specific memory CD8+ T cells in the spleen were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice than those in control mice (>40 days after primary infection (our unpublished data), we used an adoptive transfer system in which the numbers of Ag-specific cells could be defined precisely before and during immune responses. We generated memory CD8+ T cells in vivo by adoptive transfer of Ly5.1+ naive OT-I cells into C57BL6 mice (Ly5.2+) followed by infection with rLM-OVA and purified the memory OT-I cells at 40 or more days after primary rLM-OVA infection. Memory OT-I cells were of CD44high, CD122high, CD132+, CD25-, and CD69- phenotypes (data not shown), representing resting memory CD8+ T cells. Two subsets of memory CD8+ T cells based on their anatomical location, expression of cell surface markers, and effector functions have been described (30, 31). Approximately 60% of the memory OT-I cells purified from spleens of recipients expressed CD62L+, representing central memory cells (T_{CM}), and 40% were of CD62L- phenotype, representing effector memory cells (T_{EM}).

To determine the role of IL-15 in expansion of memory OT-I cells during the secondary immune response, memory OT-I cells were labeled with CFSE and adoptively transferred i.v. into naive IL-15 KO, IL-15 Tg, and C57BL6 hosts. To compare expansion of memory CD8+ T cell subsets after secondary infection, it was critical to demonstrate that total number of memory CD8+ T cells was the same after adoptive transfer to recipient mice. At 24 h after adoptive transfer, total number of memory OT-I cells recovered from spleen in IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). Then these mice were challenged with a lethal dose of rLM-OVA and the proliferation of memory OT-I cells was analyzed after infection (Fig. 2A). As shown in Fig. 2B, none of the memory OT-I cells had divided by day 3 infection when transferred into naive IL-15 Tg, IL-15 KO, or C57BL6 mice, showing kinetics similar to the kinetics of naive OT-I cells after primary infection (Figs. 1B and 2B). A massive expansion of OT-I cells occurred in all groups on day 5 after Listeria infection. Thus, during the secondary response, there was no obvious difference in the CFSE profiles of memory OT-I cells in IL-15 Tg and IL-15 KO mice compared with those of control mice. These results suggest that IL-15 is not essential for proliferation of memory CD8+ T cells during secondary immune response.

To determine the role of IL-15 in activation of memory CD8+ T cells during secondary immune response, we analyzed the expression of granzyme B in memory OT-I cells in naive IL-15 Tg and IL-15 KO mice after rLM-OVA infection. The expression levels of intracellular granzyme B in memory OT-I cells were significantly lower in IL-15 KO mice but higher in IL-15 Tg mice than those in control mice on day 2 after rLM-OVA infection (p < 0.05, Fig. 2C and D). On day 5 after reinfection, intracellular expression levels of granzyme B in the dividing memory OT-I cells in IL-15 Tg and IL-15 KO mice became comparable to those in control mice. We further analyzed the ability of transferred memory OT-I cells to produce IFN-γ upon restimulation with peptide on days 2 and 5 after rLM-OVA infection. The proportions of
intracellular IFN-γ production by OT-I cells in the spleens from IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). These results demonstrate that IL-15 are involved in the induction of granzyme B in nondividing memory CD8+ T cells at the early stage of secondary immune response.

To directly detect cytotoxic activity of OT-I cells in vivo, we measured the ability of naive and memory OT-I cells to eliminate fluorescent-labeled spleen cells pulsed with OVA257–264 peptides at the early stage after Listeria infection. Naive or memory OT-I cells were adoptively transferred into naive IL-15 Tg mice, IL-15 KO mice, and control C57BL/6 mice that were infected with rLM-OVA 24 h later. We confirmed that naive IL-15 Tg mice, IL-15 KO mice, and control C57BL/6 mice had not developed any in vivo CTL activities on day 2 after rLM-OVA (data not shown). Consistently, none of the OVA257–264 peptide-pulsed donor cells in any group of mice into which naive OT-I cells had been transferred were cleared from the spleens on day 2 after infection (Fig. 2E). In contrast, on day 2 after infection ~35% of OVA257–264 peptide-pulsed donor cells were cleared from the spleens of C57BL/6 mice into which memory OT-I cells had been transferred. In correlation with the expression of intracellular granzyme B of memory OT-I cells, in vivo CTL activity levels of memory OT-I cells were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice than those in control mice (Fig. 2E). These results suggest that IL-15 provides early activation of Ag-specific memory CD8+ CTL via induction of cytotoxic molecules.

Expansion and activation of memory CD8+ T cells transferred into immunized IL-15 KO or IL-15 Tg hosts in secondary response to L. monocytogenes

As shown above, we examined the expansion and activation of memory CD8+ T cells in the specific situation of secondary immune response by using an adoptive transfer system of memory OT-I cells into naive hosts. To examine the role of IL-15 in expansion and activation of memory OT-I cells in more physiological conditions of secondary immune response, we transferred memory OT-I cells into IL-15 Tg, IL-15 KO, or control C57BL/6 hosts that had been immunized with rLM-OVA and subsequently rechallenged with rLM-OVA (Fig. 3A). As shown in Fig. 3B, memory OT-I cells began to proliferate in all groups of immunized mice on day 2 after rLM-OVA reinfecion. This expansion was much faster than that of memory OT-I cells transferred into naive hosts after secondary infection. A massive expansion of OT-I cells

reinfecion and stained for expression of CFSE and intracellular granzyme B of memory OT-I cells. Dot plots are gated on donor cells (Ly5.1+) and the number indicated is the percentage of donor cells stained positive for granzyme B. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. D, Intracellular expression of granzyme B in OT-I cells transferred into naive IL-15 KO, IL-15 Tg, or control mice on day 2 after infection. Data were obtained from three separate experiments, and each value shown is the mean ± SD for three experiments.

FIGURE 2. IL-15 plays an important role in the induction of cytotoxic molecules of Ag-specific memory CD8+ T cells during secondary immune response in naive hosts. A. Purified naive OT-I cells (Ly5.1+) were adoptively transferred into naive C57BL/6 hosts that were infected with rLM-OVA 24 h later. At 40 or more days after primary infection, CD8+ T cells from these mice were purified by negative selection using MACS and labeled with CFSE. CFSE-labeled CD8+ T cells containing 5 × 10^5 memory OT-I cells were adoptively transferred i.v. into naive IL-15 KO, IL-15 Tg, and control mice that were infected with rLM-OVA 24 h later. B, CFSE fluorescence of splenic OT-I cells was analyzed by flow cytometry by staining with anti-CD8 mAb, anti-Ly5.1 mAb, and OVA K b tetramer at indicated time points. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on Ly5.1+CD8+ cells. C. Splenocytes from IL-15 KO, IL-15 Tg, or control mice harboring CFSE-labeled memory OT-I cells were isolated at indicated days before and after

reinfecion and stained for expression of CFSE and intracellular granzyme B of memory OT-I cells. Dot plots are gated on donor cells (Ly5.1+CD8+), and the number indicated is the percentage of donor cells stained positive for granzyme B. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. D, Intracellular expression of granzyme B in OT-I cells transferred into naive IL-15 KO, IL-15 Tg, or control mice on day 2 after infection. Data were obtained from three separate experiments, and each value shown is the mean ± SD for five mice (n, p < 0.05). E, Memory or naive OT-I cells (5 × 10^5) were adoptively transferred into naive IL-15 KO mice, IL-15 Tg mice, or control C57BL/6 mice that were infected with rLM-OVA 24 h later. As target cells, spleen cells from naive mice (Ly5.1+Ly5.2+) were pulsed with OVA peptides or left unpulsed and then injected i.v. into each infected mouse harboring memory or naive OT-I cells on day 2 after rLM-OVA infection. Histograms are gated on Ly5.1+Ly5.2+ cells in the spleen from infected mice. The values in the right corner of each panel represent the percentage of specific killing compared with nonpulsed cells. Data were obtained from three separate experiments, and each value shown is the mean ± SD for three experiments.
ory OT-I cells. DOT plots are gated on donor cells (Ly5.1).

Splenocytes from IL-15 KO, IL-15 Tg, and control mice harboring mem-

FIGURE 3. IL-15 plays an important role in the induction of cytotoxic molecules of Ag-specific memory CD8+ T cells during secondary immune response in immunized hosts. A. CFSE-labeled memory OT-I cells (Ly5.1+) generated in C57BL/6 mice were adoptively transferred i.v. into IL-15 KO, IL-15 Tg, and control mice that had been immunized with rLM-OVA 40 or more days previously. At 24 h after adoptive transfer, these mice were challenged with a lethal dose of rLM-OVA. B. CFSE fluorescence of splenic OT-I cells was analyzed by flow cytometry by staining with anti-CD8 mAb, anti-Ly5.1 mAb, and OVA Kb tetramer at indicated time points. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on Ly5.1+ CD8+ cells. C. Splenocytes from IL-15 KO, IL-15 Tg, and control mice harboring memory OT-I cells were isolated at indicated days before and after reinfection and stained for expression of CFSE and intracellular granulysin B of memory OT-I cells. Dot plots are gated on donor cells (Ly5.1+ CD8+), and the number indicated is the percentage of donor cells stained positive for granulysin B. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. D. Intracellular expression of granulysin B in OT-I cells transferred into immunized IL-15 KO, IL-15 Tg, or control mice on day 2 after secondary infection. Data were obtained from three separate experiments, and each value shown is the mean +SD for five mice (*, p < 0.05).

Effects of exogenous IL-15 on activation of memory CD8+ T cells and protection at the early stage after secondary infection

On day 40 after rLM-OVA infection, memory OT-I cells could be generated and maintained in IL-15 KO mice into which naive OT-I cells had been transferred, albeit at lower degrees than those in control mice (our unpublished data). To test directly whether IL-15 is involved in the induction of granulysin B in memory CD8+ CTL after reinfection, we examined the effects of in vivo administration of rIL-15 on expression of intracellular granulysin B in memory CD8+ T cells after secondary infection in an IL-15-deficient environment. IL-15 KO mice were subjected to transfer of naive OT-I cells followed by rLM-OVA infection, and the IL-15 KO mice were rechallenged 40 days later with a lethal dose of rLM-OVA. rIL-15 was injected i.p. at 0 and 24 h after rechallenge with rLM-OVA (Fig. 4A). On day 2 after secondary infection, splenocytes were prepared and intracellular granulysin B staining was performed. As shown in Fig. 4B, strong induction of granulysin B was found in memory OT-I CTL from rIL-15-treated IL-15 KO mice compared with that in memory OT-I CTL from PBS-treated IL-15 KO mice after secondary infection. In contrast, there were no significant differences in the proportions of memory OT-I CTL between rIL-15-treated IL-15 KO mice and PBS-treated IL-15 KO mice on day 2 after reinfection (data not shown). Intracellular expression levels of granulysin B in endogenous CD8+ T cells were also significantly increased in rIL-15-treated IL-15 KO mice compared with those in PBS-treated IL-15 KO mice on day 2 after secondary infection. We next measured in the vivo cytolytic activities of memory OT-I cells in rIL-15-treated IL-15 KO mice on day 2 after infection. In vivo CTL activity levels were significantly higher in rIL-15-treated IL-15 KO mice than in PBS-treated IL-15 KO mice after secondary infection (Fig. 4C). Moreover, to evaluate antilisterial immunity of memory CTL after treatment with rIL-15, the bacterial growth was determined in the spleen and liver from both rIL-15-treated IL-15 KO mice and PBS-treated IL-15 KO mice after secondary infection (Fig. 4C). Moreover, to evaluate antilisterial immunity of memory CTL after treatment with rIL-15, the bacterial growth was determined in the spleen and liver from both rIL-15-treated IL-15 KO mice and PBS-treated IL-15 KO mice after secondary infection. As shown in Fig. 4D, both PBS-treated naive IL-15 KO mice and rIL-15-treated naive IL-15 KO mice had high bacterial loads in the spleens and livers on day 2 after infection. In contrast, immunized IL-15 KO mice exhibited antilisterial immunity, as shown by a reduction in bacterial counts in the spleens and livers after rechallenge. Administration of rIL-15 resulted in 10- to 30-fold reduction in bacterial loads in the spleens and livers of immunized IL-15 KO mice compared with occurred in all groups on day 5 after Listeria infection. Thus, during secondary Listeria infection, there was no obvious difference in the CFSE profiles of memory OT-I cells in IL-15 Tg and IL-15 KO mice compared with those of control mice. These results suggest that not only in the specific situation in naive hosts but also in a physiological situation in immunized hosts, IL-15 is not essential for proliferation of memory CD8+ T cells during secondary infection.

We next analyzed the expression of granulysin B in memory OT-I cells in immunized IL-15 KO and IL-15 KO hosts after rLM-

OVA re-infection. The expression levels of intracellular granulysin B in memory OT-I cells not dividing were significantly lower in IL-15 KO mice than in control mice on day 2 after rLM-OVA reinfection (p < 0.05, Fig. 3, C and D). Intracellular expression levels of granulysin B in the dividing memory OT-I cells in immunized IL-15 Tg and IL-15 KO mice became comparable to those in control mice on day 5 after reinfection. Taken together, these results demonstrate that IL-15 plays an important role in rapid elicitation of effector functions in non-dividing memory CD8+ T cells at the early stage after secondary infection.
those in the case of PBS administration. These results suggest that IL-15 plays an important role in the induction of effector functions in Ag-specific memory CD8\(^+\) CTL following re-exposure to microbes.

IL-15 has been reported to directly up-regulate expression of cytotoxic molecules such as granzyme B and perforin that are closely correlated with cytotoxicity effector function of human CD8\(^+\) memory cells in vitro (25). Therefore, we next investigated whether in vivo administration of rIL-15 alone can induce cytotoxic activity of memory OT-I cells. C57BL/6 mice harboring memory OT-I cells were injected i.p. with various dose of rIL-15 (Fig. 5A), and the expression levels of intracellular granzyme B and the cytolytic activity levels of splenic memory OT-I cells at 24 h after administration of various doses of rIL-15 were examined. As shown in Fig. 5B, upper panel, memory OT-I cells contained low levels of granzyme B before rIL-15 treatment, but high intracellular levels of granzyme B in memory OT-I cells had been induced at 24 h after a single administration of 2 \(\mu\)g rIL-15. Furthermore, injection of 2 \(\mu\)g rIL-15 twice induced ~80% of expression levels of intracellular granzyme B in memory OT-I cells in the absence of TCR triggering, and this up-regulation occurred in a dose-dependent manner (Fig. 5B, lower panel). Intracellular expression levels of granzyme B in endogenous CD8\(^+\) T cells were also significantly increased after administration of rIL-15. In correlation with the expression of intracellular granzyme B in memory OT-I cells, in vivo CTL activity was significantly increased in rIL-15-treated mice compared with that in PBS-treated control mice at 24 h after a single administration of 2 \(\mu\)g rIL-15 (Fig. 5C). These results suggest that the ability to induce granzyme B in response to IL-15 is independent of prior Ag challenge.

Discussion

In the present study, we examined the roles of IL-15 in expansion and activation of Ag-specific naive and memory CD8\(^+\) T cells by direct comparison of naive and memory CD8\(^+\) T cells that exhibit the same Ag specificity for OVA\(_{257–264}\) in experiments on adoptive transfer into IL-15 Tg mice and IL-15 KO mice after infection with rLM-OVA. The absolute numbers of and the frequencies of division of naive OVA\(_{257–264}\)-specific CD8\(^+\) T cells in IL-15 Tg mice and IL-15 KO mice were almost the same as those in control C57BL/6 mice after primary infection with
rLM-OVA, confirming that IL-15 is not essential in priming naive CD8+ T cells for expansion and differentiation into effector CTL following microbial infection. In contrast, in vivo CTL activity levels of memory OVA257–264/K°-specific CD8+ T cells were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice at the early stage of secondary immune response, well before the division of memory CD8+ T cells occurred. Moreover, in vivo administration of exogenous IL-15 confers robust protection against reinfection via induction of a cytotoxic molecule in memory CD8+ T cells. These results suggest that IL-15 plays an important role in early activation of Ag-specific memory CD8+ T cells following secondary infection with microbes.

It is notable finding that in vivo CTL activity levels of memory OT-I cells were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice at the early stage of reinfection, well before the division of memory CD8+ T cells occurred. Perforin/granzyme-mediated cytolysis is the major pathway involved in lysis of target cells infected with intracellular pathogens. It has been reported that perforin-mediated cytolysis is an essential effector function in CD8+ T cell-mediated secondary resistance to L. monocytogenes (32, 33). We demonstrated that in correlation with in vivo CTL activity levels, the expression levels of granzyme B in memory OT-I CD8+ T cells were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice at the early stage after secondary infection. IL-15 has been reported to directly up-regulate expression of cytotoxic molecules such as granzyme B and perforin that are closely correlated with cytotoxicity effector function of CD8+ memory cells in vitro (25). We showed in the present study that in vivo administration of exogenous IL-15 alone could induce up-regulation of intracellular granzyme B in memory CD8+ T cells in C57BL/6 mice. There have been several lines of evidence for IL-15 production by nonlymphoid cells after infection with various microbes (34–42). A sufficiently high concentration of IL-15 produced by macrophages and epithelial cells might induce up-regulation of cytotoxic molecules in Ag-specific memory CD8+ T cells at the early stage after secondary infection with microbes and contribute to rapid elimination of reinvading microbes.

Two subsets of memory CD8+ T cells based on their anatomical location, expression of cell surface markers, and effector functions have been described (30, 31). Memory CD8+ T cells expressing homing receptors such as CD62L and CCR7, which allow efficient homing to LN, are termed TECM whereas memory T cells lacking these LN homing receptors, which are located in nonlymphoid tissues, are termed TEM. TECM have been reported to produce few effector molecules but to have a high proliferative capacity in response to IL-2/IL-15 in autocrine and/or paracrine manners (43). In contrast, TEM cells, which have greater cytolytic effector functions, facilitate their entry into infected tissues and play a role as the first line of host defense against re-exposure to microbes (30). However, the TEM population has little homeostatic proliferative potential, and this subset therefore does not seem to be a permanent memory population (43). Although we did not separate CD8+ TEM and TECM from memory OT-I cells in the spleen, IL-15 may affect mainly the function of CD8+ TEM because intracellular granzyme B was up-regulated in memory CD8+ T cells well before cell division occurred at the early stage after secondary infection. CD8+ TEM, which reside mainly in nonlymphoid tissues, serve as the first line of host defense against microbial invasion.

It has been reported that memory CD8+ T cells expressing a Tg αβ TCR specific for the male Ag expanded more than did their naive counterparts and that they accumulated much faster in recombination activating gene-2-deficient female mice (44). In contrast, a recent study has suggested that there was no significant difference between naive and memory CD8+ T cells in their proliferative capacities after LCMV infection in naive normal hosts using a system of adoptive transfer of CD8+ T cells from P14 Tg mice (specific for the GP-33 LCMV epitope) (11). We also found no difference between kinetics of the division of naive and memory OT-I cells transferred into naive hosts after rLM-OVA infection (Figs. 1B and 2B). Thus, there may not be a marked difference between naive and memory CD8+ T cells in their proliferative capacities in vivo after Ag re-exposure in naive hosts. However, in physiological conditions of secondary immune response, the help of memory CD4+ T cells in expansion of memory CD8+ T cells must be considered. Tanchot and Rocha (45) reported that CD4+ T cells are required for expansion of memory CD8+ T cells but that they are no longer needed for their function. Consistent with this finding, we found that in vivo depletion of CD4+ T cells completely inhibited the early expansion of memory OT-I cells in immunized hosts after rLM-OVA reinfection (our unpublished data). These results suggest that memory CD4+ T cells are indispensable for early expansion of memory CD8+ T cells after secondary infection and that memory CD8+ T cells may not expand in an autocrine manner during secondary infection. In is most likely that IL-2 derived from CD4+ T cells is important for expansion of memory CD8+ T cells during secondary immune responses. However, Tuma et al. (46) reported that CD40L/CD40 signaling is required for long-lasting protective immunity by transferred memory CD8+ T cells against Listeria infection. Therefore, it is possible that both IL-2 and CD40L provided by activated CD4+ T cells may be required for rapid expansion of memory CD8+ T cells during secondary immune responses. Additional experiments are needed to clarify these possibilities.

In conclusion, IL-15 plays important roles not only in maintenance of memory CD8+ T cells by homeostatic proliferation in the absence of Ag but also in the early activation of memory CD8+ T cells as secondary effector cells when microbes invade again. In vivo administration of rIL-15 to enhance cytotoxic activities of Ag-specific memory CD8+ T cells may be used for controlling microbial infection in vaccinated hosts and treating patients with chronic viral and bacterial infection or malignancy.

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References
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